Rolling of Human Bone-Metastatic Prostate Tumor Cells on Human Bone Marrow Endothelium under Shear Flow Is Mediated by E-Selectin

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ABSTRACT

Prostate tumor cells preferentially adhere to bone marrow endothelial cells (BMECs) compared with endothelial linings from other tissue microvessels, implicating the importance of BMEC adhesion in the predilection of prostate tumor metastasis to bone. E (endothelial)-selectin, which functions as an initiator of leukocyte adhesion to target tissue endothelium, is constitutively expressed on BMECs, suggesting that prostate tumor cells could use this adhesive mechanism to initiate their migration into bone. In this report, we demonstrate for the first time that human bone-metastatic prostate tumor cells roll on human BMECs under physiological flow conditions. We show that these dynamic adhesive interactions are dependent on the expression of BMEC E-selectin and sialylated glycoconjugates on bone-metastatic prostate tumor cells. We also establish the importance of both glycoprotein(s) and glycosphingolipid structures displaying sialyl Lewis X epitopes as potential E-selectin ligands on bone-metastatic prostate tumor cells. Coexpression of sialylated glycoproteins and glycolipids on bone-metastatic prostate tumor cells triggers robust E-selectin binding activity, which is identical to that observed on human hematopoietic progenitor cells. By Western blot analysis, we identify candidate E-selectin glycoprotein ligands; distinct sialyl Lewis X (or HECA-452 antigen)-bearing membrane proteins were resolved at M̄₁, 130,000 and M̄₂, 220,000 as well as others ranging from M̄₁, 100,000 to M̄₂, 220,000. Immunohistochemical analysis of HECA-452 antigen expression on normal prostate tissue and on low- and high-grade prostate adenocarcinoma shows that HECA-452 antigen expression is directly associated with prostate tumor progression and may indicate acquisition of E-selectin ligand expression. These findings provide novel insight into potential adhesive mechanisms promoting hematogenous dissemination of prostate tumor cells into bone.

INTRODUCTION

Although it is well known that human prostate tumor cells have a propensity to metastasize to bone, the mechanisms by which prostate tumor cells metastasize to bone are poorly understood (1). The predilection of prostate cancer to metastasize to bone suggests that these cellular processes are critically dependent on factors present in the bone marrow (BM) microenvironment (2, 3). Several reports have recently shown that human prostate tumor cells preferentially adhere to human BM endothelial cells (BMECs) in comparison with adhesion to human umbilical vein endothelial cells (HUVECs) (4–6), human stromal cells (5), lung endothelium (5), human aortic endothelium (7), human dermal microvascular endothelium (7), and human BM-derived extracellular matrix components (7). These studies and others show that human prostate tumor cell adherence to human BMECs under static conditions is mediated, in part, by tumor cell β₁ and β₂ integrins (4, 5, 8, 9), T-antigen (Galβ1,3GalNAc)-bearing surface carbohydrates (4, 10), and surface hyaluronan (6). More recently, results from in vivo studies and from in vitro flow chamber experiments demonstrate that homo- and heterotypic attachment of human brain-metastatic prostate DU-145 tumor cells to vascular endothelium is also largely dependent on T-antigen–gαlectin-3 interactions (11, 12). On activation by exposure to tumor cells or to de-sialylated glycoconjugates, endothelial cells mobilize gαlectin-3 to the cell surface, which enhances binding interactions to tumor cell T-antigen (12). Whereas these molecular interactions may play a role in the attachment and intravascular aggregation of brain-metastatic prostate tumor cells to dura mater vascular endothelium, the identity of adhesion molecules on human bone-metastatic prostate tumor cells that mediate the initial tethering and rolling interactions on BMECs under physiological shear stress is currently unknown.

E-selectin, which is constitutively expressed on postcapillary venules in BMECs (13), mediates tethering and rolling interactions through E-selectin ligands on hematopoietic progenitor cells (HPCs) and is critical for HPC entry into bone (14–16). We hypothesized, therefore, that E-selectin ligands on human bone-metastatic prostate tumor cells help initiate binding interactions with BMECs. In this report, we provide novel evidence of human bone-metastatic prostate tumor cell rolling on human BMECs under physiological shear stress and show that these binding interactions are dependent on BMEC E-selectin expression and previously unrecognized E-selectin ligand activities on human bone-metastatic prostate tumor cells. These E-selectin ligand activities are attributable to sialylated glycoprotein(s) and/or sialylated glycosphingolipids. On bone-metastatic prostate tumor cells exhibiting high E-selectin ligand activity, we also identify potential E-selectin glycoprotein ligand(s), which are reactive to the anti-sialyl Lewis X monoclonal antibody (moAb) HECA-452 and resolve at molecular weights ranging from 100,000 to 220,000 on reducing SDS-PAGE gels. In addition, we show that expression of HECA-452 antigen is notably deficient on normal prostate tissue and increasingly elevated from low- to high-grade prostate adenocarcinoma, indicating a potential relationship between prostate tumor progression and acquisition of E-selectin ligand expression or aggressive metastatic behavior. These results support a new mechanistic view on how circulating prostate tumor cells may initiate adhesive contact with human BMECs.

MATERIALS AND METHODS

Cells and Antibodies (Abs). Human hematopoietic KG1a cells (American Type Culture Collection, Manassas, VA) were propagated in RPMI 1640 with glutamine, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (all from Gibco/Invitrogen Corp., Grand Island, NY). Human prostate tumor MDA-PCa 2b cells derived from bone metastases (17) were maintained in BRFF-HPCI (AthenaES, Baltimore, MD), 20% FBS, and 1% penicillin/streptomycin. Other human bone-metastatic prostate tumor cell lines, PC-3, PC-3M (a metastatic variant of PC-3; Ref. 18), PC-3M Pro-4 (a highly tumorigenic variant of PC-3M; Ref. 19), and PC-3M LN-4 (a highly metastatic variant of PC-3M; Ref. 20) all from Dr. Curtis Pettaway (M. D. Anderson Cancer Center, Houston, TX), were maintained in RPMI 1640 with glutamine, 10% FBS, and 1% penicillin/streptomycin. Other human bone-metastatic prostate tumor cell lines, PC-3, PC-3M (a metastatic variant of PC-3; Ref. 18), PC-3M Pro-4 (a highly tumorigenic variant of PC-3M; Ref. 19), and PC-3M LN-4 (a highly metastatic variant of PC-3M; Ref. 20); all from Dr. Curtis Pettaway (M. D. Anderson Cancer Center, Houston, TX), were maintained in RPMI 1640 with glutamine, 10% FBS, and 1% penicillin/streptomycin. PC-R1 and PC-E1 cell lines kindly provided by Dr. Klaus Pantel (Hamburg, Germany; Ref. 20) were maintained in RPMI 1640 with glutamine, 10% FBS, 1% penicillin/streptomycin, 10 μg/ml trans-
ferrin, 5 μg/ml insulin, 10 ng/ml recombinant human epidermal growth factor, and 10 μg/ml recombinant human basic fibroblast growth factor. Human lymph node-metastatic prostate tumor cell lines, LNCaP, LNCaP Pro-5 (a more tumorigenic variant of LNCaP; Ref. 19), and LNCaP LN-3 [a highly metastatic variant of LNCaP (19); all from Dr. Pettaway] and the human brain-metastatic prostate tumor DU-145 cell line (American Type Culture Collection) were maintained in RPMI 1640 with glutamine, 10% FBS, and 1% penicillin/streptomycin.

The human BMEC line HBMEC-60, generously provided by Dr. C. Ellen van der Schoot (Sanquin Research at CLB, Amsterdam, the Netherlands; Ref. 21), was maintained in Medium 199 with HEPES and glutamine, 10% FBS, 10% human serum, 100 μg/ml G418, 5 units/ml heparin, 1 ng/ml recombinant human fibroblast growth factor, and 1% penicillin/streptomycin. Primary cultures of HUVECs were generously provided by Dr. David Jones (Department of Dermatology, Brigham and Women’s Hospital), propagated in Clonetics Endothelial Cell Growth Medium supplemented with BulletKit/1% penicillin/streptomycin, and maintained for up to 5 passages.

Laminar Flow Analysis. Tethering and rolling of prostate tumor cells on recombinant human E-selectin-immunoglobulin chimera (R&D Systems, Inc., Minneapolis, MN) were analyzed in the parallel-plate flow chamber under physiological shear stress (22). To prepare E-selectin-immunoglobulin chimera spots, E-selectin-immunoglobulin [50 ng/50 μl 0.1 M NaHCO3 (pH 9.6)] was pipetted on Ten-twenty-nine Petri dishes and allowed to adsorb overnight at 4°C. After removing E-selectin-immunoglobulin chimera solution, PBS was added and incubated for ≥2 h at 37°C to block nonspecific binding sites. Prostate tumor cells released with 0.5 mm EDTA and washed twice in PBS were suspended at 1 × 106/cm2 in HBSS, 10 mm HEPES, and 2 mm CaCl2 (HH) assay medium and infused into the chamber over selectin chimera. Cell tethering, which was defined as a reduction in forward motion below hydrodynamic velocity for a minimum of two frames (0.07 s; Ref. 23), was permitted at 0.3 dynes/cm2 for 1 min, and then stepwise increments in shear stress level of 60 dynes/cm2. Cell rolling, which was defined as ≥5 cell diameters of forward movement below hydrodynamic velocity (23), was assessed at 0.6 dynes/cm2 from the midpoint of the chamber viewing field (4 fields/selectin spot, three different experiments) at ≥100 magnification. All experiments were observed in real time and videotaped for offline analysis. Negative control experiments were performed in parallel, wherein cell binding was examined in adhesion assay medium containing 5 mm EDTA to chelate Ca2+ required for selectin binding, and cell binding was assayed on human IgG isotype control.

For cell rolling assessments on E-selectin natively expressed on human microvascular endothelium, prostate tumor cells were perfused over confluent cultures of HBMEC-60 cells or of HUVECs grown in 35 × 10-mm culture dishes (Corning Inc., Corning, NY) and stimulated for 4 h with 10 ng/ml interleukin-8 (St. Louis, MO). To determine E-selectin expression, stimulated and unstimulated endothelial cells were harvested with 0.5 mm EDTA and stained with antihuman E-selectin moAb (clone 68-5H11; BD Biosciences, Inc., San Jose, CA) for flow cytometric analysis. Non-IL-8-stimulated HBMEC-60 cells and IL-8-stimulated cells treated with 10 μg/ml neutralizing antihuman E-selectin moAb (clone 68-5H11) or isotype Ab control for 30 min at room temperature served as controls for assessing specificity of E-selectin-mediated adhesion. Cell tethering and rolling were quantified and expressed as described above from a minimum of three experiments.

Enzymatic and Metabolic Inhibitor Treatments. To examine the role of sialylated glycoprotein(s) in binding E-selectin in flow chamber assays, cells were treated with Vibrio cholerae neuraminidase (Roche Applied Sciences, Indianapolis, IN), which cleaves terminal sialic acid residues and is known for abrogating E-selectin ligand activity (0.1 unit/ml for 1 h at 37°C), or with bromelain (Sigma), a protease with broad peptide specificity known for elimination of sialic acid from glycoproteins. Cells were treated with neuraminidase or incubated with glycolipid inhibitor to assess residual sialyl Lewis X antigen.

Western Blot Analysis. Confluent cultures of human metastatic prostate tumor cells harvested with 0.5 mm EDTA or of human hematopoietic KG1a cells (control) were washed three times with ice-cold PBS. Pelleted cells were resuspended in lysis buffer (150 mm NaCl, 0.5 mm Tris base, 1 mm EDTA, 20 μg/ml phenylmethylsulfonyl fluoride, and 0.02% NaN3) containing Protease Inhibitor Cocktail Tablets (1 tablet/100 ml lysis buffer; Roche), and membrane proteins were prepared as described previously (25). Concentrations of solubilized membrane protein (lissus buffer/2% NP40) were determined by Bradford method. In preliminary experiments, protein amounts from all cell membranes were variable, nontoxic concentrations of the respective inhibitor were determined to be identical as determined by Coomassie Blue staining of resolved protein on 4–20% SDS-PAGE gradient gels. Solubilized membrane protein was diluted in reducing sample buffer and separated on 4–20% SDS-PAGE gradient gels. Resolved membrane proteins were transferred to Sequenti blot polyvinylidene difluoride membrane (Bio-Rad, Inc., Hercules, CA) and blocked in PBS for 1 h at room temperature. Blots were then incubated with rat IgM antihuman CLA HECA-452 (1 μg/ml) for 1 h at room temperature. Isotype control immuno- blots using rat IgM (1 μg/ml) were performed in parallel to evaluate nonspecific reactive proteins. After three washes with Tris-buffered saline and 0.1% Tween 20, blots were incubated with alkaline phosphatase-conjugated rabbit antirat IgM Abs (1:1000; Zymed Laboratories Inc., San Francisco, CA) for 1 h at room temperature. After several 15-min washes with Tris-buffered saline and 0.1% Tween 20, alkaline phosphatase substrate Western Blue (Promega, Madison, WI) was added to the blots. Western blots were then exposed a minimum of five times. Where indicated before SDS-PAGE/ Western blocking, solubilized membrane protein was pretreated with 0.1 μl/mem protein for 1 h at 37°C to digest terminal sialic acid residues and assess the requirement of sialic acids for moAb HECA-452 reactivity.

Immunohistochemical Analysis. Immunohistochemistry was performed on 4-μm tissue microarray sections of formalin-fixed, paraffin-embedded normal prostate tissue and prostate adenocarcinoma (Chemicon International, Inc., Temecula, CA). Prostate tumors with a Gleason score of 2–6 were designated low-grade tumors, and tumors with a Gleason score of 7–10 were designated high-grade tumors. For staining of HECA-452 antigen, 2-μm
tumor cells were enumerated and divided by total tumor cell count per field of view at ×200 magnification (0.785 mm²) and multiplied by 100 to obtain a percentage positive cell staining value. Two 2-mm cores (a minimum of four fields of view) were examined per prostate tissue specimen. Using isotype control staining as a reference for background levels, cell staining was scored as absent (<1% positive tumor cell staining), weak to moderate (≥50% positive tumor cell staining), and high (>50% positive tumor cell staining).

RESULTS

E-selectin Expressed on Human BMECs Mediates Rolling of Human Bone-Metastatic Prostate Tumor Cells. To investigate whether human prostate tumor cells roll on BMECs, we performed parallel-plate flow chamber analyses of human prostate tumor cell lines derived from bone, lymph node, or brain metastases on live confluent monolayers of human BMECs (HBMEC-60 cells). Metastatic prostate tumor cell lines from different tissues and of variable growth/metastatic potential in vivo were used in these experiments to help discriminate the putative selectivity of bone-metastatic prostate tumor cell adhesiveness with BM endothelium. HBMEC-60 cells were selected for these analyses based on their expression and maintenance of endothelial cell phenotypic markers as well as their capacity to support rolling and firm adhesive interactions with HPCs (21). Retention of these BMEC characteristics, which resemble the phenotype of primary cultures, not only benefits the understanding of molecular pathways mediating HPC homing to bone (25) but also provides a paradigm for investigations of human prostate tumor metastasis to bone.

To up-regulate cell surface expression of E-selectin, HBMEC-60 cells were first stimulated with IL-1β (Fig. 1A). At a shear stress level of 0.6 dynes/cm², we observed robust rolling of bone-metastatic prostate MDA PCa 2b tumor cells on IL-β-stimulated HBMEC-60 cells comparable with that of human hematopoietic progenitor KG1a cells (positive control; Fig. 1B). On the other hand, there were no binding interactions with nonstimulated endothelial cells or with IL-1β-stimulated endothelial cells treated with neutralizing anti-human E-selectin moAb, indicating that E-selectin is the principal adhesion receptor mediating adhesive interactions under shear stress. Furthermore, we also observed E-selectin-dependent rolling of the bone-metastatic prostate PC-3M LN-4 cell line, a metastatic variant of PC-3M, although no binding activity was observed on other bone-metastatic cell lines PC-3, PC-3M, PC-3M Pro-4, PC-R1, and PC-E1; on lymph node-metastatic LNCaP cell lines; and on the brain-metastatic DU-145 cell line (Fig. 1B). Interestingly, MDA PCa 2b cells, in contrast to other human prostate tumor cell lines, possess hallmark features of bone metastasis in patients: androgen-independent growth as well as retention of prostate-specific antigen expression and androgen sensitivity, in vivo (17). These cellular features, coincident with the previously unknown E-selectin ligand expression shown here, may be representative of a prostate tumor model, which closely mimics bone-metastasizing prostate tumor cells in humans.

To show whether the high rolling activity of MDA PCa 2b cells on HBMEC-60 E-selectin was an artifact of the cell model, we assayed MDA PCa 2b rolling activity on E-selectin natively expressed by HUVECs. When stimulated with either IL-1β or tumor necrosis factor α, primary cultures of HUVECs are a common biological model for demonstrating E-selectin-dependent adhesive interactions of human tumor cells (24, 26). We found that positive control (KG1a cells) and MDA PCa 2b cells exhibited E-selectin-dependent rolling activity on IL-1β-stimulated HUVECs (Fig. 2) similar to that observed on IL-1β-stimulated HBMEC-60 cells (Fig. 1B). Non-IL-1β-stimulated HU-
VECs and IL-1β-stimulated HUVECs treated with neutralizing anti-E-selectin moAb did not support any rolling activity (Fig. 2), and negative control PC-3 cells did not display any E-selectin-dependent binding activity, as demonstrated previously in Fig. 1B. These data corroborate observations of MDA PCa 2b cells rolling activity on HBMEC-60 cell E-selectin, showing that E-selectin function as naturally expressed after IL-1β stimulation was identical on HBMEC-60 cells and HUVECs. Furthermore, results show that there was no enhanced binding capacity of MDA PCa 2b cells to BMEC E-selectin compared with binding to HUVEC E-selectin, suggesting that other adhesive interactions and chemokine signaling events between circulating prostate tumor cells and BMECs are also important for promoting BM-specific metastasis.

Sialylated Glycoprotein and Glycolipid Structures Function as E-selectin Ligands on Human Prostate Tumor Cells. Prior studies elucidating E-selectin ligand identity on HPCs, skin homing T-cells, and colon tumor cells indicate that terminal sialic acid residues are critical for ligand activity (22–26). To determine whether sialyl Lewis X structures (which contain terminal α2,3 sialic acid residues) were expressed on prostate tumor cell lines used in these studies, flow cytometry experiments were performed with antihuman sialyl Lewis X moAb HECA-452. Results revealed that all bone-metastatic cell lines (MDA PCa 2b, PC-3, PC-3M, PC-3M Pro-4, PC-3M LN-4, PC-R1, and PC-E1) and brain-metastatic cell line DU-145 expressed high levels of sialyl Lewis X (positive cell staining > 98%), whereas lymph node-metastatic cell lines (LNCaP, LNCaP Pro-5, and LNCaP LN-3) expressed lower levels of sialyl Lewis X (positive cell staining < 57%; Table 1). Accordingly, to elucidate the role of terminal sialic acids on E-selectin ligand(s) expressed by human prostate tumor cells, cells were treated with V. cholerae neuraminidase and assayed for ligand activity in the flow chamber. Because neuraminidase treatments unmask galactose residues and could potentially expose de-sialylated oligosaccharide structures to galectin-3 binding on HBMEC-60 cells (10–12), ligand activities were assessed over purified human E-selectin-immunoglobulin chimera. Preliminary flow chamber experiments were performed to determine the appropriate concentration of chimera and shear stress level that supported a frequency of rolling tumor cells similar to that observed on E-selectin natively expressed on HBMEC-60 cells. We found that prostate tumor cell lines displayed rolling activities similar to those observed on HBMEC-60 cell E-selectin, whereas neuraminidase markedly reduced E-selectin ligand activity on all human prostate tumor cells compared with untreated cells (Fig. 3; statistically different from untreated control, P < 0.001). There was no ligand activity on human IgG-coated plastic or on E-selectin-immunoglobulin in the presence of 0.5 mM EDTA (data not shown). Incidentally, unlike binding to HBMEC-60 cell E-selectin, LNCaP and LNCaP Pro-5 cells showed some rolling activity on E-selectin-immunoglobulin, which may be presented at a more optimal density for tethering and rolling under flow conditions. Unlike the role of terminal galactose residues in attachment of brain-metastatic DU-145 cells to vascular endothelium (10–12), these data indicate that terminal sialic acids on bone-metastatic prostate tumor cells are critical for initial tethering and rolling on E-selectin.

To help dissect the potential E-selectin-binding determinants on the cell surface, tumor cells were treated with a metabolic inhibitor of glucosylceramide biosynthesis (PPPP or PPMP) for 96 h (24) and/or then treated with protease (0.1% bromelain; Ref. 25). Before incubations with non-growth-inhibitory concentrations of glycolipid inhibitors, tumor cells were first treated with neuraminidase to remove all preformed sialic acid residues and ligand activity on the cell surface and to ensure for analysis of de novo synthesized E-selectin-binding structures. As shown in Fig. 4A, neuraminidase treatment markedly reduced rolling of prostate tumor cells, and when cells were regrown in diluent control (DMSO) for 96 h, all E-selectin ligand activities were restored. Where indicated, incubations were also followed by protease treatments to analyze the contribution of glycoproteins on E-selectin-mediated rolling activity. MDA PCa 2b cell rolling was reduced by 38%, and LNCaP Pro-5 cell rolling was abolished by 75% (statistically different from diluent control, P < 0.001; Fig. 4A). Glycolipid inhibitor treatments on MDA PCa 2b (15 μM PPMP) and LNCaP Pro5 (5 μM PPPP) cells also caused a significant reduction in E-selectin ligand activity (32% and 100%, respectively; statistically different from diluent control, P < 0.001; Fig. 4A). Combined glycolipid inhibitor and proteolytic treatments on MDA PCa 2b cells further lowered E-selectin ligand activity (by 63%), although a substantial amount of residual E-selectin ligand activated persisted (37%). Flow cytometric analysis of sialyl Lewis X on MDA PCa 2b cells revealed that protease or glycolipid inhibitor treatments lowered sialyl Lewis X expression (mean channel fluorescence of 602 in untreated diluent control compared with mean channel fluorescence of 845 in treated). Additional experiments showed that neuraminidase treatment markedly reduced sialyl Lewis X expression (mean channel fluorescence of 177 in treated compared with mean channel fluorescence of 519 in untreated control).

### Table 1: Expression of sialyl Lewis X on human metastatic prostate tumor cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Percentage of positive cell staining (MCF)$^{b}$ for sialyl Lewis X antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone-metastatic cell lines</td>
<td></td>
</tr>
<tr>
<td>MDA PCa 2b</td>
<td>98 (797)</td>
</tr>
<tr>
<td>PC-3</td>
<td>100 (327)</td>
</tr>
<tr>
<td>PC-3M</td>
<td>100 (1021)</td>
</tr>
<tr>
<td>PC-3M Pro-4</td>
<td>100 (337)</td>
</tr>
<tr>
<td>PC-3M LN-4</td>
<td>100 (261)</td>
</tr>
<tr>
<td>PC-R1</td>
<td>99 (484)</td>
</tr>
<tr>
<td>PC-E1</td>
<td>100 (674)</td>
</tr>
<tr>
<td>Lymph node-metastatic lines</td>
<td></td>
</tr>
<tr>
<td>LNCaP</td>
<td>39 (28)</td>
</tr>
<tr>
<td>LNCaP Pro-5</td>
<td>21 (14)</td>
</tr>
<tr>
<td>LNCaP LN-3</td>
<td>57 (11)</td>
</tr>
<tr>
<td>Brain-metastatic lines</td>
<td></td>
</tr>
<tr>
<td>DU-145</td>
<td>100 (472)</td>
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</table>

$^{b}$ Flow cytometric analysis of sialyl Lewis X on human metastatic prostate tumor cell lines with anti-sialyl Lewis X monoclonal antibody HECA-452 (1 μg/ml). Data represent the percentage of positive cell staining (based on the number of cells staining greater than negative isotype control cell staining) and mean channel fluorescence (MCF).
These findings indicated that sialylated glycosphingolipids are critical for E-selectin binding activity of PC-3M LN-4 cells and that aggressive prostate tumors exhibiting elevated proteolytic activities may promote tumor cell adhesiveness to BMECs and elevate bone-metastatic potential.

To identify potential E-selectin glycoprotein ligands on human bone-metastatic prostate MDA PCa 2b cells, we immunoblotted preparations of membrane protein with moAb HECA-452. Prior studies using moAb HECA-452 as an indicator of sialyl Lewis X-bearing glycoproteins have shown that HECA-452-reactivity correlates with E-selectin binding function of CD44 and PSGL-1 on human HPCs (25), PSGL-1 on human skin-homing T cells (27) and L-selectin on human neutrophils (28). We also analyzed HECA-452 antigen on membrane proteins isolated from PC-3M LN-4 cells to help substantiate the observed role of sialylated glycosphingolipids as E-selectin ligands on PC-3M LN-4 cells as described above. As expected, MDA PCa 2b membrane protein(s) stained with moAb HECA-452, whereas membrane protein(s) from PC-3 cells, which lacked E-selectin ligand activity, and PC-3M LN-4 cells, which lacked protein-dependent E-selectin ligand activity, did not display HECA-452 antigen (Fig. 5A). HECA-452-stained membrane protein(s) on MDA PCa 2b cells were identified as a prominent molecular species at M, 130,000 and M, 220,000 and a number of minor bands ranging from M, 100,000 to M, 220,000 (Fig. 5A). To show that these membrane proteins were dependent on sialylation for moAb HECA-452 recognition, we pre-treated membrane protein with neuraminidase. As shown in Fig. 5B, HECA-452 reactivity of positive control KG1a membrane protein and MDA PCa 2b membrane protein was completely eliminated, which corroborates cell binding data and the dependence of terminal sialylation (or sialyl Lewis X epitopes) for E-selectin glycoprotein ligand activity. Collectively, these results showed that prostate tumor membrane glycoproteins expressing HECA-452 antigen correlate with high E-selectin ligand activity and that moAb HECA-452-reactive membrane proteins on MDA PCa 2b cells represent candidate E-selectin glycoprotein ligands.

HECA-452 Antigen Expression Is Associated with Prostate Tumor Progression. To determine whether HECA-452 antigen was related to prostate tumor progression and to test our hypothesis that E-selectin ligand expression is up-regulated on aggressive tumor cells in high-grade tumors, we performed immunohistochemical analysis on human tissue microarrays containing benign prostate tissue sections as well as low- and high-grade prostate tumor tissue sections using moAb HECA-452. Prior studies using immunohistochemical approaches with other anti-sialyl Lewis X Abs show that up-regulation of sialyl Lewis X expression is associated with hormone-resistant aggressive prostate cancer and poor prognosis, suggesting that sialyl Lewis X may be a useful prognostic indicator of patients with metastatic disease (29). We believed that immunohistochemical analysis with moAb HECA-452, specifically, would help corroborate these findings and distinguish cells with an aggressive metastatic phenotype expressing E-selectin ligand(s). We found that HECA-452 antigen expression on normal prostate tissues (n = 17) was notably deficient; only 12% of tissues showed a weak to moderate (<50% positive epithelial cell staining) staining level (Table 2). However, in low-grade prostate tumors (n = 39; Gleason score, 2–6), 49% of tumors showed weak to moderate and high (>50% positive tumor cell staining) staining levels (Table 2). In high-grade tumors (Gleason score, 7–10), HECA-452 antigen was detected at a weak to moderate and
high level of expression in 79% of tumors (Table 2). Moreover, 38% of high-grade tumors exhibited high staining levels, whereas only 15% of low-grade tumors stained at a high level. Representative photomicrographs of moAb HECA-452-stained prostate tissue illustrate the observed incremental level of HECA-452 antigen from normal tissue to low- and high-grade tumor tissues (Fig. 6). These results support previous studies reporting the prognostic value of sialyl Lewis X expression in predicting metastatic potential of prostate
HECA-452. Isotype control blots were performed in parallel to confirm the specificity of difluoride membrane, and blotted with anti-sialyl Lewis X rat monoclonal antibody. The chemokine receptor CXCR4 is functionally expressed on human growth factors that promote metastasis within a particular tissue site. Indeed, there are microenvironmental chemotactic, motility, and metastasis in a nude mouse model of experimental metastasis (11).–arrest, and retention on the microvessel wall (11, 12, 30). Interference antigen displayed by membrane proteins (B). These experiments were performed a of HECA-452 antigen. As shown, neuraminidase completely eliminated HECA-452 membranes were resolved on reducing 4–20% SDS-PAGE gradient gels, transferred to polyvinylidene difluoride membrane, and blotted with anti-sialyl Lewis X rat monoclonal antibody HECA-452. Isotype control blots were performed in parallel to confirm the specificity of HECA-452 reactivity. Human hematopoietic KG1a cell membrane protein (10 μg/lane) was included in this analysis to confirm positive HECA reactivity and help illustrate the molecular weights of known E-selectin ligands, CD44 (Mr 130,000 and Mf 220,000). Please note the presence of distinct HECA-452-reactive glycoprotein(s) at Mr 130,000 and Mf 220,000 and a number of minor stained proteins resolving from Mr 100,000 to Mf 220,000 in membrane protein from MDA PCa 2b cells (A). In B, membrane protein from positive control KG1a cells (1 μg) and MDA PCa 2b cells (40 μg) was pretreated with 0.1 unit/ml neuraminidase before Western blot analysis of HECA-452 antigen. As shown, neuraminidase completely eliminated HECA-452 antigen displayed by membrane proteins (B). These experiments were performed a minimum of five times.

Fig. 5. Western blot analysis of sialyl Lewis X expression on human bone-metastatic prostate tumor membrane proteins. Membrane proteins (40 μg/lane) from MDA PCa 2b cells (high, protease-sensitive E-selectin ligand activity), PC-3M LN-4 cells (low, protease-resistant E-selectin ligand activity), and PC-3 cells (no E-selectin ligand activity) were resolved on reducing 4–20% SDS-PAGE gradient gels, transferred to polyvinylidene difluoride membrane, and blotted with anti-sialyl Lewis X rat monoclonal antibody HECA-452. Isotype control blots were performed in parallel to confirm the specificity of HECA-452 reactivity. Human hematopoietic KG1a cell membrane protein (10 μg/lane) was included in this analysis to confirm positive HECA reactivity and help illustrate the molecular weights of known E-selectin ligands, CD44 (Mr 100,000) and PSGL-1 (Mr 130,000 and Mr 220,000). Please note the presence of distinct HECA-452-reactive glycoprotein(s) at Mr 130,000 and Mr 220,000 and a number of minor stained proteins resolving from Mr 100,000 to Mr 220,000 in membrane protein from MDA PCa 2b cells (A). In B, membrane protein from positive control KG1a cells (1 μg) and MDA PCa 2b cells (40 μg) was pretreated with 0.1 unit/ml neuraminidase before Western blot analysis of HECA-452 antigen. As shown, neuraminidase completely eliminated HECA-452 antigen displayed by membrane proteins (B). These experiments were performed a minimum of five times.

discussed above, the results demonstrated that moAb HECA-452-reactive membrane proteins were sensitive to neuraminidase, which supported results from cell binding experiments. These results indicate that moAb HECA-452-reactive mem-

prostate tumors (29) and show a clear association between prostate tumor grade and HECA-452 antigen expression levels.

**DISCUSSION**

Several recent studies show that human tumor cells exhibit rolling adhesions on microvascular endothelium (11, 12, 30–32), indicating that these dynamic shear-resistant interactions may preclude firm attachment, *trans*-endothelial migration, and expansion in situ of metastatic tumor cells. There are convincing data demonstrating human brain-metastatic prostate tumor cell rolling on dura mater microvascular endothelium through T-antigen–galectin-3 binding interactions, which subsequently promote homotypic aggregation, firm cell arrest, and retention on the microvasculature (11, 12, 30). Interference of T-antigen–galectin-3 interactions with a sugar mimetic of T-antigen results in a lower incidence and frequency of human breast cancer metastasis in a nude mouse model of experimental metastasis (11). Indeed, there are microenvironmental chemotactic, motility, and growth factors that promote metastasis within a particular tissue site. The chemokine receptor CXCR4 is functionally expressed on human prostate tumor cells and supports transendothelial migration through BMECs (33), indicating its putative role in the migration of circulating metastatic prostate tumor cells into bone. However, understanding the molecular mechanism by which tumor and endothelial cells initiate contact in blood flow may also offer new insight into the organotropism of tumor metastasis and perhaps reveal novel therapeutic targets for antimetastatic drug development.

In this report, we show heretofore unrecognized E-selectin ligand activities on human metastatic prostate tumor cells. By using human BMECs to examine the expression of E-selectin ligand activities, we determined whether other known adhesive mechanisms responsible for preferential adhesion of prostate tumor cells to BMECs could promote cell attachment or rolling behavior under shear flow. Under physiological flow conditions present in BM (34), E-selectin ligands supported prostate tumor cell tethering and rolling on E-selectin expressed on BMECs. Most notably, the bone-metastatic prostate tumor MDA PCa 2b cell line expressed robust E-selectin ligand activity, which was found to be attributable to both sialyl Lewis X-bearing glycoprotein(s) and glycosphingolipids. PC-3M LN-4 cells, originally derived from bone metastases (PC-3 cells), also displayed E-selectin ligand activity, although the major E-selectin-binding determinants appeared to be exclusively on sialylated glycosphingolipids. Furthermore, although these binding interactions were dependent on terminal sialic acids, expression of sialyl Lewis X itself did not correlate directly with the capacity of tumor cells to bind E-selectin as suggested previously (35). As reported here, sialyl Lewis X expression was necessary but not sufficient for prostate tumor cell E-selectin ligand activity, suggesting that specific sialyl Lewis X-bearing structures mediate ligand activity.

It is evident from a number of prior studies that HECA-452 antigen expression correlates with E-selectin binding function of human hematopoietic cell membrane proteins (25, 27, 28). For example, human HPC E-selectin ligands, HCELL and PSGL-1, require the presence of HECA-452 antigen for functional activity, which is critical for initiating HPC contact with BMECs under blood flow (25). To identify potential E-selectin glycoprotein ligand(s), we performed Western blot analysis of HECA-452 antigen on human prostate tumor MDA PCa 2b cell membrane protein. HECA-452 immunoblots revealed distinct glycoprotein(s) at Mr 130,000 and Mr 220,000, which comigrates with PSGL-1 (25), as well as other minor HECA-452-reactive membrane proteins ranging from Mr 100,000 to Mr 220,000. These moAb HECA-452-reactive membrane proteins were sensitive to neuraminidase, which supported results from cell binding experiments. These results indicate that moAb HECA-452-reactive membrane proteins were preferentially E-selectin glycoprotein(s), HCELL and PSGL-1, required in the presence of sialyl Lewis X-binding determinants. Furthermore, although these binding interactions were dependent on terminal sialic acids, expression of sialyl Lewis X itself did not correlate directly with the capacity of tumor cells to bind E-selectin as suggested previously (35). As reported here, sialyl Lewis X expression was necessary but not sufficient for prostate tumor cell E-selectin ligand activity, suggesting that specific sialyl Lewis X-bearing structures mediate ligand activity.

**Table 2: Immunohistochemical analysis of HECA-452 antigen in normal human prostate tissue and in low- and high-grade human prostate tumors**

<table>
<thead>
<tr>
<th>Normal prostate tissue and prostate tumor tissue</th>
<th>HECA-452 antigen expression*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal prostate tissue (n = 17)</td>
<td>Absent</td>
</tr>
<tr>
<td>Low-grade tumors (n = 39)</td>
<td>(Gleason score, 2-6)</td>
</tr>
<tr>
<td>High-grade tumors (n = 24)</td>
<td>(Gleason score, 7-10)</td>
</tr>
</tbody>
</table>

* Using isotype control staining as a reference for background levels, cell staining was graded as absent (≤3%) positive tumor cell staining), weak to moderate (≤50% positive tumor cell staining), or high (>50% positive tumor cell staining).
brane proteins expressed on MDA PCa 2b cells that are conspicuously absent on E-selectin ligand-deficient PC-3 cells or on PC-3M LN-4 cells expressing protease-resistant E-selectin ligand activity represent candidate E-selectin glycoprotein ligands. Investigations in elucidating the identity of these E-selectin ligand scaffolds on human metastatic prostate tumor cells are currently ongoing.

Immunohistochemical analysis of HECA-452 antigen expression on normal prostate epithelium and on low- and high-grade prostate adenocarcinoma. Immunohistochemical analysis was performed on prostate tissue microarray sections using anti-sialyl Lewis X monoclonal antibody HECA-452. As illustrated at ×200 magnification, HECA-452 antigen expression was absent on normal prostate epithelium but was conspicuously found on a small number of tumor cells (arrowheads) in low-grade tumor tissue (Gleason score, 2–6) and expressed on a large percentage of tumor cells in high-grade tumor tissue (Gleason score, 7–10). Please note in the enlarged panel (at ×400 magnification) that HECA-452 antigen is expressed on high-grade prostate tumor membrane structures. Bar = 100 μm.

Fig. 6. Representative photomicrographs of HECA-452 antigen expression on normal prostate epithelium and on low- and high-grade prostate adenocarcinoma. Immunohistochemical analysis was performed on prostate tissue microarray sections using anti-sialyl Lewis X monoclonal antibody HECA-452. As illustrated at ×200 magnification, HECA-452 antigen expression was absent on normal prostate epithelium but was conspicuously found on a small number of tumor cells (arrowheads) in low-grade tumor tissue (Gleason score, 2–6) and expressed on a large percentage of tumor cells in high-grade tumor tissue (Gleason score, 7–10). Please note in the enlarged panel (at ×400 magnification) that HECA-452 antigen is expressed on high-grade prostate tumor membrane structures. Bar = 100 μm.

Enlarged view of distinct HECA-452 expression on high grade prostate adenocarcinoma.

brane proteins expressed on MDA PCa 2b cells that are conspicuously absent on E-selectin ligand-deficient PC-3 cells or on PC-3M LN-4 cells expressing protease-resistant E-selectin ligand activity represent candidate E-selectin glycoprotein ligands. Investigations in elucidating the identity of these E-selectin ligand scaffolds on human metastatic prostate tumor cells are currently ongoing.

Immunohistochemical analysis of HECA-452 antigen expression on normal prostate tissue and on low- and high-grade prostate adenocarcinomas reveals a direct association between expression level and tumor grade. Normal prostate epithelial cells were largely negative for HECA-452 antigen, whereas high expression levels were more frequently found on poorly differentiated tumor cells. Because HECA-452 antigen expression correlates with prostate tumor cell E-selectin ligand activity, tumor cells stained with moAb HECA-452,
particularly in high-grade tumors, may represent aggressive cells likely to intravasate and metastasize to bone. Of note, immunohistochemical data using other anti-sialyl Lewis X moAbs show that sialyl Lewis X is directly correlated with Gleason score (36) and metastatic potential of prostate cancer (29, 36–38). High sialyl Lewis X expression on aggressive, hormone-resistant prostate cancer compared with the lack of sialyl Lewis X expression on benign epithelium and low expression on carcinoma in situ is indicative of the prognostic value of sialyl Lewis X assessment (38). Although sialyl Lewis X could be an important marker for predicting the metastatic potential of prostate cancer, coexpression of HECA-452 antigen and E-selectin-binding determinants on specific membrane scaffolds would implicate them as potential therapeutic targets in the prevention of prostate tumor progression and metastasis.

In summary, these results provide a new perspective into the molecular mechanism mediating shear-resistant adhesive interactions between metastatic prostate tumor cells and BMECs, which constitutively express E-selectin in vivo. We believe that these binding events initiate or lead to other secondary adhesive mechanisms through galectin-3, hyaluronan, and immunoglobulin superfamily members as well as trigger chemokine–chemokine receptor interactions (i.e., SDF-1α/CXCR4) that help confer BM-specific migration. Additional studies elucidating the expression of E-selectin ligand(s) on bone metastases compared with expression on primary prostate tumors or on prostate tumor metastases in other non-bone tissues will need to be performed to help validate our findings. Moreover, investigating the metastatic potential of E-selectin ligand-positive and -negative human prostate tumor cell lines orthotopically implanted into severe combined immunodeficient mice will help broaden our understanding of the role of E-selectin ligands in prostate tumor metastasis.

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