Osteonectin Promotes Prostate Cancer Cell Migration and Invasion: A Possible Mechanism for Metastasis to Bone1

Karin Jacob, Mukta Webber, Dafna Benayahu, and Hynda K. Kleinman2

Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, Maryland 20892-4370 [K. J., H. K. K.]; Departments of Zoology and Medicine, Michigan State University, East Lansing, Michigan 48824 [M. W.]; and Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel [D. B.]

ABSTRACT

The mechanism underlying the “organ-specific” metastasis of prostate cancer cells to the bone is still poorly understood. It is not clear whether the cells only invade the bone and proliferate there or whether they invade many tissues but survive mainly in the bone (“seed and soil”). Extracts from various organs were used as chemotaxants in the in vitro chemotaxis and invasion assays. Results show that, in comparison with extracts of other tissues, bone extracts promote a 2- to 4-fold increase in chemotaxis by human prostate epithelial cells and a 4-fold increase in the invasive ability of human prostate carcinoma cells. The purified active factor from bone and from marrow stromal-cell-conditioned medium is a low glycosylated osteonectin that specifically promotes the invasive ability of bone-metastasizing prostate (and breast) cancer cells but not that of non-bone-metastasizing tumor cells. It does not stimulate the growth of prostate cancer cells in vitro or in vivo. Because osteonectin specifically enhances matrix metalloproteinase activity in prostate and breast cancer cells (and not in other tumor cell types), we conclude that prostate cancer cell metastasis to the bone is, in part, mediated by the ability of osteonectin to promote migration, protease activity, and invasion.

INTRODUCTION

Prostate cancer is the second leading cause of death from cancer in men in both Europe and the United States, and the primary cause of death is invasion and metastasis (1). Over 70% of men have lymph-node metastases at the time of diagnosis (2–5). During metastasis, prostate cancer cells migrate to the pelvic lymph nodes and then to bone in the pelvic and lumbar vertebral column (2, 4, 6). The frequency of bone metastasis is much higher than predicted by random tumor-cell dissemination (7).

The mechanism of organ-specific metastasis in vivo is poorly understood. The “seed and soil” hypothesis proposes that tumor cells prefer to colonize an organ that serves as a fertile soil (8). Alternatively, prostate cancer cells may be specifically attracted by factors released from the bone and “migrate” preferentially to it (9). It has been shown that prostate cancer cells adhere well to bone marrow-derived endothelial cells and to osteoblasts (10, 11) and that their proliferation can be stimulated by CM from osteoblast-like cells (12).

Using extracts from various organs as chemotaxants in vitro chemotaxis and invasion assays, we find that prostate cancer cells migrate preferentially to bone extracts as well as to CM-MSC. Using these two sources, we have purified bone-osteonectin as the factor that specifically enhances matrix metalloproteinase activity and invasion by human prostate cancer cells. Osteonectin belongs to a family of small, calcium- and collagen-binding proteins. We propose that the low glycosylated osteonectin that we purified is a promoter of prostate cancer cell metastasis to the bone.

MATERIALS AND METHODS

Cells and Culture Conditions. The human prostate cancer cell lines DU-145 and PC-3 and the breast cancer cells MDA-MB-231 (a gift from Dr. Rick Thompson, Lombardi Cancer Center) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum and antibiotics. The human prostate epithelial cell lines PWR-IE (13; Ad-12/SV-40 immortalized), RWPE-1 (13, 14; HPV18-immortalized), RWPE-2 (13, 14; derived from RWPE-1 by Ki-ras transformation), as well as the cell lines WPE1-NA22, WPE1-NB11, WPE1-B14, and WPE1-B26 (derived from RWPE-1 after N-methyl-N-nitrosourea transformation) were used. All seven of the lines were maintained in keratinocyte-serum-free medium (K-SFM, with epidermal growth factor and bovine pituitary extract, Life Technologies, Inc., Gaithersburg, MD), B16-F10 mouse melanoma cells (obtained from the American Type Culture Collection, Rockville, MD), HT1080 fibrosarcoma cells, and NIH 3T3 ras-transformed cells were maintained in DMEM with 10% fetal bovine serum and antibiotics (Life Technologies, Inc.).

Preparation of Tissue Extracts. Crushed bone, brain, kidney, liver, lung, and spleen from 8-week-old C57/BL mice were extracted as described previously (15) and dialyzed against PBS. Human bone was obtained from an 88-year-old male (femur, MDRI, Philadelphia) and a 45-year-old female (knee).

CM-MSC. Femurs from 5-week-old rats were flushed with DMEM, and the detached cells were seeded in 6-well plates at a density of 1 × 104 cells/well. After 1 week, 18.7% of the attached cells were trypsinized, and the most adherent ones were passaged. Confluent monolayers were washed twice with PBS and incubated with serum-free DMEM for 48 h, and CM was collected.

Invasion Assays. Chemotaxis and invasion were examined using a 48-well Boyden chamber assay (Neuro Probe Inc, Cabin John, MD; Ref. 16). Organ extracts and fractions from the purification steps were used as chemoattractants at 1 μg/μl and 1 ng/μl, respectively. RPMI 1640 and NIH 3T3-CM were used as negative and positive controls. Polycarbonate filters (12-μm pore size; Neuro Probe Inc., Cabin John, MD), coated with Matrigel (500 μg/filter) or collagen IV (500 μg/filter) were used. Confluent cultures from various cell lines were incubated with 0.2% EDTA in PBS, resuspended in RPMI 1640 with 0.1% BSA, and 50,000 cells/well were seeded. The chambers were incubated at 37°C for 4 h and then fixed and stained (16, 17). The cells on the lower surface of the filter were counted (2 fields/well) under a microscope using a ×10 objective. Each condition was assayed in triplicate, and each experiment was repeated at least three times. To examine the ability of anti-osteonectin antibody (Hematological Inc., Essex, VT) to block the invasive effect, bone-osteonectin (25 ng/well) was mixed with the antibody at different concentrations before testing.

Proliferation Assay. DU-145 and PC-3 cells were seeded at 5000 cells/well in 96-well plates. Different bone extracts at a final concentration of 0.05 μg/μl or unsupplemented RPMI 1640 were added after 12 h. Medium containing fresh extract was changed every 48 h. Growth was assessed using the MTS proliferation assay (Promega Corp., Madison, WI).

Purification. Bone extract of a femur from an 88-year-old human male and 48-h CM-MSC cultures were used as the starting materials. After a 60% ammonium sulfate precipitation at 4°C overnight followed by centrifugation,
the supernatant was discarded, and the pellet was solubilized in 1:100 of the starting volume in HEPES buffer. The protein mixture was then separated on a 10% TBE-Urea gel and extracted with a gel eluter (Bio-Rad, Hercules, CA). All of the fractions of different molecular size were tested for their invasion-enhancing activity, and the active fractions were further separated by SDS-PAGE on a 10% gel for protein staining.

**Sequencing.** The factor was purified as described above from 100 ml of CM. The band of interest was excised from a 10% gel after Coomassie staining, tryptic-digested, and identified using MALDI-MS and protein sequencing after HPLC digestion (Keck Foundation, Yale University, New Haven, CT).

**Western Blot Analysis.** Active fractions (2-μg and 1-μg protein), nonactive fractions 7 and 14 (2 μg), platelet osteonectin (0.5 μg; Hematological Inc.) and SPARC (1 μg; Sigma, St. Louis MO) were run on a 10% SDS polyacrylamide gel, transferred to nitrocellulose (NOVEX, San Diego, CA), and probed with a monoclonal anti-osteonectin antibody at a 1:1000 dilution (Hematological Inc.). Horseradish-peroxide-coupled antimouse IgG antibody was used at a dilution of 1: 2000 (Amersham, Arlington Heights, IL).

**Deglycosylation.** Deglycosylation was carried out with Glykosidase F (Boehringer, Ingelheim) for 16 h at 37°C followed by a denaturation with SDS and β-mercaptoethanol for 30 min at 37°C.

**Zymogram.** Cells (1.5 × 105) were plated in 6-well plates and incubated with serum-free medium (0.5 ml/well) for 24 h with or without osteonectin. CM was collected after a 72-h incubation. Samples (20 μl) from DU-145 and PC-3 prostate cancer cells, MDA-MB-231 breast cancer cells, B16-F10 mouse melanoma cells, and HT1080 fibrosarcoma cells were run on a 10% Tris-glycine gel containing 0.1% gelatin (Novex). The gel was treated with renaturating buffer (30 min), developing buffer (30 min + 37°C overnight; Novex), and 0.25% Coomassie (30 min) and then was destained (50% methanol, 10% acetic acid).

**RESULTS**

**Bone Extracts Promote Invasion and Chemotaxis by Prostate Cancer Cells.** Bone extracts and extracts from other tissues were used as chemotactants to measure their ability to enhance invasion and chemotaxis. Bone extracts induced a 3-fold or greater increase in invasion by DU-145 cells, as compared, for example, with brain extract and other tissue extracts (Fig. 1a). Similar results were obtained with PC-3 cells (data not shown). These data demonstrate that bone contains significant migration and chemoinvasion promoting factor(s) for prostate cancer cells.

CM-MSC derived from rat long bones also induced more than a 3-fold increase in the invasive ability of DU-145 cells, which was comparable with that induced by the bone extracts (Fig. 1a). These results show that MSCs also produce migration and invasion-enhancing factor(s) for prostate cancer cells.

The chemotactant activity of the bone and of other tissue extracts was further examined, using PC3 prostate cancer cells and several other human prostate epithelial cell lines, as well as a HT1080 fibrosarcoma cell line and a B16-F10 mouse melanoma cell. The prostate cell lines include two immortalized, nontumorigenic cell lines (PWR-1E and RWPE-1) and five tumorigenic cell lines (RWPE-2, WPE1-NA22, WPE1-NB11, WPE1-NB14, and WPE1-NB26), showing different tumorigenicity and invasive ability (Ref. 13, 14 and unpublished data). All of these cell lines are derived from PWR-1E, and they are tumorigenic and androgen receptor-positive and prostate-specific antigen-positive. For all of the prostate cell lines, bone extract was the most potent chemotactant, and it induced a 2- to 4-fold increase in the invasive ability of DU-145 cells, which was significantly greater than that of other tissue extracts (Student’s t test, P < 0.0007). Effects of four different bone extracts (0.05 μg/ml medium) on the growth of DU-145 cells after a 4-day treatment: m, RPMI 1640 only, negative control; m-b, mouse long bone; h-b, human knee bone; h-h, human calcified bone; bm, human bone marrow.

**Table 1. A comparison of the chemotactant activity of different tissue extracts for human prostate epithelial cell lines.** Numbers represent fold increase relative to the negative control (RPMI-1640).

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Bone</th>
<th>Brain</th>
<th>Liver</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
</thead>
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<tr>
<td>Tumorigenic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU-145</td>
<td>1.9</td>
<td>1.1</td>
<td>0.6</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>PC-3</td>
<td>1.9</td>
<td>0.5</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>WPE1-NB26</td>
<td>1.9</td>
<td>1.6</td>
<td>0.1</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>RWPE-2</td>
<td>4.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>WPE1-NB11</td>
<td>2.2</td>
<td>1.3</td>
<td>0.2</td>
<td>2.0</td>
<td>1.5</td>
</tr>
<tr>
<td>J3WPE1-NB14</td>
<td>2.4</td>
<td>0.7</td>
<td>0.6</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>WPE1-NA22</td>
<td>2.9</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>1.5</td>
</tr>
<tr>
<td>HT 1080</td>
<td>1.6</td>
<td>2.1</td>
<td>0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>B16-F10</td>
<td>2.5</td>
<td>6.0</td>
<td>2.3</td>
<td>2.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Nontumorigenic:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PWR-1E</td>
<td>2.0</td>
<td>1.0</td>
<td>0.5</td>
<td>2.0</td>
<td>0.9</td>
</tr>
<tr>
<td>RWPE-1</td>
<td>2.5</td>
<td>0.8</td>
<td>0.8</td>
<td>1.7</td>
<td>1.5</td>
</tr>
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</table>
increase in their cell migration (Table 1). Some of these seven cell lines also showed chemotaxis to the lung extract (Table 1). HT1080 cells were most strongly attracted by brain extract but bone extract also had some activity. B16-F10 cells showed a very high response to brain extract but migrated also to bone, liver, and lung extracts. These studies show that bone extracts are more potent chemoattractants for prostate cells than other tissue extracts and that other non-bone-metastasizing cells do not show this organ specificity in their invasive activity.

Cell Proliferation Is Not Enhanced by Bone Extracts. Different bone extracts from mouse and human sources were examined for DU-145 cell proliferation in vitro. No differences in growth were observed when compared with medium alone (Fig. 1b). The same was observed for purified osteonectin. Similar results were obtained using an in vivo approach. Nude mice were injected s.c. with PC-3 tumor cells mixed with Matrigel or collagen I and either bone or liver extract. No differences in tumor growth (data not shown) were observed among these groups. These data demonstrate that the chemoattractive activity of bone does not contain a proliferation-inducing activity for prostate cancer cells.

The Chemoattractive Factor Is Osteonectin. Bone extract and CM-MSC were used as sources for purifying the active factor. Ninety percent of the invasion and migration-promoting activity was recovered after a 60% ammonium sulfate cut. The proteins in this fraction were separated on a 10% tris-borate-EDTA-Urea gel and extracted with a gel eluter according to molecular weight. Each fraction was tested in a chemoinvasion assay. Fraction 4 contained the major activity (Fig. 2a). SDS-PAGE analysis revealed that a $M_r$ 33,000 band is the major component of the active fraction.

Table 2. Enhancement of invasion of bone metastasizing cells by osteonectin

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>b-ON/neg</th>
<th>p-ON/neg</th>
<th>OP/neg</th>
<th>BMP-4/neg</th>
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</thead>
<tbody>
<tr>
<td>DU-145</td>
<td>4.0</td>
<td>3.5</td>
<td>0.8</td>
<td>1.1</td>
</tr>
<tr>
<td>PC-3</td>
<td>4.0</td>
<td>3.5</td>
<td>1.8</td>
<td>1.5</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>6.0</td>
<td>4.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>B16-F10</td>
<td>0.3</td>
<td>1.1</td>
<td>3.2</td>
<td>2.2</td>
</tr>
<tr>
<td>HT1080</td>
<td>1.0</td>
<td>0.3</td>
<td>2.3</td>
<td>1.6</td>
</tr>
<tr>
<td>3T3 ras</td>
<td>1.0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$p$-ON, platelet osteonectin.
$OP$, osteopontin.
$BMP$, bone morphogenetic protein.
$neg$, negative.
protein copurified with this activity (Fig. 2b) and was maximally active at about 1 ng/μl.

The identity of the M, 33,000 protein was determined by MALDI-MS, in which a tryptic digest of the gel-eluted band yielded 48 monoisotopic peptide masses. Of these masses, 16 matched within 0.015% error to predicted tryptic peptides expected from rat and mouse osteonectin/SPARC/BM40. And secondly the identity was determined by protein sequencing after HPLC separation of the tryptic digest of the protein band in the gel. The first 14 amino acids of one of the HPLC peaks showed a 100% match with a similar fragment of the purified factor from bone and CM-MSC is slightly smaller than human platelet osteonectin, but both proteins have the same size core protein after deglycosylation and with the established sequence for osteonectin/SPARC/BM40. The first 14 amino acids of one of the HPLC peaks showed a 100% match with a similar fragment of parallel-treated commercial osteonectin and with the established sequence for osteonectin/SPARC/BM40. The purified factor from bone and CM-MSC is slightly smaller than human platelet osteonectin, but both proteins have the same size core protein after deglycosylation (Fig. 3a).

The M, 33,000 protein was tested for cross-reactivity with a monoclonal antibody against human osteonectin by Western blot analysis (Fig. 3b). It reacted with the antibody (Lanes 1 and 2), whereas nonactive bone-derived fractions (Lanes 3 and 4) did not. Human platelet-osteonectin and rat SPARC, respectively, were used as positive controls (Lanes 5 and 6).

The chemoattractant activity of the purified factor (b-ON) was blocked by a monoclonal antibody against osteonectin/SPARC/BM40 in the in vitro chemoinvasion assay using PC-3 cells (Fig. 3c). With 500 times more antibody than antigen, 80% of the invasive activity of b-ON could be blocked with the specific antibody. An almost complete blocking could be observed with 1000 times more antibody than antigen, whereas a control IgG1 antibody only decreased the activity by 25% (data not shown). These data demonstrate that the activity of the purified factor can be blocked by an anti-osteonectin antibody.

The specificity of b-ON (bone osteonectin), as well as of platelet osteonectin, osteopontin, and BMP-4, for promoting the invasion of bone-metastasizing tumor cells versus non-bone-metastasizing cells, was investigated by the in vitro invasion assay. The bone-metastasizing cell lines used were two prostate cancer cell lines, DU-145 and PC-3, and the breast cancer cell line MDA-MB-231. The non-bone-metastasizing cell lines consisted of B16-F10 mouse melanoma, HT1080 fibrosarcoma, and the ras-transfected NIH-3T3 cell lines. Both prostate cancer cell lines showed a 4-fold increase in invasion when b-ON was the chemoattractant (Table 2), as compared with the negative control (Fig. 2). A slightly smaller increase in invasion was observed when platelet-osteonectin was used as the chemoattractant. The breast cancer cell line showed a 6-fold increase in response to b-ON and a 4-fold increase in response to platelet-ON. Two other bone-derived proteins, osteopontin and BMP-4, showed only very low chemoattractive activity for both prostate and breast cancer cells. The non-bone-metastasizing B16-F10, HT-1080, and ras-transfected 3T3 cells showed no significant response to either form of osteonectin but showed some response to osteopontin and BMP-4.

**Osteonectin Enhances MMP Activity.** Because osteonectin increased invasion through basement membrane Matrigel, we determined whether osteonectin would regulate protease activity. When PC-3 or DU-145 human prostate carcinoma cells were exposed to osteonectin 10 μg/ml for 72 h, there was a marked increase in the MMP-2 activity (M, 72,000 type IV collagenase/gelatinase A proenzyme and M, 59,000 –68,000 active forms) in the CM (Fig. 4). In the breast cancer cell line MDA-MB-231, osteonectin also induced an increase in MMP-2 activity, whereas in the non-bone-metastasizing HT-1080 and B16-F10 cells, osteonectin did not induce any change in MMP activity. These results demonstrate that osteonectin is a specific inducer of collagenase activity in those cancer cells that preferentially metastasize to the bone.

**DISCUSSION**

Prostate cancer has a tendency to metastasize preferentially to the bone. To metastasize to specific organs, cancer cells in the circulatory system must be specifically induced to attach and extravasate at distant sites (18–22). In addition to the increased ability of cancer cells to attach to bone-derived endothelial cells (10, 11), it seems that the metastasis to the bone may also be influenced by chemoattractant factors produced in the bone. We have demonstrated that bone extracts are potent chemoattractants over extracts from other tissues (Table 1), and that they increase migration and invasion by prostate cancer cell lines (Fig. 1; Tables 2). These observations support the model in which factors, specifically released by the bone, attract prostate and breast cancer cells preferentially to that site. We have purified and identified a low glycosylated osteonectin, from bone, which acts as a chemoattractant and promotes invasion by bone-metastasizing prostate and breast carcinoma cells.

It is well known that MMP activity shows a correlation with the invasive and metastatic ability of cancer cells (23). It was, therefore, interesting to find that osteonectin specifically induced activation of MMPs, especially that of MMP-2, in bone-metastasizing human prostate and breast cancer cells (Fig. 4). We also want to note that there was no change in MMP activity in the non-bone-metastasizing HT1080 fibrosarcoma and B16-F10 mouse melanoma cells after incubation with osteonectin. Activation of MMP-2 by osteonectin in human breast cancer cells was also recently observed by Gilles et al. (24). These results suggest that osteonectin is a naturally occurring enhancer of MMP activity as well as of breast and prostate cell migration, and hence, a promoter of invasion for these cells.

Our results show that bone extracts are strong chemoattractants for prostate epithelial cells in general. This is demonstrated by the observation that bone extracts and osteonectin not only enhanced the invasion and migration by DU-145 and PC-3 cells, but they were also chemoattractants for five additional human prostate tumorigenic cell lines as well as two nontumorigenic cell lines (PWR-1E and RWPE-1). These results suggest that osteonectin may be a general chemoattractant for bone-metastasizing epithelial cells, such as those of prostate and breast origin. This is further supported by the observation that osteonectin did not affect the invasiveness or MMP activity of non-
bone-metastasizing HT1080 fibrosarcoma, B16-F10 melanoma, and ras-transfected 3T3 cell lines (Table 2; Fig. 4). It has been previously shown that bone and brain extracts enhance invasion by breast cancer cells through Matrigel, however, the specific chemotactant factor had not been identified (15).

Mass spectrometry analysis (MALDI-MS) and protein sequencing of the purified factor showed a match with osteonectin. Osteonectin was first purified from bovine bone as a major noncollagenous glycoprotein, and was subsequently shown to be identical to SPARC and BM40 (25–27). Osteonectin is expressed in a variety of human tissues (28–30) and tumors (31–32). It is involved in cell-matrix interactions and angiogenesis (32). It was recently reported that decreased SPARC expression suppresses the tumorigenicity of human melanoma cells in nude mice (33). SPARC also seems to increase MMP activity in monocytes and breast cancer cell lines (24, 34). These data suggest that osteonectin/SPARC/BM40 has a variety of biological activities.

We have isolated the low glycosylated form of osteonectin that seems to be highly abundant in bone and shows specific migration, MMP, and invasion-promoting activities for bone-metastasizing cancer cells. Western blot and MALDI-MS analyses showed that b-ON is a slightly smaller protein than platelet-osteonectin but it is more potent in its chemoattractant activity (0.8 ng/µl b-ON compared with 2 ng/µg platelet osteonectin). A heterogeneity in structure and function of osteonectin in terms of glycosylation has been described (35). Several forms of osteonectin have been identified, which suggests that a family of osteonectin-related proteins exists (36–38). A possible regulatory mechanism for prostate cancer metastasis to the bone via osteonectin could also depend on the amount and/or affinity of the cellular receptors for these proteins, about which nothing is known.

The observations reported in this paper have important implications in the discovery and development of agents that might be used in the treatment of prostate cancer. For example, the specificity of osteonectin-induced enhancement of invasion is supported by the observation that when the chemoattractant activity of bone-osteonectin was blocked with an osteonectin-specific antibody, a marked inhibition of invasion by DU-145 cells occurred (Fig. 3c). Generation of an antibody, which can specifically block prostate cancer cell metastasis to the bone, is under investigation. Such an antibody could provide a valuable tool for the treatment of prostate cancer and other bone-metastasizing cancers by blocking invasion and metastasis.

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