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Hyaluronan Synthase Elevation in Metastatic Prostate Carcinoma Cells Correlates with Hyaluronan Surface Retention, a Prerequisite for Rapid Adhesion to Bone Marrow Endothelial Cells

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Bone marrow is the primary site of metastasis in patients with advanced stage prostate cancer. Prostate carcinoma cells metastasizing to bone must initially adhere to endothelial cells in the bone marrow sinusoids. In this report, we have modeled that interaction in vitro using two bone marrow endothelial cell (BMEC) lines and four prostate adenocarcinoma cell lines to investigate the adhesion mechanism. Highly metastatic PC3 and PC3M-LN4 cells were found to adhere rapidly and specifically (70–90%) to BMEC-1 and tHBMEC bone marrow endothelial cells, but not to human umbilical vein endothelial cells (15–25%). Specific adhesion to BMEC-1 and tHBMEC was dependent upon the presence of a hyaluronan (HA) pericellular matrix assembled on the prostate carcinoma cells. DU145 and LNCaP cells were only weakly adherent and retained no cell surface HA. Maximal BMEC adhesion and HA encapsulation were associated with high levels of HA synthesis by the prostate carcinoma cells. Up-regulation of HA synthase isofoms Has2 and Has3 relative to levels expressed by normal prostate corresponded to elevated HA synthesis and avid BMEC adhesion. These results support a model in which tumor cells with up-regulated HA synthase expression assemble a cell surface hyaluronan matrix that promotes adhesion to bone marrow endothelial cells. This interaction could contribute to preferential bone metastasis by prostate carcinoma cells.

Remote metastases in prostate cancer often occur in bone marrow, suggesting tissue or endothelial cell-specific factors may contribute to prostate cancer metastasis to bone (2, 5).

Bone marrow endothelial cells (BMEC)1 maintain a specialized endothelium that must allow cell trafficking in and out of the bone marrow (3, 4, 6). In addition to regulating the egress of mature myeloid and lymphoid cells, BMEC selectively allow transmigration of progenitor cells from a circulating population, indicating that specific receptors regulate the movement of cells through the endothelium (7). Studies have shown that the transmigrating cells move directly through an endothelial cell in a process that involves specific adhesive interactions (Ref. 3 and references therein). BMEC constitutively express adhesion receptors such as VCAM-1 (8, 9), E-selectin (9), and P-selectin (10), which are not expressed in large vessel endothelia unless activated by cytokines (6). Specific adhesive interactions between hemopoietic cells and BMEC thought to be important for homing include endothelial lectins and progenitor glycoproteins (11–14); VCAM/VLA-4 (11–13), and CD44/hyaluronan (15). Initial adhesion may be mediated primarily through selectins and glycoproteins. BMEC-associated chemokines such as SDF-1 (stromal-derived factor) can then stimulate integrin ligation, leading to progenitor arrest (16, 17).

Because the bone marrow microvasculature presents the first site of interaction for circulating tumor cells metastasizing to the bone marrow, it is likely that mechanisms of metastasis may parallel those employed by homing progenitors. In fact, tumor cells have been shown to bind and transmigrate through BMEC (17–19). The adhesion molecules implicated in these processes, CD44/hyaluronan, VLA-4/VCAM, and LFA-1/ICAM, are also involved in homing and extravasation of circulating lymphocytes and progenitor cells.

Hyaluronan (HA) is a ubiquitous high molecular weight glycosaminoglycan polymer required for growth, development, cell motility, and cushioning of joints (20, 21). Elevated levels of HA are associated with various pathologies, such as arthritis, inflammation, and several cancers (22–24), including prostate (25, 26). Melanoma cells selected for high expression of HA were more metastatic when injected into nude mice than cells
that expressed low amounts of HA (27). Furthermore, overexpression of HA biosynthetic enzymes in tumor cell lines has been shown to increase tumorigenicity and metastatic potential (28, 29).

To investigate the molecular mechanism of initial adhesion to bone marrow endothelium, we modeled adhesion in vitro using the bone marrow endothelial cell lines BMEC-1 and trHBMEC and four prostate adenocarcinoma cell lines, PC3, PC3M-LN4, DU145, and LNCaP. Highly metastatic PC3 and PC3M-LN4 cells adhered rapidly to BMEC-1 but not to large vein endothelial cells (HUVEC). DU145 and LNCaP cells, in contrast, were poorly adhesive to endothelial cells. Maximal BMEC adhesion was inhibited by addition of excess exogenous hyaluronan, and by hyaluronidase digestion of pericellular HA, found assembled specifically on PC3 and PC3M-LN4 cells. Presence of pericellular HA was correlated with elevated levels of HA synthesis and expression of HA synthase. Our data relate HA synthase overexpression to metastatic potential of prostate tumor cells and represent the first report of such a correlation. Collectively, our results implicate tumor cell-associated HA and up-regulation of HA synthase in prostate cancer progression and may directly impact metastatic potential or preferential tissue colonization of individual tumor cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—PC3, DU145, and LNCaP human prostate adenocarcinoma cell lines were purchased from ATCC (Manassas, VA). PC3 and DU145 cells were maintained in MEM supplemented with 10% FBS, 1 mM sodium pyruvate and non-essential amino acids. LNCaP cells were cultured in RPMI containing 10% FBS. The PC3 derivative cell line, PC3M-LN4, was kindly provided by Dr. Isaiah J. Fidler (M. D. Anderson Hospital Cancer Center, Houston, TX), and was maintained in the media described above for PC3 cells. Prostate carcinoma cells were plated 2 days prior to experiments and used at 70% confluence. The BMEC-1 human bone marrow endothelial cell line was a gift from Dr. S. Rafii (Cornell University Medical Center, New York, NY) and were maintained in M199 containing 20% FBS (30). trHBMEC human bone marrow endothelial cells were a gift from Dr. Karin Schweitzer (Free University Hospital Amsterdam, The Netherlands) and were maintained in RPMI containing 10% FBS (11). HUVEC were purchased from Clonetics and cultured in endothelial cell growth medium, EGM-2, as recommended by the vendor. Human bone marrow stromal cells (31) were cultured and generously provided by Nisha Shah and Dr. Tucker LeBien (University of Minnesota, Minneapolis, MN). Aggrecan was prepared as previously described (32) from Swarm rat chondrosarcoma.

Cell Adhesion Assay—Subconfluent prostate carcinoma cells were plated 2 days prior to experiments and used at 70% confluence. The BMEC-1 human bone marrow endothelial cell line was a gift from Dr. S. Rafii (Cornell University Medical Center, New York, NY) and were maintained in M199 containing 20% FBS (30). trHBMEC human bone marrow endothelial cells were a gift from Dr. Karin Schweitzer (Free University Hospital Amsterdam, The Netherlands) and were maintained in RPMI containing 10% FBS (11). HUVEC were purchased from Clonetics and cultured in endothelial cell growth medium, EGM-2, as recommended by the vendor. Human bone marrow stromal cells (31) were cultured and generously provided by Nisha Shah and Dr. Tucker LeBien (University of Minnesota, Minneapolis, MN). Aggrecan was prepared as previously described (32) from Swarm rat chondrosarcoma.

Cell Adhesion Assay—Subconfluent prostate carcinoma cells were plated in adhesion medium containing the appropriate concentration of HA, and immediately added to the endothelial cell monolayers (30,000 cells/well).

Particle Exclusion Assay—Pericellular HA matrices were visualized as described previously (33). Briefly, prostate carcinoma cells cultured in 48-well plates overnight prior to the assay were treated for 25 min in the absence or presence of 16 units/ml Streptomyces hyaluronidase in phenol red-free MEM with 0.1% BSA at 37 °C. This medium was removed and cells were incubated 90 min with 2 mg/ml aggrecan in MEM/0.1% BSA at 37 °C. The aggrecan solution was removed and 1 x 10^6 glutaraldehyde-fixed sheep red blood cells (Accurate Chemical and Scientific Corp.) in PBS/1% BSA were added, allowed to settle for 15 min and then viewed with phase-contrast microscopy. The HA matrix was evidenced by halos surrounding the cells from which the fixed erythrocytes were excluded. Representative cells were photographed at 400x magnification. To quantify matrix retention, outlines of matrices and cellular boundaries from 20 individual cells of each type were traced and relative areas calculated using IMAGE software (National Institutes of Health). Relative matrix areas from similar tracings of each cell type following the following three experiments were subtracted, and the HA matrix thickness was reported as the ratio of matrix area to cell area for each cell type. A ratio of 1 indicates complete absence of pericellular clearing.

HA Synthesis Quantitation—The concentration of HA in cell culture supernatants was determined in a competitive binding assay (34). 96-well Immulon microtiter plates were coated with human umbilical cord HA at 25 μg/ml in 200 mM carbonate buffer (pH 9.6) for 4 h at 37 °C. Excess HA was removed with four washes of PBS/0.05% Tween 20. PC3M-LN4, DU145, and LNCaP cells were plated overnight in 12-well plates. 24-h conditioned culture media were harvested, and cell counts were determined by trypsin release and manual counting in a hemacytometer. Serial dilutions of cell culture supernatant (100 μl of total volume in PBS/Tween 20) were combined with 100 μl of a 1 μg/ml solution of biotinylated hyaluronic acid-binding protein (Seikagaku) and incubated in the HA precoated wells at room temperature overnight. The plate was washed 4x with PBS/Tween 20, developed using an avidin-biotin HRP system (Vector Laboratories ABC-HRP Kit PK-4000) and visualized with OPD (Sigma P8287) as substrate and read at 490 nm. HA concentration was interpolated from a standard curve generated by plotting HA standards against absorbance values. The mean HA concentration for each sample of culture supernatant was calculated, and the results were normalized to cell number. Data are presented as mass of HA (in μg) per 10^6 cells.

Determination of HA Synthase Expression—HA synthase isoforms and relative level of expression in prostate carcinoma cell lines was semi-quantitatively assayed by RT-PCR. Poly(A)^+ RNA was isolated from subconfluent PC3M-LN4, PC3, DU145, and LNCaP cell lines with the Oligotex mRNA isolation kit (Qiagen) and quantitated by Ribogreen fluorescence (Molecular Probes). Normal prostate poly(A)^+ RNA was purchased from CLONTECH. 25 ng of each mRNA template was reverse-transcribed with an oligo(dt) primer using the Superscript II first strand cDNA synthesis kit (Life Technologies, Inc.). PCR oligonucleotides specific for Has1, Has2, and Has3 messages were designed from the sequence data base; exact sequences are given in Table I along with relative positions in the reported sequences and expected product sizes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with each set of primers to standardize conditions using the AmpliTaq Gold kit (Perkin Elmer) as follows: 3 min at 95 °C; 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 30 s polymerization at 72 °C; 5 min final extension at 72 °C. 15 μl of each reaction was electrophoresed on a 3% agarose gel, stained with ethidium bromide, and digitally photographed. To determine relative expression levels, digital images were integrated using Molecular Analyst software, and band intensities were normalized to the corresponding GAPDH band. Levels are reported as the fold expression relative to that determined for normal prostate.
PC3M-LN4 Cells Adhere Rapidly and Specifically to BMEC-1 Bone Marrow Endothelial Cells—Prostate adenocarcinoma cells have been reported to adhere preferentially to bone marrow-derived microvascular endothelial cells relative to endothelial cells from a large vein source (HUVEC). This preference implies a specific intercellular recognition process dictated in part by heterogeneous expression of endothelial cell surface adhesion receptors. To investigate the molecular interactions underlying this process, we initially chose PC3M-LN4, a human prostate adenocarcinoma cell line derived from PC3 cells. This subline was clonally selected for enhanced metastatic propensity in mice and, in particular, was shown to be capable of metastasis to bone upon intracardial injection (35). We determined a time course for adhesion of PC3M-LN4 cells to BMEC-1 bone marrow endothelial cells and compared it with adhesion to HUVEC. Within 10 min, 70% of the cells were adherent on BMEC-1 relative to 16% on HUVEC (Fig. 1). After 30 min, nearly 100% of the cells adhered to BMEC-1 compared with only 25% on HUVEC. PC3M-LN4 cells, therefore, demonstrate preferential rapid adhesion to BMEC-1 bone marrow microvascular endothelial cells.

Hyaluronan Presented by the Prostate Carcinoma Cells Mediates Adhesion to BMEC-1—Several surface-borne adhesion molecules have been implicated in homing of circulating cells to the bone marrow, including VLA-4 (α4β1 integrin), LFA-1 (αβ2 integrin), CD44 proteoglycan, and the high molecular weight glycosaminoglycan hyaluronan (HA). Pretreatment of PC3M-LN4 cells with blocking antibodies directed against α4, β1, αL, or β2 integrin subunits, or against CD44, had no effect on adhesion of these cells to BMEC-1 (data not shown). To assess the relevance of HA to prostate carcinoma cell adhesion, we pretreated PC3M-LN4 or BMEC-1 cells individually or simultaneously with hyaluronidase (HAase) enzyme and assayed initial rapid adhesion at a 12-min time point. As above, ~70% of PC3M-LN4 cells were adherent at this time point in the absence of enzymatic digestion (Fig. 2). Pretreatment of BMEC-1 cells with HAase had no effect on this adhesion. However, treatment of PC3M-LN4 or both cell types reduced adhesion to about 35%, indicating that cell surface HA promotes this rapid intercellular interaction. Furthermore, the HA required for maximum adhesion is carried by the prostate carcinoma cells.

HA-mediated Adhesion of Prostate Carcinoma Cells Is Specific for Bone Marrow-derived Endothelial Cells—Because endothelial cell types of different origin exhibit differences in HA binding (36), we assayed the specificity of hyaluronidase-sensitive prostate tumor cell adhesion to endothelial cells. PC3M-LN4 cells were preincubated in the absence or presence of hyaluronidase and allowed to adhere to BMEC-1, HUVEC, or bone marrow-derived stromal cells (BMSC). Consistent with the above results, PC3M-LN4 cells adhered rapidly to BMEC-1 and adhesion was inhibited by 50% in the presence of HAase (Fig. 3). Cells adhered weakly to HUVEC, and adhesion was not inhibited by enzyme treatment. Although adhesion of PC3M-LN4 cells to BMSC was efficient and rapid, it was not inhibited by HAase digestion, suggesting cell surface HA was not mediating this adhesion. Collectively, these results imply that heterogeneous expression of HA receptors among endothelial cell types of different origin exhibit differences in HA binding (36).

### RESULTS

**TABLE I**

RTP-PCR primers

| Oligo sequences | Sequence position | Product length |
|----------------|------------------|---------------|
| HAS 1          |                  |               |
| Forward        | 5’ CTAGGATCCGTGCTTGACTCGGAC 3’ | 737 | 246 |
| Reverse        | 5’ GCTAAGCTCTTGAAGGACCGCTG | 963 |   |
| HAS 2          |                  |               |
| Forward        | 5’ GTATCGATTGTGGTTTACAATC 3’ | 1441 | 207 |
| Reverse        | 5’ GACCCATGTCAATTGTGTGC | 1648 |   |
| HAS 3          |                  |               |
| Forward        | 5’ GTGACGTTATTAGTGCGGCCCT 3’ | 839 | 414 |
| Reverse        | 5’ GTCGACAGTGAAGGACCG | 1253 |   |
| GAPDH          |                  |               |
| Forward        | 5’ TGAGGTCCGGAGTCACCGGATTG 3’ | 44  | 982 |
| Reverse        | 5’ CATGTTGGCCATGAGGTCACCAC | 1026 |   |

**FIG. 1.** Time course for adhesion of PC3M-LN4 prostate carcinoma cells to BMEC-1 and HUVEC. Calcein-AM-labeled PC3M-LN4 cells were added in a single cell suspension to confluent BMEC-1 (closed symbols) or HUVEC (open symbols) monolayers in a 48-well plate. At the indicated times, wells were washed to remove nonadherent cells, and adherent cells were lysed and quantified in a fluorescence plate reader. Results are presented as the mean percentage of input cells from triplicate wells ± standard error of the mean (S.E.).

**FIG. 2.** HA on the PC3M-LN4 cells is required for adhesion to BMEC-1. BMEC-1 monolayers and/or calcein-AM-labeled PC3M-LN4 cell suspensions were treated with 16 units/ml hyaluronidase for 25 min. The hyaluronidase was removed, and the PC3M-LN4 cells were added to the BMEC-1 for 12 min at 37 °C. Nonadherent cells were removed by washing, and adherent cells were quantified in a fluorescence plate reader. Each bar represents the mean of triplicate wells assayed ± S.E., reported as percentage of input cells.
Enzymatic removal of HA inhibits PC3M-LN4 adhesion to BMEC-1 but not HUVEC or BMSC. Calcein-AM-labeled PC3M-LN4 suspensions were pretreated for 25 min at 37 °C in the absence (solid bars) or presence (open bars) of 16 units/ml Streptomyces hyaluronidase. The cells were diluted 5-fold with adhesion medium and added to BMEC-1, HUVEC, or BMSC monolayers in a 48-well plate for 12 min at 37 °C. Nonadherent cells were removed by washing, and adherent cells were lysed and quantified in a fluorescence plate reader. Each bar represents the mean ± S.E. of quadruplicate wells assayed, reported as a percentage of input cells. Each assay was repeated three times.

Rapid, Specific Prostate Carcinoma Cell Adhesion to Bone Marrow Endothelial Cells Is Differentially Sensitive to Hyaluronidase and Exogenous Hyaluronan—To examine whether HA would generally promote adhesion of prostate carcinoma cell lines to bone marrow endothelial cells, we measured HAase-sensitive BMEC-1 adhesion of the commercially available cell lines PC3, DU145, and LNCaP. As demonstrated above with PC3M-LN4 cells, about 60–70% of PC3 cells adhered to BMEC-1 monolayers and adhesion was inhibited 40–50% by HAase treatment (Fig. 4A). In contrast, DU145 and LNCaP adhered very poorly (about 25%), and the low level of adhesion was not altered by HAase treatment. None of the cell lines adhered well to HUVEC, and adhesion to HUVEC was not sensitive to HAase (Fig. 4A, inset). These results were replicated in similar experiments using another human bone marrow endothelial cell line, trHBMEC. As presented in Fig. 4B, PC3 and PC3M-LN4 cells exhibited comparable levels of HAase-sensitive adhesion to trHBMEC, whereas very few DU145 or LNCaP cells were adherent, and their adhesion was unaffected by HAase treatment. HA, therefore, is required for maximal rapid interaction between prostate carcinoma cells and bone marrow endothelial cells.

To further establish the requirement for direct HA recognition by the BMEC as a component of preferential adhesion to prostate carcinoma cells, we pretreated trHBMEC monolayers with increasing concentrations of exogenous high molecular weight HA. PC3M-LN4 cells were incubated with or without HAase, which was then removed. Cells were resuspended in the respective concentrations of HA and added to the BMEC. As before, about 80% of the cells were adherent after 12 min, and adhesion was reduced to 35% by HAase digestion (Fig. 5). Preincubation of the BMEC with 10 μg/ml of HA modestly increased adhesion of untreated cells, but rather strikingly increased adhesion of HAase-treated cells to about 60%. However, a dose-dependent inhibition was observed at higher concentrations, with adhesion almost completely inhibited at 500 μg/ml HA. This effect was more greatly manifested in HAase-treated cells, suggesting the residual adhesion observed for these cells may be due to incompletely digested HA or HA resynthesis during the time of the assay. When DU145 cells were similarly incubated with pretreated trHBMEC (Fig. 5, inset graph), no effect of exogenous HA was observed. This confirms that the inhibitory effect of HA is not occurring indirectly by destabilizing the BMEC monolayer, and that other receptor interactions are promoting the less rapid adhesion of DU145 cells to BMEC.

Prostate Carcinoma Cell Surface HA Retention Correlates to BMEC Adhesion—If HA was mediating the differential BMEC adhesion observed among the various prostate carcinoma cell lines, then these differences should be manifested in varied levels of HA retained on the surface of prostate carcinoma cells. Therefore, we used a particle exclusion assay to visualize the cell surface HA. In these experiments, surface-associated HA was first amplified by addition of aggrecan, a large multivalent proteoglycan that specifically associates with HA at the cell surface, surrounding the cell with a highly hydrated gel-like envelope. HA is thereby detected as a pericellular clear zone upon addition of fixed red blood cells, which cannot settle directly at the cell surface. Cells that lack HA do not exhibit these cleared halos, and the blood cell particles contact the cell perimeter. PC3M-LN4 cells were surrounded by a large matrix...
Prostate Carcinoma Cell HA Synthesis Correlates to Presence of a Pericellular HA Matrix and Adhesion to BMEC—Elevated levels of HA have been reported to correlate with progression of several tumor types, including prostate. Furthermore, high levels of HA synthesis are necessary and sufficient for production of a pericellular HA coat. To address the possibility that the prostate carcinoma cells used in our study could be synthesizing large amounts of HA but differentially retaining it at the cell surface, we quantitated the HA synthesized by each cell line. HA synthesis occurs at the plasma membrane, concurrent with extrusion of HA from the cell, such that the majority of cellular HA is shed into the culture medium. Overnight culture media from each cell type were accordingly analyzed for HA content by a competitive binding assay and HA level was normalized to cell count (Fig. 7). PC3 and PC3M-LN4 cell culture supernatants were found to contain high levels of HA (~5 and 6 μg/10⁶ cells, respectively, Table II) whereas DU145 culture medium had very little (1 μg/10⁶ cells), and levels in LNCaP culture were virtually undetectable. These results are consistent with high levels of HA synthesis contributing to cell surface HA retention, promoting adhesion to bone marrow endothelial cells.

Elevated Levels of HA Synthase Expression by Prostate Carcinoma Cells Correlate to HA Production and Adhesion to BMEC—HA synthesis is catalyzed by one or more isoforms of three homologous HA synthase enzymes: Has1, Has2, and Has3. We used RT-PCR to determine initially which isoform(s) were expressed by each prostate carcinoma cell line, in an attempt to correlate elevated HA synthesis and BMEC adhesion with presence of a specific message. We were unable to detect Has1 message in any cell line using any set of oligonucleotide primers, although we could amplify a product from a Has1 cDNA control plasmid (data not shown). However, expression of Has2 and Has3 was detectable in most of the cell lines. Because the presence or absence of message for a particular isoform did not appear to correlate with either HA matrix formation or BMEC adhesion, we developed a semi-quantitative approach to look at relative message levels. Equal amounts of input mRNA from normal prostate (CLONTECH) and each of the prostate carcinoma cell lines were reverse-transcribed and used as templates for PCR amplification of Has2 (Fig. 8A, lanes 8–14) or Has3 (Fig. 8B, lanes 8–14), concurrent with a GAPDH housekeeping control message (Fig. 8, lanes 2–6). Product yields were normalized to GAPDH and presented as fold expression relative to normal prostate in Table II.

Results of this assay showed that PC3 (lanes 4 and 10) and PC3M-LN4 cells (lanes 3 and 9) expressed higher levels of both Has2 and Has3 than DU145 (lanes 5 and 11), or LNCaP (lanes 6 and 12), consistent with higher levels of HA synthesis and rapid adhesion to BMEC by those cell lines. Has2 was virtually absent in DU145 cells and undetectable in normal prostate (lanes 2 and 8) or LNCaP cells. Has3 expression was dramatically increased in the most highly metastatic line, PC3M-LN4, followed by PC3 and DU145. Has3 expression was very low in normal prostate or LNCaP cells. Although DU145 cells appeared to express significant levels of Has3, this cell line was found to be a heterogeneous population in which about 2–10% carry surface associated HA (in contrast to PC3 and PC3M-LN4, in which >99% of the cells carry abundant surface HA). This was visualized by phase contrast microscopy at the level of individual cells using biotinylated HA binding protein, followed by streptavidin-HRP detection with diaminobenzidine precipitation (data not shown). The apparent Has3 cDNA level amplified from this cell line may be anomalously high due to those specific cells and not representative of the overall phenotype of the cell line, which probably expresses lower message levels. Collectively, these results demonstrate that HA synthesis correlates very well to HA synthase expression level, which in...
Relative to cell number in the original culture. Bound HA binding protein was detected, following extensive washes, supernatants were harvested and cells were trypsin-released and overnight then given fresh culture medium. After an additional 24 h, equal numbers of prostate carcinoma cells were seeded corresponding ratio for HAase treated cells was subtracted. A value of 1 denotes a cell completely lacking pericellular matrix.

TABLE II

|                  | PC3M-LN4 | PC3      | DU145     | LNCaP     |
|------------------|----------|----------|-----------|-----------|
| HA coat          | 1.94 ± 0.03 | 1.89 ± 0.04 | 1.02 ± 0.02 | 1.01 ± 0.01 |
| HA synthesis     | 6.38 ± 1.01 | 4.74 ± 0.61 | 1.19 ± 0.41 | 0.64 ± 0.21 |
| HAS expression   |          |          |           |           |
| Has2             | 2        | 6        | 0.2       | 0.1       |
| Has3             | 20       | 17       | 10        | 5.5       |
| BMEC adhesion    | 71 ± 2   | 65 ± 8   | 24 ± 2    | 28 ± 3    |
| Metastatic potential | High    | High     | Moderate  | Low/none  |

a Pericellular clear zones and cell perimeters of 20 individual cells were traced in NIH IMAGE, the relative ratio was calculated, and the corresponding ratio for HAase treated cells was subtracted. A value of 1 denotes a cell completely lacking pericellular matrix.

b Values presented are those plotted in Fig. 7 (in micrograms per 10^6 cells).

c Derived from Fig. 8. Data are shown as -fold expression relative to levels in normal prostate, normalized to GAPDH.

d Presented as percentage of input cells. The value for PC3M-LN4 cells is extracted from Fig. 2, and the other values from Fig. 4A.

e Cited in “Discussion.”

**FIG. 7.** HA synthesis and secretion by prostate carcinoma cells in culture. Equal numbers of prostate carcinoma cells were seeded overnight then given fresh culture medium. After an additional 24 h, supernatants were harvested and cells were trypsin-released and counted. HA-containing culture media were serially diluted, incubated with biotinylated HA binding protein, and applied to HA-coated plates. Bound HA binding protein was detected, following extensive washes, with streptavidin-HRP conjugate and OPD substrate, and quantified spectrophotometrically. Total HA in the culture media was determined by interpolation from a concurrent HA standard curve and plotted relative to cell number in the original culture.

**DISCUSSION**

Bone metastasis is an eventuality of advanced stage prostate cancer that results in severely reduced quality of life and ultimate morbidity. Metastasis is preceded by initial adhesion of circulating tumor cells to endothelial cells lining the vasculature of the secondary site. Because prostate carcinoma metastasizes to bone, we modeled this initial event using prostate carcinoma cell lines and the transformed bone marrow sinusoidal endothelial cell lines, t_rHBMEC and BMEC-1. In this study we demonstrate that highly metastatic prostate carcinoma cells adhere to bone marrow endothelial cells through pericellular hyaluronan (HA). Unlike reports of other model system interactions requiring this molecule, we find that the prostate carcinoma cells present the HA recognized by the BMEC. The HA-mediated adhesion shows endothelial source specificity, because PC3M-LN4 cells do not adhere rapidly to HUVEC, another endothelial cell type. Furthermore, HA-mediated adhesion exhibits specificity among bone-derived cell types: Although adhesion of PC3M-LN4 cells to bone marrow stromal cells is rapid, HA does not appear to be involved, suggesting other receptor interactions contribute to this process. Up-regulation of HA synthase in prostate tumor cells may promote bone marrow metastasis by specifically arresting those cells on the bone endothelium.

The bone marrow microvasculature is a specialized network of venules and fenestrated sinusoids permeable to low molecular weight fluorophores but impenetrable by larger macromolecular conjugates and cellular bodies (10). However, the bone marrow as the site of hemopoiesis must be capable of progenitor cell flux across its protective endothelial layer, a function not required or desired in other types of endothelium. Endothelial cell types exhibit heterogeneity in cytokine response (11, 37), receptor expression (9, 11, 36), and signaling pathways (38). In the absence of specific stimuli, endothelial heterogeneity alone is able to influence homing of circulating progenitor cells to the bone marrow through differential expression of selectins (10, 39, 40), glycoproteins (14), and VCAM-1 and CD44 (15). Receptor expression and adhesion of circulating leukocytes to sites of inflammation is further regulated through endothelial activation by inflammatory cytokines (41, 42). These cell surface differences may translate into preferential adhesion of circulating tumor cells to endothelial cells in specific tissues.

Transformed BMEC lines recently developed have facilitated exploration of the mechanisms by which endothelial adhesion receptors may dictate tumor preference for specialized endothelia. Results presented in this study demonstrate that prostate cancer cells adhere rapidly to bone marrow but not large vein endothelial cells. This is in agreement with observations by other investigators that prostate adenocarcinoma cell lines...
adhere preferentially to cell cultures enriched for BMEC over components of the bone marrow or hepatic endothelial cells (2). In another study, adhesion of prostate carcinoma cells to a cell line immortalized from isolated BMEC was shown to be inhibited by preincubation of BMEC with monoclonal anti-LFA-1 antibodies or polyclonal anti-galectin-3 (43), but it was unclear which cell type expressed LFA-1 because neither has been previously reported to do so. We have used two transformed bone marrow endothelial cell lines, BMEC-1 (30) and trHBMEC (11), which express the same cell adhesion molecules and synthesize the same cytokines as the primary endothelial cells with minor differences in level of expression, to determine that HA on the prostate tumor cells mediates adhesion to BMEC. However, this does not exclude the possibility of other interactions. We expect that, like progenitor arrest, adhesion and migration of prostate tumor cells on BMEC involves multiple adhesive interactions that may or may not be interdependent.

HA is a ubiquitous polysaccharide component of extracellular and cell-associated matrices (44, 45), essential for growth and motility. HA is required for normal ductal branching in the developing prostate gland (46), underscoring its vital role in cell migration (47). In some cell types, this requirement entails assembly of an HA pericellular matrix for proliferation and migration (33, 48). Cell-associated HA may facilitate growth and motility by stimulating detachment of rounded, dividing cells and the trailing edges of migrating cells, respectively. HA exhibits further functional complexity as an adhesion molecule involved in recruitment of circulating lymphocytes to inflamed tissues through the action of cell surface HA receptors (38). The structure of HA, consisting of many thousand repetitions of a disaccharide motif, is well-suited to serve as a multivalent ligand for coordinate binding by many simultaneous adhesion receptors or for providing a scaffold for cellular movement.

Cellular behaviors contributing to cancer progression include unrestricted growth, motility, and ability to circulate and colonize new tissues. Because HA is a normal component of such processes, it is not surprising that elevated levels correlate with cancer progression (21, 49, 50). High levels of serum HA, for example, correlated with disseminated carcinoma in general (51, 52) and, specifically, with tumor progression to metastatic disease in malignant lymphoma (53) and breast carcinoma (54). In human breast carcinoma, HA is more concentrated in areas where the tumor is invading into the surrounding tissue (22), and elevated stromal and cell-associated HA correlates with malignancy (55). Elevated stromal and epithelial HA are also indicative of poor survival rate in patients with ovarian and colorectal cancers (23, 24). In animal models, tumor cells with high levels of HA expression were more metastatic than cells expressing lower levels of HA (27, 29, 56, 57). Interestingly, both high and low HA-expressing cells have the same growth rate in vitro and at the primary injection site (27). This correlation of HA with metastasis but not growth rate suggests that HA may be more critical to endothelial adhesion and/or the infiltration of the cells into tissues.

PC3M-LN4 cell adhesion to BMEC in vitro was both enhanced and inhibited by the addition of high molecular weight HA. At low concentrations comparable to those secreted into the culture medium of the cells during growth, adhesion of hyaluronidase-treated tumor cells to BMEC was significantly enhanced by HA preincubation. HA prebinding by the BMEC may restore adhesion by replacing the cross-bridging ligand normally presented by the prostate tumor cells via its own cell surface HA receptors. When BMEC were precoated with higher levels of HA, PC3M-LN4 cell adhesion was almost entirely precluded, regardless of HAase treatment. This suggests that BMEC and prostate HA receptors have been saturated and are no longer able to cross-link. HA has been previously reported to enhance/inhibit intercellular adhesion in this fashion in development of chick limb buds (58). Rapid intercellular adhesion was synergistically inhibited by HAase treatment and high exogenous HA, suggesting the incomplete HAase effect is probably due to HA reassembly during the assay. By contrast, DU145 cell adhesion was not affected by addition of HA at any concentration, and therefore, these cells most likely lack active cell surface HA binding proteins.

PC3 (59) and PC3M-LN4 (35) prostate carcinoma cells are highly metastatic in mouse models and are shown in this report to produce a dense pericellular HA matrix that mediates adhesion to bone marrow endothelial cells. DU145 (60) and LNCaP (61) cell lines, by contrast, are poorly metastatic in mice, produce very little HA, and do not adhere well to BMEC. It is worth noting the origins and characteristics of the four cell types: PC3 is from a human bone metastasis; its derivative, PC3M-LN4, metastasizes to mouse bone; DU145 and LNCaP are from a human brain and a human lymph node metastasis, respectively, and their interaction with bone has never been documented. The correlation between high metastatic potential as reported in the literature, up-regulated HA synthesis and expression of HA biosynthetic enzymes is summarized in Table II. This correlation is consistent with a putative role for HA as a component of prostate cancer metastasis. In fact, HA overproduction is thought to be directly involved in prostate cancer progression. Histological sections of normal adult prostate tissue demonstrate the presence of HA in the prostate stroma (25, 46). In cancerous human prostates, HA expression levels are increased on the carcinoma cells and correspond to dedifferentiation of the cancer (25, 26).

Synthesis and secretion of HA is catalyzed in vertebrates by a family of three HA synthases: Has1 (62), Has2 (63), and Has3 (64), each of which is capable of conferring HA synthesis and pericellular HA retention to transfected cells (for a review of HA synthases, see Ref. 65). HAS expression is ubiquitous, but isoforms exhibit temporal and tissue-specific distribution. Targeted disruption of the has2 gene is an embryonic lethal mutation in mice, which fail to produce HA essential for pericellular endothelial cell migration and endothelial/mesenchymal transformation during cardiac development (66). Has2 is also specifically up-regulated in response to wounding in a mesothelial cell model (67). Because HAS expression is critical during periods of normal tissue remodeling, understanding its dysregulation in tumors may be important in controlling tumor growth and metastasis. HAS expression is regulated by glucocorticoids (68), growth factors such as platelet-derived growth factor (69), transforming growth factor β1 (70), and pro-inflammatory cytokines (71). Expression of HAS appears to correlate directly to HA synthesis (69), suggesting regulation occurs at the level of transcription. To date, there is no evidence for post-transcriptional mechanisms. Elevated HA in tumor cells is, therefore, probably a reflection of HAS gene expression. In support of this, we have determined that Has2 and Has3 are strongly up-regulated in highly metastatic prostate tumor cells. HAS up-regulation in prostate cancer progression may be dictated in part by factors such as those described above, produced and secreted by prostate stromal or epithelial cells.

HA synthase enzymes have been implicated in tumorigenesis and metastasis in mouse models. Overexpression of Has2 in fibrosarcoma cells yields significantly larger subcutaneous tumors (28). Mammary carcinoma cells transfected with has1 were more metastatic than control cells (29). In a melanoma model, tumor cells selected for high cell surface expression of HA were highly tumorigenic and metastatic, whereas tumor
cells bearing little or no surface HA, although equally tumorigenic, did not metastasize (27). In the latter model, however, has isoform expression was not characterized. Our data present the first characterization of has expression in prostate carcinoma cells and reveal a possible correlation of Has3 overexpression with tumor cell metastatic potential. Collectively, these results suggest involvement of HA in tumor growth and metastasis, and imply that specific HA synthase isoforms and/or expression levels of those isoforms may mediate these processes.

Both Has2 and Has3 are capable of synthesizing HA with an average molecular mass of 1–2 million Da (72), the average size of the exogenous HA used to enhance/inhibit adhesion (Fig. 5). This would suggest that the products of both enzymes are capable of supporting intercellular adhesion. DU145 cells, however, appear to express elevated Has3 but synthesize little HA and retain no matrix. One possible explanation may be that Has3 message is transcribed but not translated in these cells or that the protein made is inactive. Alternatively, there may be a requisite maximum threshold of HAS expression for maintenance of a pericellular matrix. Levels of Has3 expression sufficient to promote matrix retention may occur in only a subset of DU145 cells, with the remaining cells expressing it at lower levels. This may also be the case for Has2 expression. If production of an HA matrix enhances arrest in the bone marrow sinusoids, the cell population would have significantly diminished propensity to do so, relative to PC3 or PC3M-LN4 cells, which could then translate to reduced bone metastatic proclivity. Both enzymes are up-regulated in highly metastatic cells, which may implicate HAS up-regulation in prostate cancer progression. Has3 overexpression most consistently corresponds to aggressive potential, but intrinsic heterogeneity within the cell lines renders assignment of such a correlation premature.

Another factor to consider is that DU145 cells may lack surface receptors to anchor the matrix, because exogenous HA could restore adhesion of HAase-inhibited PC3M-LN4 cells, which are normally able to maintain an HA coat, but not enhance adhesion of DU145 cells. It is important to recognize that, although HA produced by prostate tumor cells may facilitate metastasis to bone marrow by initially attaching to the endothelium in the bone marrow, changes in matrix-associated HA binding proteins could also modify prostate tumor cell behavior. Because HA is secreted as a free glycosaminoglycan and is not attached to a core protein, its retention at the cell surface is achieved through accessory proteins such as versican, which contribute to the matrix, specifically binding and cross-linking the multivalent HA into a dense network (33, 48). Elevated levels of versican are associated with prostate carcinoma progression, but it is not known if its HA binding properties are responsible (73).

However, in addition to the HA binding proteins in the extracellular matrix, HA is specifically recognized by a widely expressed transmembrane receptor, CD44. Bone marrow and umbilical vein endothelial cells BMEC-1, trHBMEC, and HUVEC express cell surface CD44 but exhibit different affinity for PC3M-LN4 cells. Preincubation of BMEC-1 cells with various reported anti-CD44 blocking monoclonal antibodies failed for PC3M-LN4 cells. Preincubation of BMEC-1 cells with various reported anti-CD44 blocking monoclonal antibodies failed to impact prostate tumor cell interaction (data not shown). Nonetheless, regulation of CD44 activation state with respect to HA binding occurs on many levels and probably contributes to endothelial adhesive preference. CD44 and HA interactions may be important for metastasis. CD44-mediated migration and invasion of glioma cell lines is stimulated by HA (74) and inhibiting CD44/HA interactions in vivo inhibits metastasis (75, 76). HA clusters CD44 resulting in stimulation of signal transduction pathways and engagement of adhesion molecules such as integrins (77), which could either strengthen adhesion or lead to transmigration of the prostate cells through the endothelium.

Alternatively, HA binding proteins on the surface of BMEC-1 other than CD44 may contribute to rapid adhesion. One candidate protein is the receptor for HA-mediated motility (RHAMM), which has not been reported on BMEC surfaces but has been shown to mediate differential HA binding by endothelial cells of different vascular origin (36). It is therefore possible that differences in vascular endothelial RHAMM expression could be promoting the recognition of prostate carcinoma cells bearing surface-associated HA. Other recently described cell surface HA receptors include the lymph vessel endothelial-specific LYVE-1 (78), and the HA receptor for endocytosis thought to be specific for clearance of circulating HA through the liver and spleen (79).

In this report, we have surveyed established prostate carcinoma cell lines for common molecular interactions governing preferential adhesion to bone marrow endothelial cells and discovered a correlation between metastatic potential and elevated HA synthase. We have delineated a mechanism in which prostate cancer cells adhere to bone marrow endothelial cells via tumor cell-associated HA. It will be important to extend these findings to establish whether this adhesive interaction contributes to prostate cancer metastasis. With this goal, we are currently manipulating HA levels on prostate carcinoma cells in HA synthase transfectants, which should enable us to determine if HA changes the metastatic potential of prostate carcinoma cells in an animal model and whether a specific HA synthase isoform is responsible. Furthermore, this approach will allow us to study the impact of HA overproduction on activation of adhesion receptors and signal transduction pathways in HA-mediated adhesion of prostate carcinoma cells to bone marrow endothelium.

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