

Cancer Research

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

Charles J. Dimitroff, Mirna Lechpammer, Denise Long-Woodward, et al.

Cancer Res 2004;64:5261-5269.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/64/15/5261>

Cited Articles This article cites by 37 articles, 20 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/64/15/5261.full.html#ref-list-1>

Citing articles This article has been cited by 13 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/64/15/5261.full.html#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

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

Charles J. Dimitroff,¹ Mirna Lechpammer,³ Denise Long-Woodward,¹ and Jeffery L. Kutok²

¹Harvard Skin Disease Research Center, Department of Dermatology; ²Department of Pathology, Brigham and Women's Hospital, and ³Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts

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 ligand(s); distinct sialyl Lewis X (or HECA-452 antigen)-bearing membrane proteins were resolved at M_r 130,000 and M_r 220,000 as well as others ranging from M_r 100,000 to M_r 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 adhesion to human BMECs under static conditions is mediated, in part, by tumor cell β_1 and β_2 integrins (4, 5, 8, 9), T-antigen (Gal β 1,3GalNAc)-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–galectin-3 interactions (11, 12). On activation by exposure to tumor cells or to de-sialylated glycoconjugates, endothelial cells mobilize galectin-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 (mAb) 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 (19); 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 μ g/ml trans-

Received 2/25/04; revised 5/3/04; accepted 5/21/04.

Grant support: NIH Grants NCI IR21 CA102913-01 (C. J. Dimitroff), NCI IR21 CA104828-01 (C. J. Dimitroff), and NIAMS 5P30 AR042689-090040 (C. J. Dimitroff), NIAMS Harvard Skin Disease Research Center Core Grant 5P30 AR042689 and the Prostate Pathology Core of the Dana Farber/Harvard Cancer Center Grant CA06516-37.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Charles J. Dimitroff, Harvard Institutes of Medicine, Room 650, 77 Avenue Louis Pasteur, Boston, MA 02115. Phone: (617) 525-5693; Fax: (617) 525-5571; E-mail: cdimitroff@rics.bwh.harvard.edu.

ferrin, 5 $\mu\text{g/ml}$ insulin, 10 ng/ml recombinant human epidermal growth factor, and 10 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ 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 NaHCO_3 (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, FBS 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 $1 \times 10^6/\text{ml}$ in HBSS, 10 mM HEPES, and 2 mM CaCl_2 (H/H) 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 every 15 s were used to a final 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 $\times 100$ 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 Ca^{2+} 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 (IL)-1 β (Sigma, St. Louis, MO). To confirm 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-1 β -stimulated HBMEC-60 cells and IL-1 β -stimulated cells treated with 10 $\mu\text{g/ml}$ 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 eliminating E-selectin glycoprotein ligands (0.1% for 1 h at 37°C; Ref. 22). Of note, during bromelain treatments, tumor cells were vortexed every 15 min to disaggregate cells and optimize digestion of all cell surface proteins and then passed through a 70 μm filter to remove cell clumps and recounted before assay. To confirm digestion of cell surface sialic acid by neuraminidase and membrane proteins by bromelain, cells were stained with antihuman sialyl Lewis X moAb HECA-452 or with antihuman CD44 moAb A3D8, respectively, and analyzed by flow cytometry.

To determine the contribution of tumor cell surface glycolipids to E-selectin binding in flow chamber assays, cells were pretreated with neuraminidase and

then recultured in the presence of glucosylceramide synthase inhibitor D,1-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP; generously provided by Dr. Konstantinos Konstantopoulos, Johns Hopkins University) or D,1-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-propanol (PPMP; Matreya, Inc., Pleasant Gap, PA) to interfere with the reexpression of *de novo* synthesized E-selectin glycolipid ligand on the cell surface (24). Preliminary experiments were performed to determine the growth-inhibitory effects of PPPP or PPMP on each prostate tumor cell line as determined by trypan blue exclusion and establish nontoxic concentrations for analysis of glycolipid inhibition. Because drug sensitivities of PPPP and PPMP on cell lines were variable, nontoxic concentrations of the respective inhibitor were relative to each cell line. Accordingly, MDA PCa 2b cells were grown in 15 μM PPMP; PC-3, PC-3M, PC-3M Pro-4, PC-R1, PC-E1, and DU-145 cell lines were grown in 1 μM PPPP or 5 μM PPMP; PC-3M LN-4 cells were grown in 2.5 μM PPPP or 10 μM PPMP; and all LNCaP cell lines were grown in 5 μM PPPP or 10 μM PPMP. For adhesion experiments, cells were grown in DMSO diluent control or in nontoxic concentrations of glycolipid inhibitor (see above) for 96 h or, as determined in pilot experiments, the period of time after neuraminidase treatment in which E-selectin ligand activity was restored. In some cases, cells were also treated with bromelain as described above after incubation with glycolipid inhibitor to assess residual sialyl Lewis X and E-selectin glycoprotein ligand expression.

Flow Cytometry. Cells were washed twice with cold PBS/2% FBS and suspended at 10^7 cells/ml in PBS and 1% FBS. Antihuman sialyl Lewis X moAb HECA-452 (BD Biosciences), antihuman E-selectin moAb clone 68-5H11 (BD Biosciences), antihuman CD44 moAb A3D8 (Sigma), or appropriate isotype-matched control Ab (1.5 $\mu\text{g}/\text{test}$) was incubated with cells for 30 min on ice. After two washes with PBS and 2% FBS, cells were resuspended in PBS, 1% FBS, and fluorochrome-conjugated secondary Abs (2 μl) and incubated for 30 min on ice. Cells were washed twice with PBS and 2% FBS and resuspended in PBS, and flow cytometry was performed on a FACScan apparatus equipped with an argon laser tuned at 488 nm (Becton Dickinson). Cells stained with relevant isotype control Ab were subtracted from positive cell staining with test Ab to control for test Ab specificity and avoidance of false-positive results.

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 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride, and 0.02% NaN_3) 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 (lysis buffer/2% NP40) were determined by Bradford method. In preliminary experiments, protein amounts from all cell membrane preparations to be analyzed by Western blotting were shown 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 Sequi-blot polyvinylidene difluoride membrane (Bio-Rad, Inc., Hercules, CA) and blocked in FBS for 1 h at room temperature. Blots were then incubated with rat IgM antihuman CLA HECA-452 (1 $\mu\text{g/ml}$) for 1 h at room temperature. Isotype control immunoblots using rat IgM (1 $\mu\text{g/ml}$) 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 develop the blots. These experiments were performed a minimum of five times. Where indicated before SDS-PAGE/Western blotting, solubilized membrane protein was pretreated with 0.1 unit/ml neuraminidase 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-mm

microarray core sections were deparaffinized and rehydrated according to the manufacturer's protocol. Microarray sections were subjected to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) for 2 min, blocked in normal goat serum (1:20), and incubated with rat IgM antihuman sialyl Lewis X moAb HECA-452 (1:250) for 1 h at room temperature. After rinsing, microarrays sections were incubated with rabbit antirat immunoglobulin (1:10,000) for 30 min at room temperature. Antigen-Ab complexes were revealed with standardized development times, using Envision Plus Kit (Dako) and diaminobenzidine as a substrate. Incubations with rabbit antirat immunoglobulin alone were also performed to establish background staining levels. All sections were counterstained in hematoxylin. For examination of positive staining, brown-stained tumor cells were enumerated and divided by total tumor cell count per field of view at $\times 200$ magnification (0.785 mm^2) 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 ($\leq 1\%$ positive tumor cell staining), weak to moderate ($\leq 50\%$ 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-1 β (Fig. 1A). At a shear stress level of 0.6 dynes/cm^2 , 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-1 β -stimulated endothelial cells treated with neutralizing antihuman 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-1 β 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-1 β -stimulated HUVECs (Fig. 2) similar to that observed on IL-1 β -stimulated HBMEC-60 cells (Fig. 1B). Non-IL-1 β -stimulated HU-

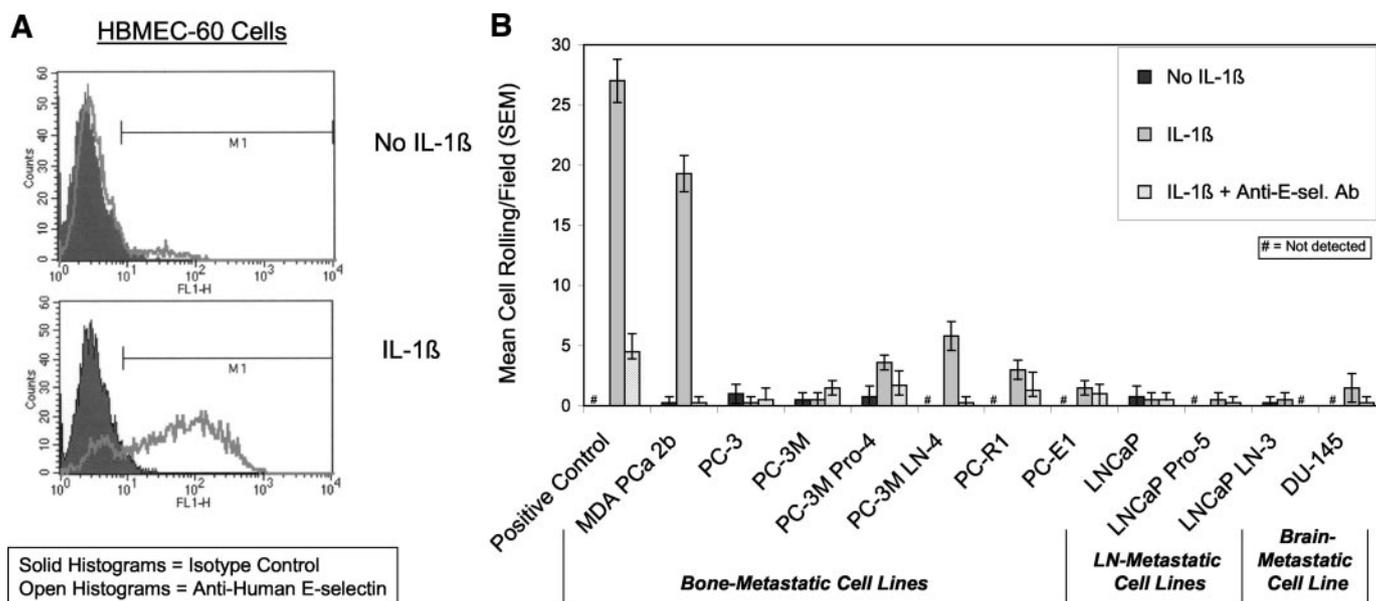


Fig. 1. Parallel-plate flow analysis of human prostate tumor cell rolling on E-selectin natively expressed by human bone marrow endothelial cells. To analyze the rolling activity of human prostate tumor cells on E-selectin on bone marrow endothelial cells, tumor cells were infused into the parallel-plate flow chamber experiments over confluent cultures of HBMEC-60 cells. To induce E-selectin expression, HBMEC-60 cultures were first pretreated with 10 ng/ml interleukin (IL)-1 β for 4 h at 37°C (A). Cell rolling assessments were determined at 0.6 dynes/cm^2 [mean cell rolling (SE) from four fields of view and three different experiments]. Compared with positive control cell rolling (KG1a cells), MDA PCa 2b and PC-3M LN-4 cells exhibited rolling activities on IL-1 β -stimulated HBMEC-60 cells, whereas no activities were observed on non-IL-1 β -stimulated HBMEC-60 cells or on anti-E-selectin antibody-treated HBMEC-60 cells stimulated with IL-1 β (B). #, no cell rolling detected.

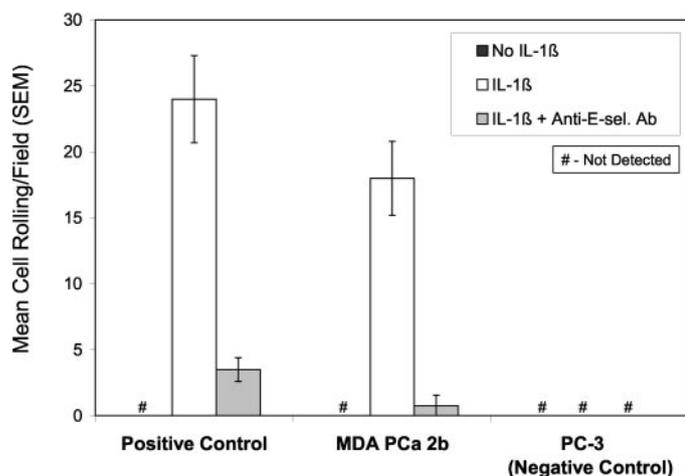


Fig. 2. Parallel-plate flow analysis of human prostate tumor MDA PCa 2b cell rolling on E-selectin natively expressed by human umbilical vein endothelial cells (HUVECs). To analyze the rolling activity of MDA PCa 2b cells on E-selectin expressed by HUVECs, tumor cells were infused into the parallel-plate flow chamber experiments over confluent cultures of HUVECs. To induce E-selectin expression, HUVEC cultures were first pretreated with 10 ng/ml interleukin (IL)-1 β for 4 h at 37°C. Cell rolling assessments were determined at 0.6 dynes/cm² [mean cell rolling (SE) from four fields of view and three different experiments]. Similar to positive control cell rolling activity (KG1a cells), MDA PCa 2b cells exhibited rolling activity on IL-1 β -stimulated HUVECs and not on non-IL-1 β -stimulated HUVECs or on IL-1 β -stimulated HUVECs pretreated with neutralizing anti-E-selectin monoclonal antibody. Negative control PC-3 cells did not display any rolling activity. #, no cell rolling detected.

VECs and IL-1 β -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 cell rolling activity on HBMEC-60 cell E-selectin, showing that E-selectin function as natively expressed after IL-1 β 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 α 2,3 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 desialylated 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 concen-

tration 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.

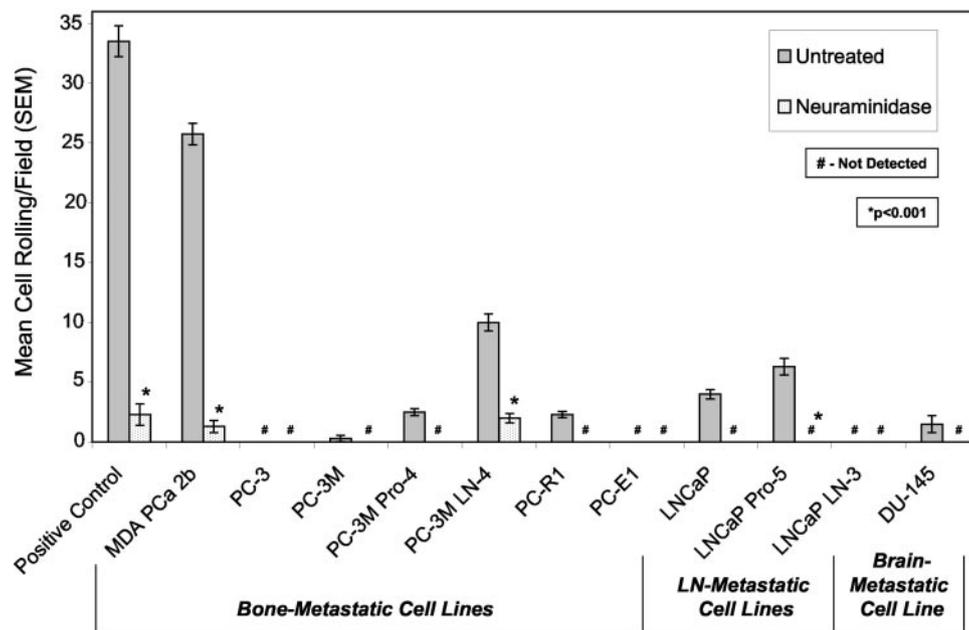
To help dissect the potential E-selectin-binding determinants on the cell surface, tumor cells were treated with a metabolic inhibitor of glucosylceramide biosynthesis (PPPP or PPMP) for 96 h (24) and/or then treated with protease (0.1% bromelain; Ref. 25). Before incubations with non-growth-inhibitory 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 μ M PPMP) and LNCaP Pro5 (5 μ M 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

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

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

^a Flow cytometric analysis of sialyl Lewis X on human metastatic prostate tumor cell lines with anti-sialyl Lewis X monoclonal antibody HECA-452 (1 μ g/test). 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).

Fig. 3. Requirement of sialylation on human prostate tumor cell rolling on human E-selectin. To examine the requirement of terminal sialic acid residues for E-selectin ligand activity, untreated or neuraminidase-treated tumor cells were infused into the parallel-plate flow chamber over purified human E-selectin-immunoglobulin chimera (1 $\mu\text{g}/\text{ml}$). Cell rolling assessments were determined at 0.6 dynes/cm² [mean cell rolling (SE) from four fields of view and three different experiments]. Compared with neuraminidase sensitivity of positive control cell rolling (KG1a cells), E-selectin ligand activities of prostate tumor cells were also sensitive to neuraminidase (*, statistically significant difference compared with untreated control, $P < 0.001$). #, no cell rolling detected.



74 and 93, respectively; Fig. 4B). However, there was also residual sialyl Lewis X expression above neuraminidase-treated levels that was not entirely abrogated by combined glycolipid inhibitor and protease treatments (Fig. 4B). These results indicate that both sialylated glycoprotein E-selectin ligand(s) and sialylated glycosphingolipids are expressed on MDA PCa 2b and LNCaP Pro-5 cells and that there are additional sialylated glycoprotein/glycolipid(s) on MDA PCa 2b cells resistant to protease or glycosylceramide synthase inhibitor treatments. Interestingly, protease treatments caused a ≥ 3 -fold increase in rolling activity of PC-3M, PC-3M Pro-4, and PC-3M LN-4 cells (Fig. 4A). This enhanced binding activity after protease treatment may have been due to the exposure of functional glycolipid ligands sterically hindered by surface proteins. Indeed, when compared with diluent control, E-selectin ligand activity on PC-3M LN-4 cells treated with glycolipid inhibitor (2.5 μM PPPP) was completely absent (Fig. 4A). Likewise, fluorescence-activated cell-sorting analysis showed that sialyl Lewis X epitopes on protease-treated PC-3M LN-4 cells were increased (mean channel fluorescence of 396 in protease-treated cells compared with mean channel fluorescence of 160 in control), whereas PPPP treatment markedly lowered sialyl Lewis X expression to neuraminidase treated levels (Fig. 4C). 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 the 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_r 130,000 and M_r 220,000 and a number of minor bands ranging from M_r 100,000 to M_r 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 ($\leq 50\%$ 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

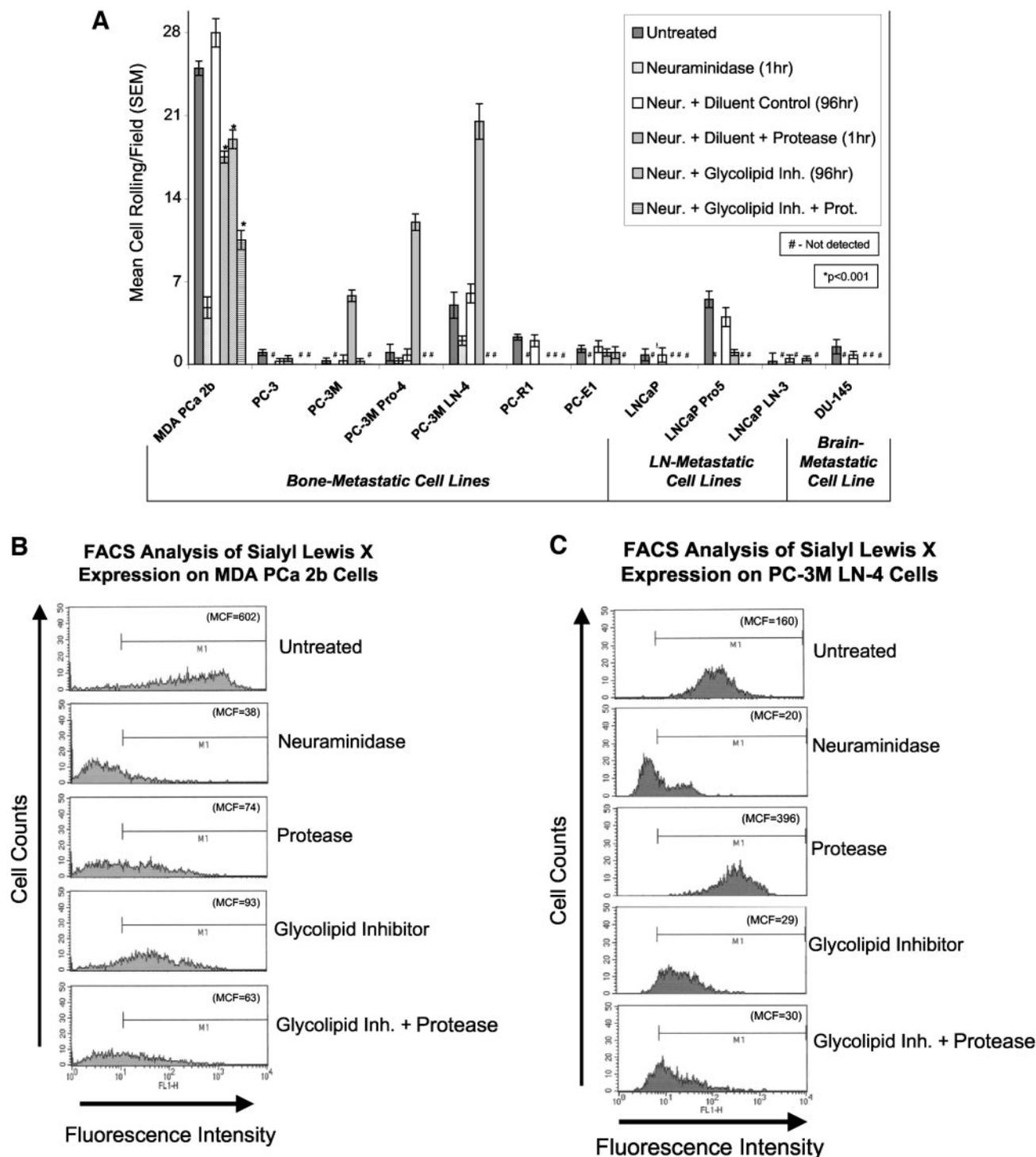


Fig. 4. Contribution of glycoprotein(s) and/or glycosphingolipids in human prostate tumor cell rolling on E-selectin. To investigate the role of glycoconjugates in E-selectin binding, tumor cells were treated with neuraminidase and recultured in diluent control or glycolipid inhibitor and then assayed for E-selectin ligand activity in the parallel-plate flow chamber. Where indicated, cells were also treated with protease after incubations with diluent control or glycolipid inhibitor. Whereas protease treatments significantly lowered MDA PCa 2b and LNCaP Pro-5 rolling on E-selectin (*, statistically different from diluent control, $P < 0.001$), rolling activities of PC-3M, PC-3M Pro-4, and PC-3M LN-4 were elevated by >3 -fold compared with untreated controls (A). Glycolipid inhibitor treatments completely ablated PC-3M LN-4 and LNCaP Pro-5 cell rolling activity (A). Fluorescence-activated cell-sorting analysis with antihuman sialyl Lewis X monoclonal antibody HECA-452 was performed on MDA PCa 2b and PC-3M LN-4 cells treated with neuraminidase, protease, glycolipid inhibitor, or both glycolipid inhibitor and protease treatments. In B, mean channel fluorescence (MCF) values indicated that neuraminidase treatment completely removed sialyl Lewis X epitopes on MDA PCa 2b cells, whereas protease and/or glycolipid inhibitor treatments caused partial reductions in sialyl Lewis X expression. In C, MCF values revealed a marked reduction in sialyl Lewis X expression after neuraminidase and glycolipid inhibitor treatments on PC-3M LN-4 cells, whereas protease treatments increased expression levels. #, no cell rolling detected.

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 illus-

trate 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

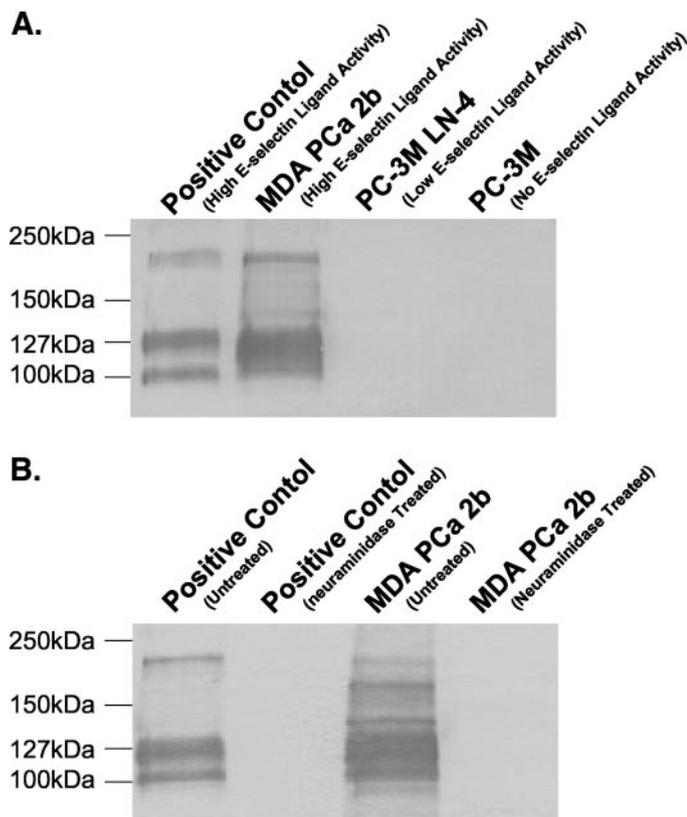


Fig. 5. Western blot analysis of sialyl Lewis X expression on human bone-metastatic prostate tumor membrane proteins. Membrane proteins (40 $\mu\text{g}/\text{lane}$) from MDA PCa 2b cells (high, protease-sensitive E-selectin ligand activity), PC-3M LN-4 cells (low, protease-resistant E-selectin ligand 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 $\mu\text{g}/\text{lane}$) was included in this analysis to control for positive HECA reactivity and help illustrate the molecular weights of known E-selectin ligands, CD44 (M_r 100,000) and PSGL-1 (M_r 130,000 and M_r 220,000). Please note the presence of distinct HECA-452-reactive glycoprotein(s) at M_r 130,000 and M_r 220,000 and a number of minor stained proteins resolving from M_r 100,000 to M_r 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.

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 microvessel wall (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 M_r 130,000 and M_r 220,000, which comigrates with PSGL-1 (25), as well as other minor HECA-452-reactive membrane proteins ranging from M_r 100,000 to M_r 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 mem-

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

For HECA-452 antigen expression analysis, deparaffinized microarrays of prostate tissues were blocked in normal goat serum (1:20) and incubated with rat IgM antihuman sialyl Lewis X monoclonal antibody HECA-452 (1:250) for 1 h at room temperature. After rinsing, sections were incubated with rabbit anti-rat immunoglobulin (1:10,000) for 30 min at room temperature. Antigen-antibody complexes were detected with standardized development times, using Envision Plus Kit and diaminobenzidine as a substrate. All sections were counterstained with hematoxylin.

Normal prostate tissue and prostate tumor tissue	HECA-452 antigen expression ^a		
	Absent	Weak to moderate	High
Normal prostate tissue ($n = 17$)	15	2	
Low-grade tumors ($n = 39$) (Gleason score, 2–6)	20	13	6
High-grade tumors ($n = 24$) (Gleason score, 7–10)	5	10	9

^a Using isotype control staining as a reference for background levels, cell staining was graded as absent ($\leq 1\%$ positive tumor cell staining), weak to moderate ($\leq 50\%$ positive tumor cell staining), or high ($> 50\%$ positive tumor cell staining).

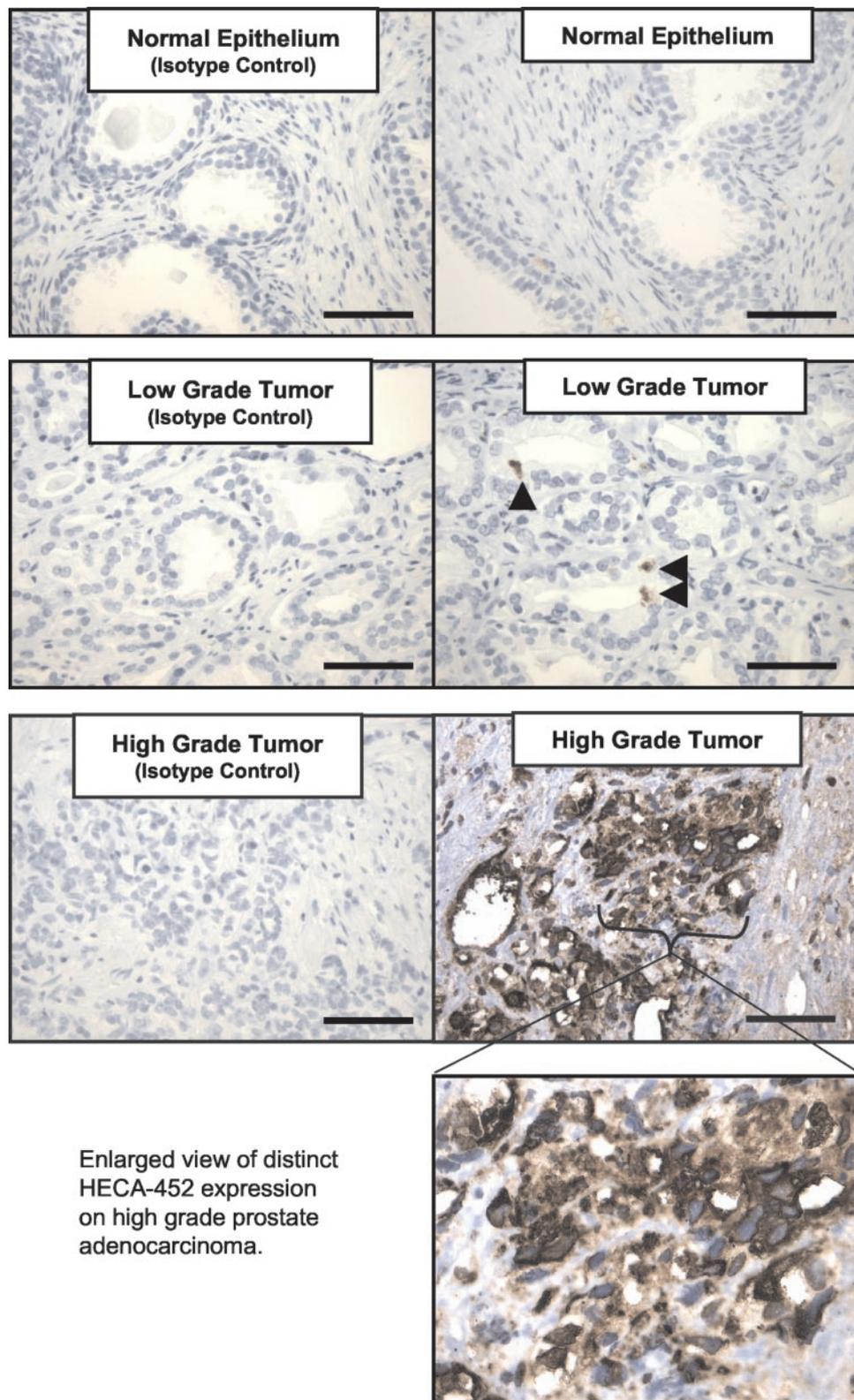


Fig. 6. Representative photomicrographs of HECA-452 antigen expression on normal prostate epithelium and on low- and high-grade prostate adenocarcinoma. Immunohistochemical analysis was performed on prostate tissue microarray sections using anti-sialyl Lewis X monoclonal antibody HECA-452. As illustrated at $\times 200$ 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 $\times 400$ magnification) that HECA-452 antigen is expressed on high-grade prostate tumor membrane structures. *Bar* = 100 μm .

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-1 α /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.

ACKNOWLEDGMENTS

We thank Drs. Robert Sackstein and Thomas S. Kupper (Department of Dermatology, Brigham and Women's Hospital) and Dr. Francis Luscinskas (Department of Pathology, Brigham and Women's Hospital) for helpful discussions and criticisms of our research work. We also thank Christine Knoblauch, Derek Cain, and Taylor Schreiber for expert technical assistance.

REFERENCES

- Lange PH, Vessella RL. Mechanisms, hypotheses and questions regarding prostate cancer micrometastases to bone. *Cancer Metastasis Rev* 1999;17:331–6.
- Reddi AH, Roodman D, Freeman C, Mohla S. Mechanisms of tumor metastasis to the bone: challenges and opportunities. *J Bone Miner Res* 2003;18:190–4.
- Keller ET, Zhang J, Cooper CR, et al. Prostate carcinoma skeletal metastases: cross-talk between tumor and bone. *Cancer Metastasis Rev* 2001;20:333–49.
- Lehr JE, Pienta KJ. Preferential adhesion of prostate cancer cells to a human bone marrow endothelial cell line. *J Natl Cancer Inst (Bethesda)* 1998;90:118–23.
- Scott LJ, Clarke NW, George NJ, et al. Interactions of human prostatic epithelial cells with bone marrow endothelium: binding and invasion. *Br J Cancer* 2001;84:1417–23.
- Simpson MA, Reiland J, Burger SR, et al. Hyaluronan synthase elevation in metastatic prostate carcinoma cells correlates with hyaluronan surface retention, a prerequisite for rapid adhesion to bone marrow endothelial cells. *J Biol Chem* 2001;276:17949–57.
- Cooper CR, McLean L, Walsh M, et al. Preferential adhesion of prostate cancer cells to bone is mediated by binding to bone marrow endothelial cells as compared to extracellular matrix components *in vitro*. *Clin Cancer Res* 2000;6:4839–47.
- Fornaro M, Manes T, Languino LR. Integrins and prostate cancer metastases. *Cancer Metastasis Rev* 2001;20:321–31.
- Cooper CR, Bhatia JK, Muenchen HJ, et al. The regulation of prostate cancer cell adhesion to human bone marrow endothelial cell monolayers by androgen dihydrotestosterone and cytokines. *Clin Exp Metastasis* 2002;19:25–33.
- Glinsky VV, Glinsky GV, Rittenhouse-Olson K, et al. The role of Thomsen-Friedenreich antigen in adhesion of human breast and prostate cancer cells to the endothelium. *Cancer Res* 2001;61:4851–7.
- Glinsky VV, Glinsky GV, Glinskii OV, et al. Intravascular metastatic cancer cell homotypic aggregation at the sites of primary attachment to the endothelium. *Cancer Res* 2003;63:3805–11.
- Glinskii OV, Turk JR, Pienta KJ, Huxley VH, Glinsky VV. Evidence of porcine and human endothelium activation by cancer-associated carbohydrates expressed on glycoproteins and tumour cells. *J Physiol* 2004;554:89–99.
- Schweitzer KM, Drager AM, van der Valk P, et al. Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. *Am J Pathol* 1996;148:165–75.
- Frenette PS, Subbarao S, Mazo IB, von Andrian UH, Wagner DD. Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. *Proc Natl Acad Sci USA* 1998;95:14423–8.
- Mazo IB, Gutierrez-Ramos J-C, Frenette PS, et al. Hematopoietic progenitor cells rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *J Exp Med* 1998;188:465–74.
- Naiyer AJ, Jo DY, Ahn J, et al. Stromal derived factor-1-induced chemokinesis of cord blood CD34(+) cells (long-term culture-initiating cells) through endothelial cells is mediated by E-selectin. *Blood* 1999;94:4011–9.
- Navone NM, Olive M, Ozen M, et al. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. *Clin Cancer Res* 1997;3:2493–500.
- Kozlowski JM, Fidler IJ, Campbell D, et al. Metastatic behavior of human tumor cell lines grown in the nude mouse. *Cancer Res* 1984;44:3522–9.
- Pettaway CA, Pathak S, Greene G, et al. Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin Cancer Res* 1996;2:1627–36.
- Putz E, Witter K, Offner S, et al. Phenotypic characteristics of cell lines derived from disseminated cancer cells in bone marrow of patients with solid epithelial tumors: establishment of working models for human micrometastases. *Cancer Res* 1999;59:241–8.
- Rood PM, Calafat J, von dem Borne AE, Gerritsen WR, van der Schoot CE. Immortalisation of human bone marrow endothelial cells: characterisation of new cell lines. *Eur J Clin Invest* 2000;30:618–29.
- Dimitroff CJ, Bernacki RJ, Sackstein R. Glycosylation-dependent inhibition of cutaneous lymphocyte-associated antigen expression: implications in modulating lymphocyte migration to skin. *Blood* 2002;101:602–10.
- Fuhlbrigge RC, King S, Dimitroff CJ, Kupper TS, Sackstein R. Direct real-time observation of E- and P-selectin-mediated rolling on cutaneous lymphocyte-associated antigen immobilized on western blots. *J Immunol* 2002;168:5645–51.
- Burdick MM, McCaffery JM, Kim YS, Bochner BS, Konstantopoulos K. Colon carcinoma cell glycolipids, integrins, and other glycoproteins mediate adhesion to HUVECs under flow. *Am J Physiol Cell Physiol* 2003;284:C977–87.
- Dimitroff CJ, Lee JY, Rafii S, Fuhlbrigge RC, Sackstein R. CD44 is a major E-selectin ligand on human hematopoietic progenitor cells. *J Cell Biol* 2001;153:1277–86.
- Dimitroff CJ, Pera P, Dall'Olio F, et al. Cell surface N-acetylneuraminic acid alpha2,3-galactoside-dependent intercellular adhesion of human colon cancer cells. *Biochem Biophys Res Commun* 1999;256:631–6.
- Fuhlbrigge RC, Kieffer JD, Armerding D, Kupper TS. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T-cells. *Nature (Lond)* 1997;389:978–81.
- Picker LJ, Warnock RA, Burns AR, et al. The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. *Cell* 1991;66:921–33.
- Jorgensen T, Berner A, Kaalhus O, et al. Up-regulation of the oligosaccharide sialyl Lewis X: a new prognostic parameter in metastatic prostate cancer. *Cancer Res* 1995;55:1817–9.
- Glinskii OV, Huxley VH, Turk JR, et al. Continuous real time ex vivo epifluorescent video microscopy for the study of metastatic cancer cell interactions with microvascular endothelium. *Clin Exp Metastasis* 2003;20:451–8.
- Khaldoyanidi SK, Glinsky VV, Sikora L, et al. MDA-MB-435 human breast carcinoma cell homo- and heterotypic adhesion under flow conditions is mediated in part by Thomsen-Friedenreich antigen-galectin-3 interactions. *J Biol Chem* 2003;278:4127–34.
- Al-Mehdi AB, Tozawa K, Fisher AB, et al. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nat Med* 2000;6:100–2.
- Taichman RS, Cooper C, Keller ET, et al. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* 2002;62:1832–7.
- Mazo IB, von Andrian UH. Adhesion and homing of blood-borne cells in bone marrow. *J Leukocyte Biol* 1999;66:25–32.
- Satoh M, Numahata K, Kawamura S, Saito S, Orikasa S. Lack of selectin-dependent adhesion in prostate cancer cells expressing sialyl Le(x). *Int J Urol* 1998;5:86–91.
- Idikio HA. Sialyl-Lewis-X, Gleason grade and stage in non-metastatic human prostate cancer. *Glycoconjugate J* 1997;14:875–7.
- Martensson S, Bigler SA, Brown M, et al. Sialyl-Lewis(x) and related carbohydrate antigens in the prostate. *Hum Pathol* 1995;26:735–9.
- Jorgensen T, Kanagasigam Y, Kaalhus O, et al. Prognostic factors in patients with metastatic (stage D2) prostate cancer: experience from the Scandinavian Prostatic Cancer Group Study-2. *J Urol* 1997;158:164–70.