Identification of Leukocyte E-Selectin Ligands, P-Selectin Glycoprotein Ligand-1 and E-Selectin Ligand-1, on Human Metastatic Prostate Tumor Cells

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Abstract

Prostate tumor cells, which characteristically metastasize to bone, initiate binding interactions with bone marrow endothelium under blood flow conditions through binding interactions with E-selectin. We hypothesized that E-selectin ligands on prostate tumor cells are directly associated with bone-metastatic potential. In this report, we elucidate the identity of E-selectin ligands on human metastatic prostate tumor cells and examine their association with prostate tumor progression and metastasis *in vivo*. To our surprise, we found that the E-selectin-binding form of P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on the human bone-metastatic prostate tumor MDA PCa 2b cell line. Interestingly, we also found that human prostate tumor cells derived from bone, lymph node, and brain metastases expressed another leukocyte E-selectin ligand, E-selectin ligand-1 (ESL-1). Immunohistochemical analysis of PSGL-1 and ESL-1 in normal prostate tissue and in localized and metastatic prostate tumors revealed that ESL-1 was principally localized to intracellular cell membrane and expressed on all normal and malignant prostate tissue, whereas PSGL-1 was notably detected on the surfaces of bone-metastatic prostate tumor cells. These findings implicate a functional role of PSGL-1 in the bone tropism of prostate tumor cells and establish a new perspective into the molecular mechanism of human prostate tumor metastasis. (Cancer Res 2005; 65(13): 5750-60)

Introduction

The tropism of prostate tumor cells to bone is a common pathologic process (1, 2) and, as evidenced by numerous reports, seems to be regulated by factors inherent to both prostate tumor cells and bone marrow microenvironment (3–6). The identity of bone-specific factors, which promote bone metastasis of prostate cancer, is currently undefined. It has been suggested that the fate of metastases formation in a particular organ is due to survival factors indigenous to that organ and that the initial seeding of tumor cells occurs by vascular lodgment (7). The presence of circulating prostate tumor cells and the conspicuous avidity of prostate tumor cells to bone marrow endothelial cells (BMEC) suggest, to the contrary, that migration of prostate tumor cells to bone may be mediated through a bone-specific homing mechanism (8–11).

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Human hematopoietic progenitor cells (HPC) require the coordinated functional expression of the chemokine receptor, CXCR4, and endothelial-selectin (E-selectin) ligands for migration to human bone marrow (12–17). Whereas CXCR4 binding to stromal cell–derived factor-1α (SDF-1α) mediates HPC migration across BMEC (13), E-selectin ligands expressed on HPC support rolling adhesive interactions on BMEC that initiate a cascade of molecular events and facilitate HPC entry into bone. HPC E-selectin ligands, P-selectin glycoprotein ligand-1 (PSGL-1) and CD44, are specialized membrane protein scaffolds bearing sialyl Lewis X epitopes (HECA-452 antigen) concomitant with E-selectin-binding activity (14). Recent *in vivo* homing experiments indicate that PSGL-1 is a major contributor of HPC E-selectin ligand activity (16, 17), highlighting the importance of BMEC E-selectin and HPC E-selectin ligand(s) in bone homing.

In the context of prostate tumor metastasis, SDF-1α mediates *trans*-BMEC migration of human prostate tumor cells, whereas CXCR4 expression is elevated on localized and bone-metastatic prostate tumors, implicating this mechanism in the bone tropism of prostate tumor cells (10, 18, 19). Recent data from our laboratory show that BMEC E-selectin helps initiate adhesion of bone-metastatic prostate tumor cells with BMEC and that HECA-452 antigen expression is associated with prostate tumor progression (20). We hypothesize, therefore, that circulating prostate tumor cells use a similar bone-homing mechanism as HPC and that acquisition of E-selectin ligand expression may correspond to a bone metastasis phenotype. Although CD44 is a viable E-selectin ligand candidate due to its noted expression on prostate tumor cells (21, 22), other leukocyte E-selectin ligands, such as PSGL-1, L-selectin, and E-selectin ligand-1 (ESL-1), represent other potential E-selectin glycoprotein ligands by virtue of their potential expression of HECA-452 antigen (14, 23–25). The identity of E-selectin ligand(s) on human prostate tumor cells are currently unknown and is the focus of our work described herein.

In this study, we investigated the identity of E-selectin glycoprotein ligands on human prostate tumor cells derived from bone, lymph node, or brain metastases. We found that PSGL-1 bearing HECA-452 antigen and E-selectin-binding determinants, otherwise known as cutaneous lymphocyte-associated antigen (CLA), was expressed on human bone-metastatic prostate tumor cells. In addition, we identified the E-selectin ligand, ESL-1, on all metastatic prostate tumor cells. Immunohistochemical analysis of ESL-1 on normal prostatic tissue and on low- and high-grade prostate tumors revealed that ESL-1 was highly expressed on all prostatic tissue and was principally localized to intracellular membranous structures. PSGL-1 expression, on the other hand, coincided with high E-selectin ligand activity on the

bone-metastatic prostate tumor MDA PCa 2b cell line and was directly associated with bone metastases. Immunohistochemical analysis showed that PSGL-1 was conspicuously detected on the surface of prostate tumor cells in bone, whereas low to negligible levels of PSGL-1 were found on normal prostate epithelium, localized prostate cancer and prostate tumor metastases in nonbone tissue. These data are the first described to date and support the notion that PSGL-1/CLA may facilitate the bone tropism of prostate cancer.

Materials and Methods

Cell lines. Human HPC KG1a cells and murine monocytic WEHI-3 cells (both from American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 with glutamine/10% fetal bovine serum (FBS)/1% penicillin/streptomycin (all from Life Technologies Invitrogen Corp., Grand Island, NY). Human prostate tumor MDA PCa 2b cells derived from bone metastases (26) were propagated in BRFF-HPCI (AthenaES, Baltimore, MD)/20% FBS/1% penicillin/streptomycin. Other human bone-metastatic prostate tumor cell lines, PC-3, PC-3M, PC-3M Pro-4, and PC-3M LN-4 (27, 28), were maintained in RPMI 1640 with glutamine/10% FBS/1% penicillin/streptomycin, whereas PC-R1 and PC-E1 cell lines (generously provided by Dr. Klaus Pantel, Hamburg, Germany; ref. 29) were cultured in RPMI 1640 with glutamine/10% FBS, 1% penicillin/streptomycin, 10 μg/mL transferrin, 5 μg/mL insulin, 10 ng/mL recombinant human epidermal growth factor, and 10 μg/mL recombinant human basic fibroblast growth factor (bFGF). Human lymph node–metastatic prostate tumor cell lines, LNCaP, LNCaP Pro-5, and LNCaP LN-3 (28), and human brain-metastatic prostate tumor DU-145 cells (American Type Culture Collection) were maintained in RPMI 1640 with glutamine/10% FBS/1% penicillin/streptomycin. Human BMEC, HBMEC-60 (kindly provided by Dr. C. Ellen van der Schoot, Sanquin Research at CLB; Amsterdam, the Netherlands; ref. 30), were cultured in Medium199 with HEPES and glutamine/10% FBS/10% human serum/100 μg/mL G418/5 units/mL heparin/1 ng/mL recombinant human bFGF/1% penicillin/streptomycin.

Parallel-plate flow analysis. For cell rolling assessments on E-selectin natively expressed on human BMEC, prostate tumor cells and (+) control KG1a cells were perfused over confluent cultures of HBMEC-60 grown in 35 × 10 mm culture dishes (Corning, Inc., Corning, NY) and stimulated for 4 hours with 10 ng/mL IL-1β (Sigma Co., St. Louis, MO) before assay as previously described (20). To confirm E-selectin expression, cells were harvested with 0.5 mmol/L EDTA and stained with anti-human E-selectin monoclonal antibody (mAb) 68-5H11 (BD Biosciences, Inc., San Jose, CA) for flow cytometric analysis. IL-1β-stimulated HBMEC-60 cells treated with 10 μg/mL neutralizing anti-human E-selectin mAb 68-5H11 for 30 minutes at room temperature was done to confirm E-selectin-mediated adhesion. Prostate tumor cells released with 0.5 mmol/L EDTA and washed twice in PBS were suspended at 1×10^6 cells/mL in HBSS/10 mmol/L HEPES/2 mmol/L $CaCl_2$ assay medium and infused into the chamber over HBMEC-60 cultures as previously described (20). Cell rolling was assessed at 0.6 dynes/cm^2 from the midpoint of the chamber-viewing field (four fields of view and three different experiments) at 100× magnification as previously described (31). All experiments were observed in real time and videotaped for offline analysis.

Immunoprecipitations and Western blot analysis. Membrane preparations of prostate tumor, WEHI-3 and KG1a cells were prepared, and membrane protein was quantified by Bradford method as previously described (14, 20). E-selectin ligand, PSGL-1 and ESL-1 were immunoprecipitated from membrane protein solubilized in 2% NP40 and precleared in Protein G-agarose with recombinant murine E-selectin/human immunoglobulin chimera (R & D Systems, Inc., Minneapolis, MN), mouse immunoglobulin G anti-human PSGL-1 mAb KPL-1 (BD Biosciences), and rabbit polyclonal antisera against ESL-1, respectively. ESL-1 antisera was generously provided by Dr. Bruce Furie (Beth-Israel Deaconess Medical Center, Boston, MA) and was also prepared by immunizing rabbits with keyhole limpet hemocyanin-conjugated peptides from amino acids 157-176 of ESL-1 (Invitrogen Corp., Carlsbad, CA). Solubilized membrane protein precleared in protein G-agarose was mixed with antibody/chimera at a mass ratio of 100:4, incubated for 18 hours on a rotator at 4°C, mixed with protein-G agarose for 2 hours at 4°C, and analyzed by Western blotting. Immunoprecipitations done with respective isotype controls were conducted in parallel to control for nonspecific protein binding. Where indicated, surfaces of WEHI-3 and prostate tumor cells were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce Biotechnology, Inc., Rockford, IL) according to manufacturer's protocol before membrane protein preparation. Biotinylated membrane protein was incubated with avidin-agarose (Vector Laboratories, Burlingame, CA) for 18 hours on a rotator at 4°C and analyzed by Western blotting.

For Western blotting, total membrane protein, biotinylated membrane protein or avidin/immunoprecipitates were subjected to reducing SDS-PAGE on 4% to 20% gradient gels, transferred to Immunoblot polyvinylidene difluoride membrane (Bio-Rad, Inc., Hercules, CA), and blotted with respective antibody. To confirm that quantified membrane protein was equivalent, identically loaded SDS-PAGE gels were prepared in parallel and stained with Coomassie blue R-250. Intensity analysis of Coomassie blue–stained protein showed that membrane protein loaded for blotting experiments was identical. Blots were first blocked in FBS and then incubated with E-selectin/immunoglobulin, anti-PSGL-1 mAb KPL-1, rat immunoglobulin M anti-human CLA mAb HECA-452 (BD Biosciences) or ESL-1 antisera (all at 1 μg/mL) for 1 hour at room temperature. Isotype control immunoblots were done in parallel to evaluate nonspecific protein binding. Blots were then incubated with respective alkaline phosphatase–conjugated secondary antibodies (all at 1:1,000; Zymed Labs., Inc., San Francisco, CA) for 1 hour at room temperature and developed with alkaline phosphatase/substrate Western Blue (Promega, Madison, WI) as previously described (20). These experiments were done a minimum of five times.

Purification and mass spectrometry of E-selectin/immunoglobulin-reactive 150-kDa membrane protein. To isolate the 150-kDa protein(s), we separated MDA PCa 2b membrane protein on reducing 4% to 20% SDS-PAGE gradient gels and analyzed the E-selectin/immunoglobulin-reactive 150-kDa protein as follows. To guide localization, excision and retention of the relevant protein, an E-selectin/immunoglobulin-immunostained blot was prepared in parallel, and the stained blot was superimposed with the corresponding gel. Excised gel fragments from corresponding gels were loaded into a single well (three gel fragments per well) of a new 4% to 20% gradient gel and subjected to reducing SDS-PAGE. This semipurification method was repeated thrice, and the last excised gel fragment was digested with trypsin and analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Molecular Biology Core Facility, Dana-Farber Cancer Institute), and the NCBInr database was searched for possible peptide matches.

Flow cytometry. Cells from suspension cultures or from adherent cultures harvested by 5 mmol/L EDTA were washed twice with cold PBS/2%FBS and suspended in PBS/1%FBS. mAb HECA-452, anti-human PSGL-1 mAb PL-2 (BD Biosciences), anti-human CD44 mAb A3D8 (Sigma), ESL-1 rabbit antisera, anti-human E-selectin mAb 68-5H11, or appropriate isotype-matched control antibody (2 μg/test) was incubated with cells for 30 minutes on ice. Following two washes, cells were incubated with fluorochrome-conjugated secondary antibody for 30 minutes on ice. After washing twice, flow cytometry was done using a FACScan apparatus equipped with an argon laser tuned at 488 nm (Becton Dickinson, San Jose, CA). Cells stained with relevant isotype control antibody were subtracted from cells stained with test antibody to control for nonspecific binding.

Immunohistochemical analysis. For ESL-1 immunohistochemical analysis, we used tissue microarrays containing 4-μm sections of 2-mm cores from formalin-fixed, paraffin-embedded normal prostate tissue and prostate adenocarcinoma (Chemicon International, Inc., Temecula, CA). Prostate tumors with a Gleason score of 2 to 6 were designated as low-grade tumors, and tumors with a Gleason score ranging from 7 to 10 were designated as high-grade tumors. Tissue microarrays were deparaffinized and rehydrated according to the manufacturer's protocol. For antigen retrieval, tissue microarrays were incubated in 10 mmol/L citrate buffer (pH 6.0) in a steam pressure cooker per manufacturer's instructions (Biocare Medical, Walnut Creek, CA). Tissue microarrays were blocked in

hydrogen peroxide and normal goat serum (1:20) and incubated with rabbit antisera against ESL-1 (1:1,000) for 1 hour at room temperature. After washing, tissue microarrays were incubated with horseradish peroxidase (HRP)–linked-anti-rabbit immunoglobulin G (Envision Plus Kit, DakoCytomation, Inc., Carpinteria, CA) for 30 minutes at room temperature. Staining was done using standardized development times and diaminobenzidine (DakoCytomation) as a substrate. All tissue microarrays were counterstained in hematoxylin. For semiquantitative analysis of cell staining, brown-stained tumor cells were enumerated and divided by total tumor cell count per field of view at 200× magnification (0.785 mm^2) and multiplied by 100 to obtain a percent 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 (≤75% positive tumor cell staining), and high (>75% positive tumor cell staining).

For PSGL-1 analysis, tissue microarrays were constructed and immunostained as follows. Tissue microarrays were generated from 30 rapid autopsies and represent prostate tumor specimens from the prostate gland and from all potential metastatic sites (32). The rapid autopsy program was approved by the Institutional Review Board of The University of Michigan. Tissue microarrays cores were assembled using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD) as previously described (33). Tissue cores from designated areas were targeted for transfer to array blocks. Tissue cores in triplicate (0.6 mm in diameter) were sampled from each representative tissue block and spaced at 0.8 mm from core-center to core-center. After construction, 4-μm sections were cut and stained with H&E to verify histologic diagnosis. All data are maintained on a relational database as previously described (34). Tissue microarrays were deparaffinized and rehydrated and then subjected to antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker for 2 minutes. For immunostaining, tissue microarrays were blocked in hydrogen peroxide and normal goat serum (1:20) and incubated with mouse anti-human PSGL-1 antibody (clone PL-2; 1:200) for 1 hour at room temperature. After washing, tissue microarrays were incubated with HRP-linked anti-murine immunoglobulin G (Envision Plus Kit, DakoCytomation), developed, and counterstained as described above.

Semiautomated quantitative image analysis. A semiautomated quantitative image analysis system, ACIS II (Chromavision, San Juan Capistrano, CA), was used to evaluate stained tissue microarrays. For immunohistochemical analysis, proprietary software for the ACIS II device was used to detect and quantify brown staining intensity and then compares this value to blue counterstain representing background. Theoretical intensity levels range from 0 to 255 chromogen intensity units. In pilot experiments for this study, reproducibility of the ACIS II system was tested and confirmed by scoring several tissue microarrays. The correlation coefficient for these experiments was $r^2 = 0.973$. Due to tissue heterogeneity and to the potential of false-positive leukocyte staining with anti-PSGL-1, a pathologist electronically circled the areas of interest on each tissue core with the ACIS II software, ensuring that intensity measurements were consistent with selected stained areas. Benign prostate tissue cores were included on each tissue microarray to help normalize each slide.

Statistical analysis. Intensity values were obtained for each tissue core and normalized within each tissue microarray. Due to potential variation of immunohistochemical staining and intensity values for each tissue microarray, data were normalized for each tissue microarray and from multiple tissue microarrays. Staining intensity values for each tissue core from a given tissue microarray were subtracted by the mean intensity for that same tissue microarray and then divided by the SD:

$$\text{Intensity}_{ij}^{\text{Normalized}} = \frac{\text{Intensity}_{ij} - \text{mean}\ (\text{Intensity}_i)}{\text{SD}\ (\text{Intensity}_i)},$$

where $j = 1, \ldots, n_i$ (n_i is the total number of cores on TMA_i). Thus, each normalized tissue microarray had a mean score of 0 and SD equal to 1. Data were then combined using this normalized scale and analyzed using the SPSS software (SPSS Systems, Chicago, IL).

Results

Analysis of candidate E-selectin ligands on human metastatic prostate tumor cells. To analyze candidate E-selectin ligands, we first confirmed the expression of E-selectin ligand activities on several bone-, lymph node–, and brain-metastatic prostate tumor cell lines in the parallel-plate flow chamber (20). We assayed tumor cell rolling on E-selectin natively expressed on BMEC. In an E-selectin-dependent manner, we found that

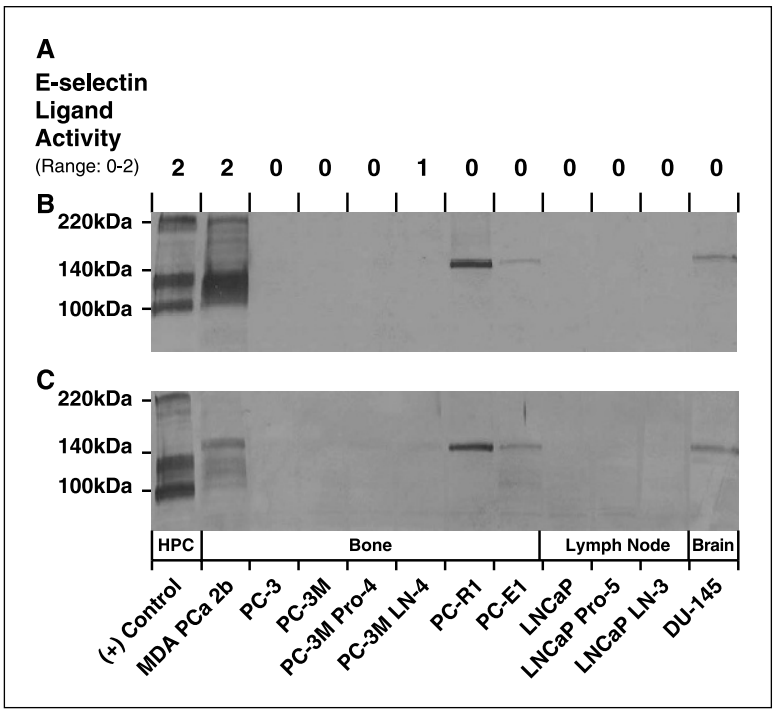


Figure 1. Candidate E-selectin ligand(s) on human metastatic prostate tumor cells. *A,* E-selectin ligand activities of human prostate tumor cell lines derived from bone, lymph node, or brain were analyzed at 0.6 dynes/cm^2 on IL-1β stimulated HBMEC-60 in the parallel-plate flow chamber. Results are expressed as a scoring range from 0 to 2 (0 = no, 1 = low, and 2 = high rolling activity) relative to (+) control E-selectin ligand activity of KG1a cells. Positive control KG1a membrane protein (10 μg/lane) and tumor membrane protein (40 μg/lane) were subjected to SDS-PAGE on 4% to 20% gradient gels, transferred to PVDF membrane, and immunoblotted with mAb HECA-452 (*B*) or with E-selectin/immunoglobulin chimera (*C*). As indicated in (*A*), (*B*), and (*C*), MDA PCa 2b cells possessed high E-selectin ligand activity and exhibited HECA-452-reactive membrane protein from 110 to 130 and at 220 kDa, while E-selectin/immunoglobulin-reactive protein was detected at ~110 to 130 and 150 kDa. All other cell lines possessed low to no activity, although PC-R1, PC-E1, and DU-145 expressed a HECA-452 and E-selectin/immunoglobulin-reactive protein at 150 kDa. Binding data and immunoblots are representative of a minimum of five experiments, and blots stained with AP-secondary antibody alone showed no detectable proteins.

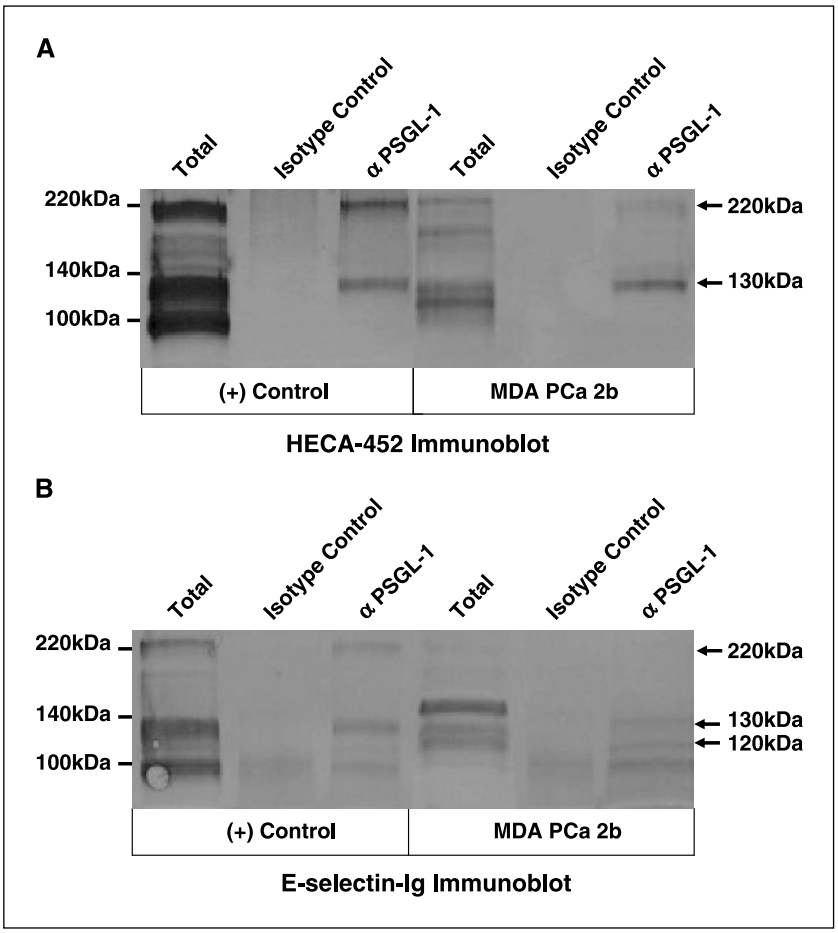


Figure 2. HECA-452-reactive and E-selectin-binding PSGL-1 is expressed on human bone-metastatic prostate tumor cells. Anti-PSGL-1 immunoprecipitates from MDA PCa 2b membrane protein were subjected to SDS-PAGE on 4-20% gradient gels, transferred to PVDF membrane and immunoblotted with mAb HECA-452 (*A*) or E-selectin/immunoglobulin (*E-selectin-Ig, B*). Compared with immunostaining of anti-PSGL-1 immunoprecipitate from KG1a membrane protein and isotype control immunoprecipitates, MDA PCa 2b cells expressed HECA-452-reactive protein at ~130 and 220 kDa and E-selectin/immunoglobulin-reactive protein at 120 and 130 kDa. Stainable protein at 100 kDa was nonspecific as isotype control immunoprecipitates also contained a 100-kDa stained protein. Immunoblots are representative of at least five experiments and isotype control blots (alkaline phosphatase-secondary antibody alone) showed no staining.

bone-metastatic MDA PCa 2b cells exhibited high rolling activity on IL-1β stimulated HBMEC-60 cultures, whereas PC-3M LN4 cells possessed low activity and all other cells lines did not support rolling adhesions (Fig. 1*A*). Flow assays done in the presence of neutralizing anti-human E-selectin antibody completely abrogated rolling activity (data not shown).

We subsequently wanted to determine the repertoire of potential E-selectin glycoprotein ligands on human metastatic prostate tumor cells. Prior studies have indicated that the reactivity of mAb HECA-452 to human HPC membrane proteins on Western blots conferred E-selectin-binding function (14, 23–25). Accordingly, we did Western blot analysis of HECA-452 antigen on membrane protein prepared from prostate tumor cell lines. As shown in Fig. 1*B*, HECA-452-reactive membrane protein from HPC KG1a cells was represented by CD44 at 100 kDa and PSGL-1 at 130 (monomer) and 220 kDa (dimer). Interestingly, prominent HECA-452-stained membrane protein from MDA PCa 2b cells was evident from 110 to 130 kDa and at 220 kDa. Of note, a single HECA-452-reactive band at 150 kDa was detectable on cell lines that did not express E-selectin ligand activity (Fig. 1*B*). To help confirm that HECA-452-reactive proteins corresponded to potential E-selectin glycoprotein ligand(s), we did Western blotting experiments on membrane protein using E-selectin/immunoglobulin chimera as a probe. Similar to HECA-452 immunostaining analysis, we found that KG1a membrane protein stained at 100, 130, and 220 kDa (representing CD44 and PSGL-1) and MDA PCa 2b membrane protein(s) was stained at 110 to 130 kDa with E-selectin/immunoglobulin. A distinct protein at 150 kDa from MDA PCa 2b cells as well as from E-selectin ligand (−) cell lines PC-R1, PC-E1, and DU-145 was also stained with E-selectin/immunoglobulin (Fig. 1*C*).

Because HECA-452-reactive PSGL-1 migrates at 120 to 130 and 220 kDa (14), we examined the expression of HECA-452 antigen on anti-human PSGL-1 immunoprecipitate from MDA PCa 2b membrane protein. HECA-452 immunostaining of anti-PSGL-1 immunoprecipitate from KG1a membrane protein clearly showed the expression and gel mobility pattern of PSGL-1 (Fig. 2*A*). To our surprise, we found that anti-human PSGL-1 immunoprecipitate from MDA PCa 2b membrane protein was also reactive to mAb HECA-452 at 130 and 220 kDa (Fig. 2*A*). By definition, HECA-452-reactivity of PSGL-1 confers E-selectin-binding activity and designation as CLA (14, 23, 31). CLA is a major E-selectin ligand on human HPC and on human skin-homing T cells (14, 23, 31). Anti-human PSGL-1 immunoprecipitate from KG1a membrane protein was similarly immunostained with E-selectin/immunoglobulin at 130 and 220 kDa, whereas anti-PSGL-1 immunoprecipitate from MDA PCa 2b cells was stained with E-selectin/immunoglobulin at 120 and 130 kDa (Fig. 2*B*). Absence of staining at 220 kDa in anti-PSGL-1 immunoprecipitate from MDA PCa 2b cells was also noted, suggesting that E-selectin-binding species were only detectable on Western blots in the monomer form. Staining of isotype control immunoprecipitates at 100 kDa and not at 120 and 130 kDa with E-selectin/immunoglobulin indicated that staining at 100 kDa was nonspecific and that staining of anti-PSGL-1 immunoprecipitate at 120 and 130 kDa was PSGL-1 (Fig. 2*B*).

Because all other prostate tumor cell lines did not express CLA, we investigated by fluorescence-activated cell sorting (FACS) analysis whether PSGL-1 polypeptide was inherently expressed on metastatic prostate tumor cells. We found that PSGL-1 polypeptide was variably expressed on all metastatic prostate tumor cell lines (5-75% positive; Table 1). As previously reported, prostate tumor cells express CD44 (21, 22); thus, we considered the possibility that CD44 could represent HECA-452-reactive membrane protein at 110 kDa on MDA PCa 2b cells. FACS analysis of CD44 showed that CD44 expression was high on most metastatic prostate tumor cell lines; however, CD44 was notably absent on MDA PCa 2b cells (Table 1). Collectively, these results showed that CLA expression and not CD44 expression was associated with robust E-selectin ligand activity on prostate tumor cells and that CLA was expressed on human bone-metastatic prostate MDA PCa 2b tumor cells. The fact that CLA expression was noted only on MDA PCa 2b cells among several other bone-metastatic prostate tumor cell lines should not weaken the mechanistic implications of these findings. For example, studies on the identification of E-selectin ligands on hematopoietic stem cells, namely CD44 (HCELL) and PSGL-1, were done using a single human HPC line (KG1a), which led to subsequent analyses and confirmation that HCELL and E-selectin-binding PSGL-1 were also expressed on freshly isolated human HPC (14).

The distinct E-selectin/immunoglobulin-reactive 150-kDa membrane protein on MDA PCa 2b cells prompted subsequent biochemical analyses to identify this protein. We did sequential SDS-PAGE purification of the gel fragments corresponding to the E-selectin/immunoglobulin-stained 150-kDa protein and analyzed resolved protein(s) in the resultant gel fragment by MALDI-TOF mass spectrometry. Estimated molecular weights of trypsin-digested peptides in this gel fragment strongly corresponded to the molecular identity of a sialylated membrane glycoprotein known as E-selectin glycoprotein ligand (ESL-1; refs. 35, 36). ESL-1 is a major E-selectin ligand on murine myelocytes and has high homology to the type 1 sialo-membrane protein of the medial cisternae of the Golgi apparatus, MG-160 (35–37). To confirm the localization of ESL-1 at 150 kDa, we immunoblotted MDA PCa 2b membrane protein and membrane protein from ESL-1-positive WEHI-3 cells with rabbit immunoglobulin G antisera raised against ESL-1. We found that ESL-1 expression was evident and colocalized with the E-selectin/immunoglobulin-reactive 150-kDa protein from MDA PCa 2b cells (Fig. 3*A*). To verify that the 150-kDa protein on MDA PCa 2b cells was ESL-1, we blotted E-selectin/immunoglobulin-purified protein with ESL-1 antisera, and alternatively, blotted anti-ESL-1 immunoprecipitate with E-selectin/immunoglobulin. As shown in Fig. 3*B*, ESL-1 was detectable in E-selectin/immunoglobulin affinity-purified eluate and not in eluate prepared in the presence of EDTA, which confirmed the requirement of Ca^{2+} for ligand binding activity. Likewise, anti-ESL-1 immunoprecipitate from WEHI-3 and MDA PCa 2b membrane protein was stained with E-selectin/immunoglobulin, whereas isotype control immunoprecipitates did not stain with E-selectin/immunoglobulin (Fig. 3*C*).

Table 1. FACS analysis of candidate E-selectin ligands on human metastatic prostate tumor cells

Cell lines	Percent positive cell staining (MCF)*	
	CD44	PSGL-1
Positive control (KG1a cells)	100 (470)	100 (36)
Bone-metastatic lines		
MDA PCa 2b	1 (20)	75 (31)
PC-3	100 (79)	6 (21)
PC-3M	100 (161)	35 (20)
PC-3M Pro-4	72 (41)	9 (18)
PC-3M LN-4	100 (51)	37 (50)
PC-R1	100 (193)	13 (17)
PC-E1	98 (365)	27 (74)
Lymph node–metastatic lines		
LNCaP	13 (50)	22 (196)
LNCaP Pro-5	21 (96)	37 (166)
LNCaP LN-3	4 (13)	34 (22)
Brain-metastatic lines		
DU-145	99 (137)	5 (31)

Abbreviation: MCF, mean channel fluorescence.
*Flow cytometric analysis of CD44 and PSGL-1 on human metastatic prostate tumor cell lines with anti-CD44 mAb A3D8 (1 μg/test) and anti-PSGL-1 mAb PL-2 (1 μg/test). Percent positive cell staining and mean channel fluorescence indicates the number of cells staining greater than negative isotype control cell staining.

To examine the presence of ESL-1 polypeptide on all metastatic cell lines, we blotted tumor cell membrane protein with ESL-1 antisera. We found that ESL-1 polypeptide, representing both functional and nonfunctional forms, was expressed all cell lines (Fig. 4*A*). However, FACS analysis of ESL-1 was relatively low to absent on most cell lines (0-13% positive), with the exception of WEHI-3 cells (91% positive) and LNCaP LN-3 cells (63% positive; Table 2). These data suggested that ESL-1 expression as detected by Western blotting experiments may be localized to intracellular membranous structures. To help validate this notion, we did Western blot analysis of ESL-1 in avidin/agarose-purified eluate of surface biotinylated membrane protein. Compared with the presence of ESL-1 in avidin-purified eluate from WEHI-3 cells, ESL-1 was relatively absent in avidin-purified eluate from prostate tumor cell lines (Fig. 4*B*). Results from these experiments indicated that ESL-1 in either E-selectin-binding or nonbinding forms was expressed on metastatic prostate tumor cells and that ESL-1/MG-160 seemed to be an intracellular membrane protein as previously reported (35–37).

***In vivo* expression of E-selectin ligand-1 on normal and malignant prostate tissue.** To investigate the role of ESL-1 as a marker of prostate tumor progression, we did immunohistochemical analysis of ESL-1 on tissue microarrays of normal prostatic tissue and of low-grade (score of 2-6) and high-grade (score of 7-10) prostate tumor tissue. Control staining experiments were done using paraffin-embedded MDA PCa 2b cell pellets to determine appropriate ESL-1 antisera concentrations for antigen-specific detection (data not shown). As summarized in Fig. 4*C*, ESL-1 expression was relatively high on all normal prostate epithelial cells and prostate tumor cells from localized cancer, as 75% of cells stained positive with ESL-1 antisera. Furthermore, we observed intense perinuclear staining, which was localized to intracellular structures of the Golgi apparatus (Fig. 4*D*). These results in collaboration with Western blot and FACS analysis of ESL-1 on metastatic prostate tumor cell lines suggested that ESL-1 was expressed as an intracellular membrane protein on all normal and malignant prostate tissue and was not associated with prostate tumor progression and metastasis.

***In vivo* expression of P-selectin glycoprotein ligand-1 in prostate cancer.** Prior immunohistochemical analysis of PSGL-1

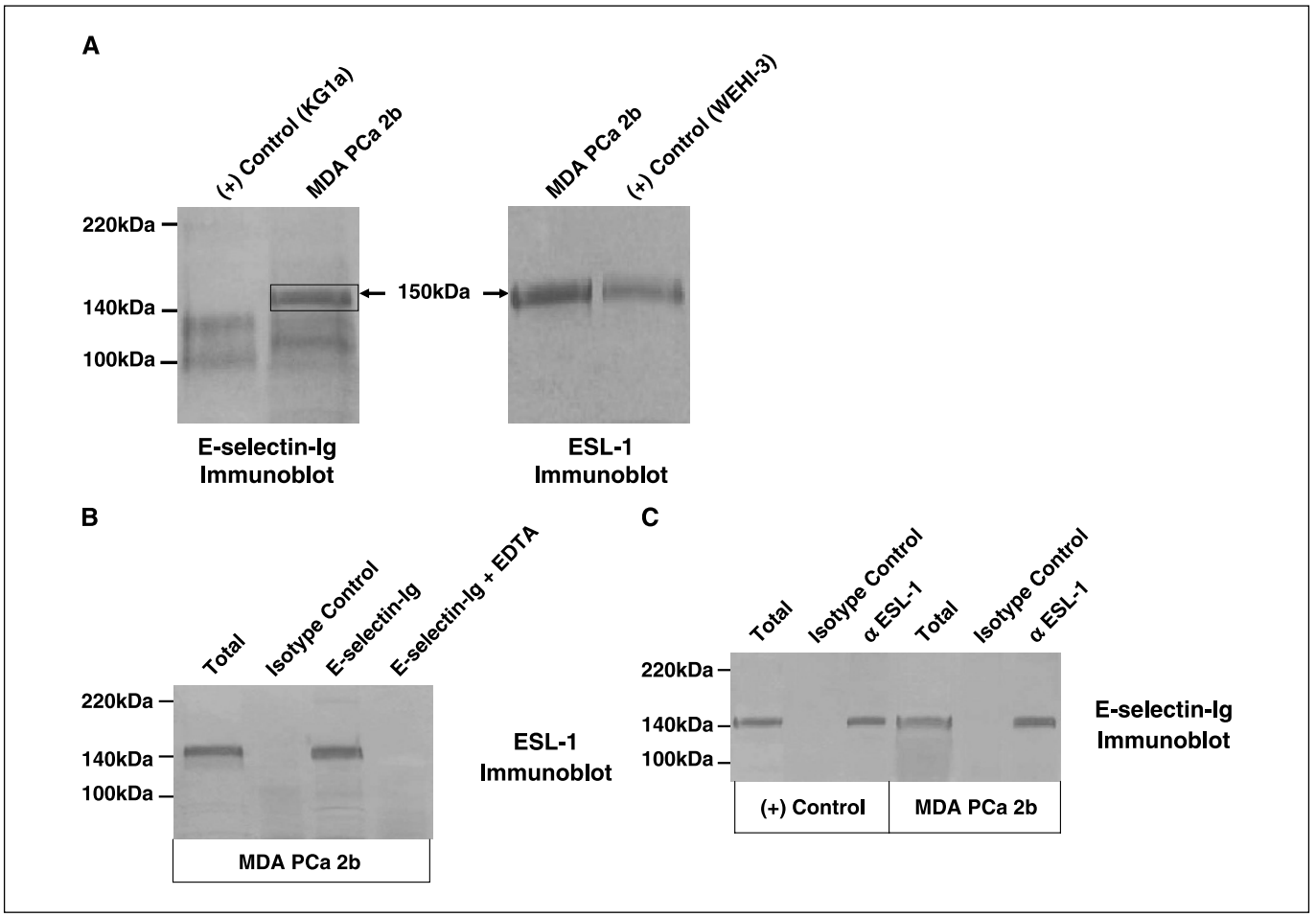


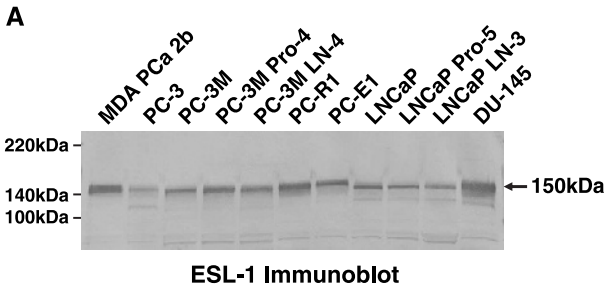
Figure 3. ESL-1 is a candidate E-selectin ligand on human bone-metastatic prostate tumor cells. Membrane protein and immunoprecipitates were resolved on 4% to 20% SDS-PAGE gels, transferred to PVDF membrane, and blotted as indicated. *A,* ESL-1 was identified from the gel fragment (box) corresponding to the E-selectin/immunoglobulin (*E-selectin-Ig*)-reactive membrane protein at 150 kDa from MDA PCa 2b cells by mass spectrometry. Immunoblot analysis of (+) control WEHI-3 and of MDA PCa 2b membrane protein (40 μg/lane) with ESL-1 antisera showed a stainable protein at 150 kDa. To determine whether ESL-1 was the E-selectin glycoprotein ligand at 150 kDa, E-selectin/immunoglobulin affinity-purified eluate from MDA PCa 2b membrane protein (300 μg) was blotted with ESL-1 anti-sera (*B*). In addition, anti-ESL-1 immunoprecipitates of MDA PCa 2b membrane protein (300 μg) were immunoblotted with E-selectin-Ig (*C*). *B,* the 150-kDa protein was detected in E-selectin/immunoglobulin eluate with ESL-1 antisera and not in the E-selectin/immunoglobulin eluate containing EDTA. *C,* the 150-kDa protein was blotted with E-selectin/immunoglobulin in membrane protein (40 μg/lane) and anti-ESL-1 immunoprecipitates from WEHI-3 and MDA PCa 2b cells. Immunoblots performed with alkaline phosphatase-secondary antibody alone did not result in background staining. Immunoblots are representative of a minimum of five experiments.

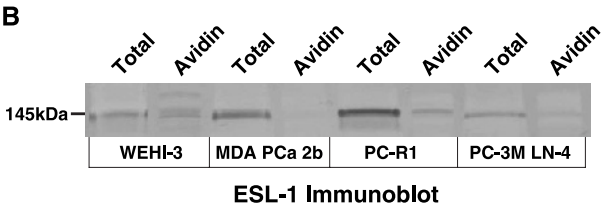
has indicated that PSGL-1 antigen is primarily found on hematopoietic cells: PSGL-1 is broadly expressed on immature HPC and on mature leukocytes, including monocytes, granulocytes, dendritic cells, and lymphocytes (38). To investigate the expression of PSGL-1 in prostate cancer, we did immunohistochemical analysis of PSGL-1 on tissue microarrays constructed from benign prostate tissue, localized prostate cancer, and metastatic deposits from a number of tissues. Preliminary immunohistochemical experiments were first done on paraffin-embedded MDA PCa 2b and (+) control KG1a cell pellets with anti-human PSGL-1 mAb PL-2 to ascertain relevant dilutions of mAb PL-2 and of secondary antibodies and development reagents for PSGL-1-specific reactivity (data not shown). Following immunohistochemistry with mAb PL-2 on tissue microarrays, benign prostate epithelium and malignant prostate tumor cells within the cores were identified by histologic examination, and staining intensities pertaining to selected prostatic tissue were normalized and analyzed using ACIS II image analysis. Of note, intravascular granulocytes were routinely detected with mAb PL-2, serving as an internal positive control for mAb PL-2 reactivity. Normalized PL-2 staining intensity data from these experiments showed that PSGL-1 expression was significantly elevated on prostate tumor cells from bone metastases compared with staining levels on benign prostate epithelial cells and on prostate tumor cells from localized cancer (independent t test, $P < 0.009$; Fig. 5*A*). There was also evidence that PSGL-1 was expressed on prostate tumor cells in lung, lymph node, liver, bladder, and soft tissue metastases (Fig. 5*B*). As illustrated in Fig. 5*C*, PSGL-1 expression on prostate tumor cells within bone-metastatic tissue was robust, whereas PSGL-1 levels on benign prostate epithelium and localized prostate cancer were largely negative. Granulocytes in blood vessels were distinctly positive for PSGL-1 (Fig. 5*C*) and PSGL-1 (+) HPC were routinely detected in bone marrow tissue.

Discussion

We have previously shown that expression of the sialyl Lewis X-like epitope, HECA-452 antigen, is associated with prostate tumor progression (20). HECA-452 antigen expression on prostate

tumor cell membrane glycoprotein(s) and not HECA-452 expression itself correlates with the capacity of human bone-metastatic prostate tumor cells to initiate adhesive contact with BMEC through E-selectin-mediated rolling adhesions (20). Because prostate cancer characteristically metastasizes to bone, we have speculated that binding interactions between E-selectin glycoprotein ligand(s) on circulating prostate tumor cells and E-selectin constitutively expressed on BMEC (39) are critical to formation of prostate tumor metastasis. The importance of E-selectin ligand/BMEC E-selectin adhesive interactions in bone tropism is not unfounded, as human HPC homing to bone in part requires the functional expression of E-selectin ligands (i.e., HCELL and





C

Immunohistochemical Analysis of ESL-1 in Normal Prostate Tissue and in Low- and High-Grade Prostate Tumors

Normal Prostate Tissue and Prostate Tumor Tissue (Gleason Scoring Range)	ESL-1 Expression*		
	Absent	Weak to Moderate	High
Normal Prostate Tissue (n=10)	0	2	8
Low Grade Tumors (n= 17) (Gleason Score, 2-6)	0	2	15
High Grade Tumors (n= 13) (Gleason score, 7-10)	0	1	12

*Using isotype control staining as a reference for background levels, cell staining was graded as absent (≤ 1% positive tumor cell staining), weak to moderate (≤ 75% positive tumor cell staining) and high (>75% positive tumor cell staining).

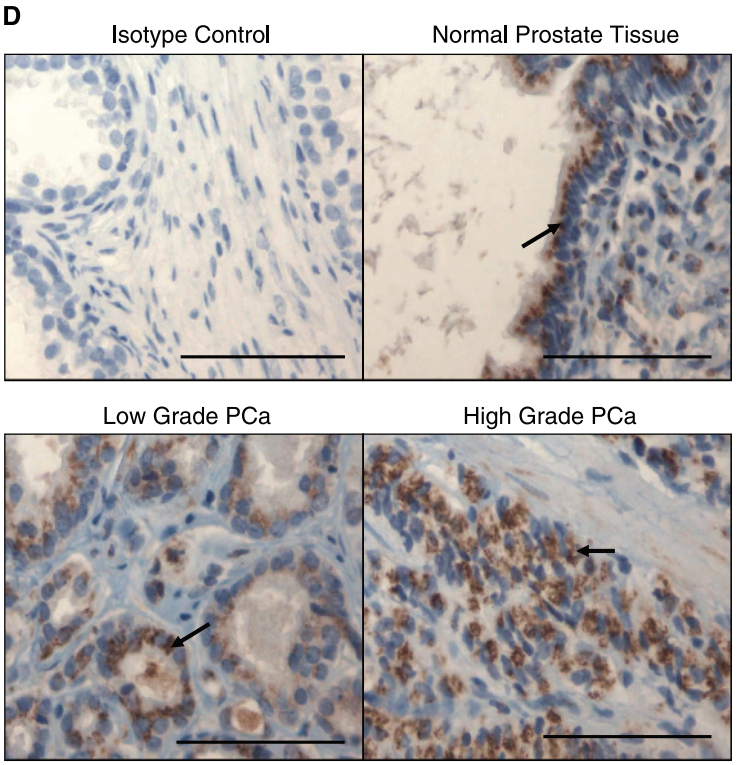


Figure 4. ESL-1 is constitutively expressed and localized to intracellular membranes of normal and malignant prostate epithelium. *A,* membrane protein (40 μg) from human prostate tumor cells were resolved on 4% to 20% SDS-PAGE gradient gels, transferred to PVDF membrane, and blotted with ESL-1 anti-sera. *B,* biotinylated membrane protein from the surface of tumor cells was affinity purified with avidin-agarose and blotted with ESL-1 anti-sera. Compared with staining of ESL-1 from total protein and from avidin-purified (+) control WEHI-3 membrane protein, ESL-1 was relatively absent in avidin-purified protein from prostate tumor cells. Immunoblots in (*A*) and (*B*) were done a minimum of five times. *C,* this table summarizes ESL-1 staining levels on normal prostatic tissue and on low and high grade prostate tumors. *D,* representative photomicrographs of immunohistochemical analysis of ESL-1 on normal prostate tissue and on localized, low- and high-grade prostate cancer showed that ESL-1 staining (in brown) was found on intracellular Golgi structures (arrows indicate perinuclear membranous structures). *Bar*, 100 μm.

Table 2. FACS analysis of ESL-1 on human metastatic prostate tumor cells

Cell lines	Percent positive cell staining (MCF)*, ESL-1
Positive control (WEHI-3 cells)	91 (46)
Bone-metastatic lines	
MDA PCa 2b	1 (11)
PC-3	14 (11)
PC-3M	13 (21)
PC-3M Pro-4	3 (1)
PC-3M LN-4	2 (1)
PC-R1	10 (9)
PC-E1	7 (8)
Lymph node–metastatic lines	
LNCaP	13 (10)
LNCaP Pro-5	9 (12)
LNCaP LN-3	62 (30)
Brain-metastatic lines	
DU-145	0 (10)

*Flow cytometric analysis of ESL-1 on human metastatic prostate tumor cell lines with rabbit immunoglobulin G antisera against ESL-1 (1 μg/test). Percent positive cell staining indicates the number of cells staining greater than negative isotype control cell staining.

PSGL-1/CLA; refs. 14–17). The coordinated expression of E-selectin ligands and chemokine receptor(s), such CCR4 on skin-homing T cells and CXCR4 on bone-homing HPC, more notably, is critical for tissue-specific migration (40–42). The fact that human prostate tumor cells express functional CXCR4 and CXCR4 expression is related to prostate tumor metastasis to bone strongly suggests that E-selectin ligands may help promote bone tropism of prostate tumor cells (10, 18, 19).

In this report, we investigated the identity of E-selectin glycoprotein ligands on human metastatic prostate tumor cells. Using the parallel-plate flow chamber, we initially ascertained the prevalence of E-selectin ligand activities on prostate tumor cell lines derived from bone, lymph node, or brain metastases. We observed that E-selectin ligand activity was notably robust on the bone-metastatic MDA PCa 2b cell line, whereas all other cell lines exhibited low to nil activity (20). Because we have previously speculated that HECA-452-reactive glycoproteins represent candidate E-selectin ligand(s) (refs. 14, 20), we first did Western blot analysis of HECA-452 antigen on membrane proteins from both E-selectin ligand (+) and (−) cell lines. Western blotting membrane protein with E-selectin/immunoglobulin chimera was also conducted to help show whether prospective HECA-452-reactive glycoprotein(s) expressed E-selectin ligand activity. We found that distinct HECA-452- and E-selectin/immunoglobulin-reactive membrane proteins from MDA PCa 2b cells were resolved at ~120 and 220 kDa as well as at the 150-kDa protein, which was also detected on cells that did not possess ligand activity. To identify E-selectin glycoprotein ligand(s) at ~120 and 220 kDa, we exercised a candidate immunoprobe approach due to the known molecular weights of leukocyte E-selectin ligand PSGL-1 (14). We did Western blot analysis of HECA-452 antigen and E-selectin ligand expression on anti-PSGL-1 immunoprecipitates from MDA PCa 2b cells and found that the E-selectin-binding glycoform of PSGL-1 was present. Subsequent FACS analysis of PSGL-1 revealed that PSGL-1 polypeptide was variably expressed at low levels on most metastatic prostate tumor cell lines. To identify the E-selectin/immunoglobulin-reactive membrane protein at 150 kDa, we did mass spectrometry on a trypsin-digested gel fragment corresponding to E-selectin/immunoglobulin-staining region at 150 kDa. We found that the molecular weights of digested peptides closely correlated with a known sialo-membrane glycoprotein, ESL-1 (also called MG-160; refs. 35–37). Western blot analysis of affinity-purified E-selectin ligand(s) with ESL-1 antisera and of anti-ESL-1 immunoprecipitates with E-selectin/immunoglobulin chimera was subsequently executed to confirm ESL-1 expression and E-selectin-binding activity. Further analysis of ESL-1 expression revealed that all metastatic prostate tumor cells expressed ESL-1 in membrane preparations, whereas ESL-1 expression was negative as determined by flow cytometry. Indeed, ESL-1 immunoblotting experiments using avid-purified membrane protein from biotinylated whole cells strongly suggested that ESL-1 was not be expressed on the cell surface. Collectively, results from these experiments showed that both leukocyte E-selectin ligands, PSGL-1 and ESL-1, were expressed on human metastatic prostate tumor cells. However, only PSGL-1 bearing HECA-452 antigen (called CLA; at 120 and 220 kDa) expressed at high levels on MDA PCa 2b cells was coincident with robust cellular E-selectin ligand activity. ESL-1, on the other hand, was expressed, either in its E-selectin-binding or non–E-selectin-binding form, on all cells independent of cellular E-selectin ligand activity. It should be noted that the MDA PCa 2b cell line, opposed to all other bone-metastatic prostate tumor cell lines, is unique in its capacity to recapitulate the molecular and growth characteristics of bone metastases from patients with late stage disease (26). Because the synthetic pathway of E-selectin ligands is governed by a coordinated set of glycosyltransferases and a distinct molecular trafficking mechanism that helps target the glycosylation of specific membrane scaffolds, we believe that MDA PCa 2b cells provided a biological tool to help dissect the potential molecular mediators of E-selectin binding to BMEC. The peculiarity of PSGL-1 and ESL-1 expression on cell lines was subsequently analyzed on localized and metastatic prostate tumor cells *in vivo*.

Immunohistochemical analysis of ESL-1 on normal and malignant prostate tissue showed that ESL-1 was notably expressed at high levels on all prostatic tissue and restricted to intracellular membranous structures. These results paralleled data from other studies showing ESL-1 or MG-160 expression on the Golgi apparatus of human cells (36, 37, 43). MG-160, first identified as a 160-kDa sialoglycoprotein of the medial cisternae of the Golgi apparatus in rat brain (44), has been detected as a Golgi membrane protein in several human fetal and adult tissues, including normal and malignant human brain tissue (43, 45). Our prior findings showing that HECA-452-reactive membrane protein contributes 40% of cellular E-selectin activity (20) coupled with evidence of intracellular localization of ESL-1 and of PSGL-1 expression on metastatic prostate tumor cells indicate that ESL-1 may not be a functional E-selectin ligand and that PSGL-1 could be a key contributor to E-selectin ligand activity on human bone-metastatic prostate tumor cells.

To investigate the relationship between PSGL-1 expression and prostate tumor progression and metastasis, we immunostained normal, benign, and malignant prostate tissue, as well as a number of metastatic lesions from various tissues. Other groups investigating PSGL-1 expression by immunohistochemical analysis have surveyed both hematopoietic and nonhematopoietic tissues (38)

and have shown that PSGL-1 staining is expressed almost exclusively on immature and mature leukocytes from peripheral blood and from within hematopoietic and nonhematopoietic tissues (38). In our analysis, with the exception of granulocytes and lymphocytes in vessels and hematopoietic tissues, we found that normal, benign, and malignant prostatic tissues were largely negative for PSGL-1 expression. However, 95% confidence intervals from normalized PSGL-1 staining intensities of prostate tumor cells in bone metastases compared with staining intensities of epithelia in benign prostate tissue and localized prostate cancer showed that PSGL-1 expression was statistically higher on prostate tumor cells in bone metastases (independent t test, $P < 0.009$). In addition, there was PSGL-1 staining on prostate tumor cells from other potential metastatic tissues, which corroborated with detection of PSGL-1 on other non–bone-metastatic prostate tumor cell lines. Because metastatic tumor tissues were not traceable to the primary prostate tumors, we could not directly ascertain the potential causal relationship between PSGL-1 expression and tumor metastasis. Whether acquisition of PSGL-1 expression occurs on specialized tumor cells within localized prostate cancer, on circulating prostate tumor cells or on prostate tumor cells within the bone marrow parenchyma is unknown and currently under investigation.

In summary, our studies elucidate potential E-selectin glycoprotein ligands on human metastatic tumor cells and show that PSGL-1/CLA is a major candidate E-selectin glycoprotein ligand on

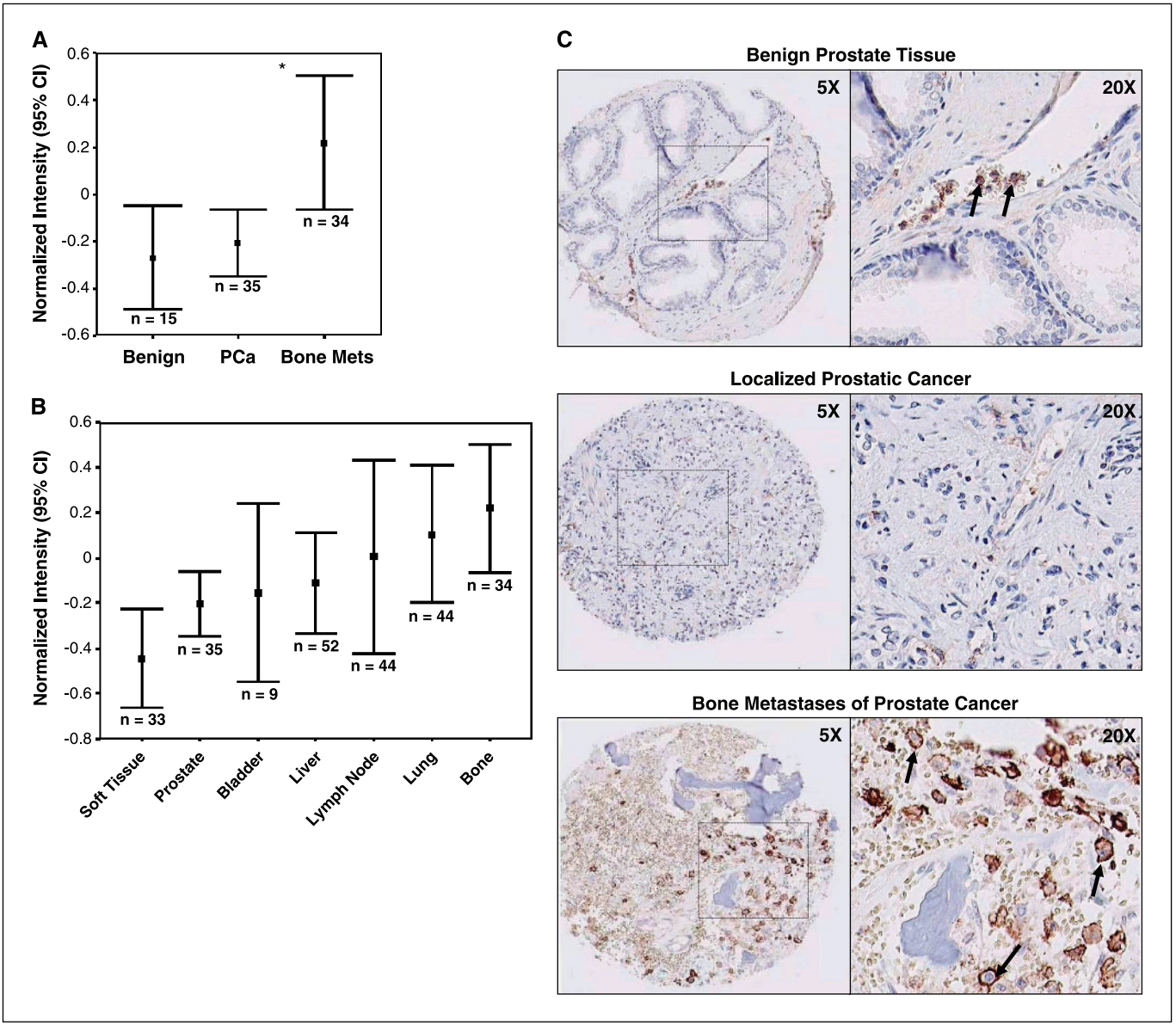


Figure 5. PSGL-1 expression is up-regulated on prostate tumor cells in bone metastasis. Tissue microarrays containing benign prostatic tissue and localized and metastatic prostate cancer were immunostained with anti-human PSGL-1 mAb PL-2. *A,* 95% confidence intervals of normalized mean staining intensity indicate that bone metastases possessed a higher level of PSGL-1 expression than on benign prostate tissue and on localized prostate cancer. *, $P < 0.009$ (independent t test). *B,* in addition, prostate tumor cells in bone possessed the highest level of PSGL-1 expression compared with expression on prostate tumor cells from other metastatic sites (n = number of core specimens). *C,* representative photomicrographs of PSGL-1 staining showed that PSGL-1 (in brown) was detectable on the surface of prostate tumor cells in bone metastases (*arrows*) and largely absent on benign prostatic epithelium and on localized prostate tumor cells. Of note, as a positive control, PSGL-1 expression on neutrophils was routinely detected in blood vessels as illustrated with arrows in the 20× magnification image of benign prostate tissue.

bone-metastatic tumor cells and PSGL-1 expression may be associated with bone metastasis. Coupled with the role of CXCR4 in prostate tumor metastasis, we speculate that bone metastasis of prostate cancer is a pathobiological outcome, which mirrors the process of HPC homing to bone. Interestingly, we found that E-selectin-binding form of CD44 (HCELL) was not expressed on metastatic prostate tumor cells as previously described on HPC (14). CD44, in the context of prostate tumor metastasis, most likely functions as a hyaluronic acid receptor mediating firm adhesion with BMEC (21, 22). The hyaluronan-binding feature of CD44 has previously been found to play a role in the trafficking of human $CD34^+$ stem cells to bone marrow (46), which further argues the mechanistic similarities between bone metastasis of prostate cancer and HPC homing to bone. Other adhesion molecules mediating prostate tumor cell binding interactions with BMEC and with bone marrow hematopoietic, osteogenic, and stromal elements are undoubtedly also required for seeding, retention, and growth of prostate tumor cells in bone (22, 47–50). We believe that broadening our understanding of the "HPC mimicry" or bone-homing behavior of prostate tumor cells will help expand our current view of the pathogenesis of bone metastasis. *In vivo* mouse experiments tailored to examine the homing efficiency of E-selectin ligand (+) or (−) human metastatic prostate tumor cells into human bone will need to be done to fully appreciate the functional role of E-selectin ligand(s). Information from these analyses will instigate the development of new therapeutic strategies for inhibiting the bone tropism of prostate tumor cells.

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