CD44 Is a Major E-Selectin Ligand on Human Hematopoietic Progenitor Cells

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Abstract. E-selectin plays a critical role in mediating tissue-specific homing of T cells into skin, and of primitive hematopoietic progenitor cells (HPCs) into bone marrow (BM). Though it is known that a glycoform of PSGL-1 (CLA) functions as the principal E-selectin ligand on human T lymphocytes, the E-selectin ligand(s) of human HPCs has not been identified. We used a shear-based adherence assay to analyze and define the E-selectin ligand activity of membrane proteins from human HPCs. Our data show that PSGL-1 expressed on human HPCs is an E-selectin ligand, and that HPCs also express a previously unrecognized E-selectin ligand, CD44. The E-selectin ligand activity of CD44 is conferred by the elaboration of sialylated, fucosylated binding determinants on *N*-glycans. This glycoform of CD44 is expressed on primitive CD34+ human HPCs, but not on more mature hematopoietic cells. Under physiologic flow conditions, this molecule mediates E-selectin–dependent rolling interactions over a wider shear range than that of PSGL-1, and promotes human HPC rolling interactions on E-selectin expressed on human BM endothelial cells. These findings offer new insights into the structural biology and physiology of CD44, and into the molecular basis of E-selectin–dependent adhesive interactions that direct homing of human HPC to BM.

Key words: hematopoietic stem cells • E-selectin • CD44 • PSGL-1 • CLA

Introduction

The selectin family of adhesion molecules consists of lectins that mediate leukocyte tethering and rolling interactions on endothelial cells. Their expression on either the leukocyte (L-selectin) or endothelium (P- and E-selectin) helps the leukocyte "slow down," enabling cells in flow to respond to chemokine/cytokine signals and, thus triggered, to firmly attach to and extravasate into a tissue site. This primary intravascular braking mechanism is controlled by the Ca^{2+}-dependent binding activity of the selectins directed against respective sialylated and fucosylated ligands expressed on leukocytes and/or endothelial cells. Selectin-mediated interactions are critical not only for the rapid and efficient recruitment of leukocytes at a site of injury (Kansas, 1996), but also for steady-state, tissue-specific homing as illustrated in (a) lymphocyte homing to peripheral lymph nodes (Kansas, 1996), (b) cutaneous tropism of human skin–homing T cells (Picker et al., 1990; Berg et al., 1991), and (c) hematopoietic progenitor cell (HPC)[1] entry into bone marrow (BM) (Frenette et al., 1998; Mazo et al., 1998; Naiyer et al., 1999). The latter two paradigms represent physiologically relevant processes that, at least in part, are dependent on the cell-adhesive interactions between vascular E-selectin, which is constitutively expressed on postcapillary venules in the skin (Weninger et al., 2000), and BM (Schweitzer et al., 1996) and its leukocyte E-selectin ligands. Therefore, the cell-specific expression of E-selectin ligands is important for understanding tissue tropisms, but our understanding of the identity and structure of these molecules in humans is incomplete.

Rolling of human HPCs on immobilized E-selectin has been observed in flow chamber studies, with higher efficiency rolling of primitive CD34+ "stem" cells compared with CD34− HPCs (Greenberg et al., 2000). The nature of these E-selectin ligands on human HPCs has not been elucidated. To date, the only E-selectin glycoprotein ligand identified on HPCs is the 220-kD sialomucin-like protein,

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[1]*Abbreviations used in this paper:* AML, acute myelogenous leukemia; AP, alkaline phosphatase; BM, bone marrow; BMEC-1, BM endothelial cell line-1; CLA, cutaneous lymphocyte antigen; HA, hyaluronic acid; HCELL, hematopoietic cell E-/L-selectin ligand; HPC, hematopoietic progenitor cell; OSGE, O-sialoglycoprotein endopeptidase; PSGL-1, P-selectin glycoprotein ligand 1; PVDF, polyvinylidene difluoride; VCAM, vascular cell adhesion molecule; VLA, very late antigen.

P-selectin ligand glycoprotein 1 (PSGL-1). Notably, a putative E-selectin ligand known as ESL-1 has been described in murine leukocytes (Steegmaier et al., 1995), but its function and expression in human cells is uncertain. Though PSGL-1 can function as a ligand for all three selectins (Asa et al., 1995; Tu et al., 1996), the E-selectin ligand activity of PSGL-1 depends on appropriate post-translational glycosylation of the core protein. When decorated with the relevant sialylated, fucosylated oligosaccharides detected by the rat monoclonal antibody mAb HECA-452, expression of PSGL-1 on subsets of memory T cells confers the ability of these cells to enter cutaneous sites through interactions with E-selectin (Picker et al., 1990, 1991a; Rossiter et al., 1994; Fuhlbrigge et al., 1997). Forms of PSGL-1 on T cells lacking HECA-452 epitope(s) do not bind E-selectin, and for this reason HECA-452–reactive carbohydrates on PSGL-1 are designated as cutaneous lymphocyte antigen (CLA) (Fuhlbrigge et al., 1997). Though it is well recognized that human CD34+ HPCs express PSGL-1 (Zannettino et al., 1995; Levesque et al., 1999; Sackstein and Dimitroff, 2000) and that human CD34+ HPCs bind to E-selectin expressed on human BM endothelial cells (Schweitzer et al., 1996; Naiyer et al., 1999; Rood et al., 1999), there is neither direct evidence that PSGL-1 on human HPCs is an E-selectin ligand, nor any information on the identity of another potential human HPC E-selectin glycoprotein ligand(s).

Because expression of HECA-452 epitope(s) is functionally correlated with E-selectin ligand activity, we sought to determine whether glycoproteins bearing HECA-452 antigen(s) on human HPCs could function as E-selectin ligands. Using a novel blot-rolling adherence assay developed in our laboratory, we demonstrate that several HECA-452–reactive glycoproteins expressed on human HPCs function as E-selectin ligands. We show that human HPCs express the CLA form of PSGL-1, and that an HECA-452–reactive glycoform of CD44 functions as the major E-selectin glycoprotein ligand on human HPCs. The E-selectin binding determinants on CD44 are displayed on sialofucosylated N-linked carbohydrates, and expression of this structure is restricted to primitive CD34+ human BM cells. Expression of this CD44 glycoform on human HPCs promotes functional E-selectin–mediated "rolling" interactions on primary BM microvascular endothelial cells over a wider shear range than that of PSGL-1 alone. These findings offer a new perspective on the biology of CD44, and implicate a role for this molecule as a BM "homing" receptor on primitive human HPCs.

Materials and Methods

Cells, Membrane Protein Preparation, SDS-PAGE, and Western Blotting

Human hematopoietic cell lines (KG1a, HL60, RPMI-8402, and K562) and the BM endothelial cell line-1 (BMEC-1) (Candal et al., 1996) were propagated in RPMI 1640/10% FBS/1% penicillin/streptomycin (Life Technologies). Fresh circulating leukemia blasts were isolated by Ficoll-Hypaque (1.077–1.0800 g/ml; ICN Biomedicals) density gradient centrifugation from the peripheral blood of patients, where they represented >80% of all circulating leukocytes. Normal human BM cells were extracted from vertebral bodies of cadaveric organ donors that were obtained with the consent of donor families and through the cooperation of the New England Organ Bank. BM mononuclear cells were isolated by Ficoll-Hypaque (1.077–1.0800 g/ml) density gradient centrifugation, and were separated into CD34+ and lineage+/CD34− subpopulations using either a negative cell selection StemSep™ human progenitor enrichment column (StemCell Technologies Inc.), or a positive selection for CD34+ cells or other subpopulations of BM cells (monocytes [CD14+], granulocytes [CD15+], B cells [CD19+], or T cells [CD3+]) by immunomagnetic beading separation (Miltenyi Biotec). CD34+/CD44+, CD34+/CD44−, and CD34−/CD44+ cell populations were isolated by cell sorting on a MoFlo apparatus (Cytomation) using fluorochrome-conjugated anti-CD34 mAb (HPCA-2) (Becton Dickinson) and anti-CD44 mAb (Hermes-1) (Jalkanen et al., 1986; antibody was a gift from Dr. Brenda Sandmaier, Fred Hutchinson Cancer Research Center, Seattle, WA).

Membrane preparations of HPCs were isolated as described previously (Sackstein and Dimitroff, 2000). For SDS-PAGE and Western blotting, membrane preparations were diluted in reducing sample buffer and separated on 6–9% SDS-PAGE gels. Where indicated, membrane proteins were also treated with *N*-glycosidase F (8 U/ml for 24 h; Roche Molecular Biomedicals) or *Vibrio cholerae* neuraminidase (0.1 U/ml H/H/Ca^{2+} for 1 h at 37°C; Roche Molecular Biochemicals) as described previously (Sackstein and Dimitroff, 2000). Resolved membrane proteins were transferred to Sequi-blot polyvinylidene difluoride (PVDF) filter membrane (Bio-Rad Laboratories) and blocked with PBS/Tween 20/20% FBS for 1 h at 4°C. Blots were incubated with rat mAb HECA-452 (1.2 μg/ml PBS; PharMingen) or anti–PSGL-1 mAb 4H10 (a gift from Dr. Ray Camphausen, Genetics Institute, Cambridge, MA) for 1 h at room temperature. Isotype control immunoblots using either rat IgM or mouse IgG were performed in parallel to evaluate nonspecific reactive proteins. After three washes with PBS/0.1% Tween 20, blots were incubated with either alkaline phosphatase (AP)-conjugated rabbit anti–rat IgM Abs (1:400) or AP-conjugated goat anti–mouse IgG (1:8,000), depending on the primary Ab. AP substrate, Western Blue (Promega), was then added to develop the blots.

Immunoprecipitation Studies

Membrane protein preparations of human hematopoietic cell lines or of normal human BM HPCs or leukemic blasts were solubilized in 2% NP-40 and precleared in Protein G Agarose (Life Technologies). Protein preparations (lysate) were incubated with anti–PSGL-1 antibody 4H10 or anti-CD44 mAb Hermes-1 (each at 3 μg), or with mouse/rat IgG isotype controls (3 μg) for 2 h at 4°C. The antibody–lysate mixture was added to 30 μl of Protein G Agarose and incubated for 1 h at 4°C under constant rotation. Immunoprecipitates were washed three times with lysis buffer (2% NP-40/1% BSA), diluted in reducing sample buffer, subjected to 6 or 9% SDS-PAGE, transferred to PVDF membrane, and immunostained with mAbs HECA-452 or 4H10. For exhaustive immunoprecipitation of CD44, supernatants from each round of antibody–lysate incubation were reincubated with Hermes-1 (3 μg in each of three rounds), and immunoprecipitates from each round were prepared as described above.

Parallel Plate Flow Chamber Studies Using Cell Monolayers

Under defined shear stress, E-selectin–mediated adhesive interactions were examined between hematopoietic cell monolayers and suspensions of CHO-E (CHO cells stably transfected with full-length cDNA encoding human E-selectin) in flow. CHO-E and empty vector constructs (mock-transfected CHO cells [CHO-Mock]) were maintained in MEM with 10% FBS and 1% penicillin/streptomycin (Life Technologies), and HAM's F-12 with 5% FCS and 1% penicillin/streptomycin, respectively (Cellgro, Inc.) CHO-E cell tethering and rolling on hematopoietic cell monolayers was visualized by video microscopy in real time using the parallel plate flow chamber. Before experimentation, CHO-E cells were harvested with 5 mM EDTA, washed twice in HBSS, and suspended at 10^7/ml in HBSS/10 mM Hepes/2 mM $CaCl_2$ (H/H/Ca^{2+}). Negative control groups were prepared by adding either 5 mM EDTA to the H/H assay buffer (to chelate Ca^{2+} required for binding), treating CHO-E cells with anti–E-selectin Abs (clone 68-5H11) (10 μg/ml; BD PharMingen), or using CHO-empty vector transfectants (CHO-mock). To prepare hematopoietic cell monolayers >90% confluent, suspensions of cells (KG1a, HL60, K562, or RPMI 8402) at 2×10^6/ml RPMI 1640 without sodium Bicarbonate/2% FBS were cytocentrifuged in 6-well plates at 5×10^6/well, and then fixed in 3% glutaraldehyde. Reactive aldehyde groups were blocked in 0.2 M lysine and plated cells were suspended in H/H/Ca^{2+}. In parallel, cells were also pretreated with either *V. cholerae* neuraminidase (0.1 U/ml in H/H/

Ca^{2+} for 1 h at 37°C) or O-sialoglycoprotein endopeptidase (OSGE) (60 μg/ml in H/H/Ca^{2+} for 1 h at 37°C; Accurate Chemicals), respectively. Cell monolayers were placed in the parallel plate flow chamber and CHO-E/-mock cells were perfused into the chamber. After allowing the CHO-E or CHO-mock cells to come into contact with the cell monolayers, the flow rate was adjusted to exert shear stress of 2.8 dyn/cm^2. The number of CHO-E/-mock cell rolling on each monolayer was measured in one frame of five independent fields under 100× magnification from multiple experiments. A minimum of three experiments was performed and results were expressed as the mean ± SD.

Alternately, using freshly isolated human primary BMECs cultured as previously described (Rafii et al., 1994), BMECs from subcultures not older than passage 5 were seeded at 10^5 cells/well in 6-well plates, and when 90–100% confluent, were stimulated with IL-1α (40 U/ml) for 4 h to upregulate the surface expression of E-selectin (expression of which was measured by flow cytometric analysis). Live cultures were then placed in the parallel plate flow chamber and hematopoietic cells (10^7/ml in H/H/Ca^{2+}) were perfused into the chamber over the BMECs. Hematopoietic cell tethering and rolling was visualized at 2.8 dyn/cm^2. Non–IL-1α–activated BMECs and IL-1α–activated BMECs treated with 10 μg/ml anti–E-selectin mAb (clone 68-5H11) served as controls for assessing specificity of E-selectin–mediated adhesion. Cellular rolling was quantified and expressed as described above.

Using Immobilized Immunoprecipitates

CD44 was immunoprecipitated from untreated cell lysates or from cell lysates treated with *N*-glycosidase F (8 U/ml), *V. cholerae* neuraminidase (0.1 U/ml), or α-L-fucosidase (80 m U/ml), and PSGL-1 was immunoprecipitated from untreated cell lysates as described previously (Dimitroff et al., 2000; Sackstein and Dimitroff, 2000). CD44 or PSGL-1 immunoprecipitates were spotted onto plastic Petri dishes, fixed in 3% glutaraldehyde, and incubated in 0.2 M lysine to block unreactive aldehyde groups, and nonspecific binding was prevented by incubating in 100% FBS for 1 h at room temperature. Where indicated, fixed spots were also treated with *V. cholerae* neuraminidase (0.1 U/ml assay medium), which was overlaid onto the spots and incubated at 37°C for 1 h. The protein dishes were placed in the parallel plate flow chamber and CHO-E, CHO-P (CHO stably transfected with human cDNA encoding full- length P-selectin), CHO-P cells treated with function-blocking anti–P-selectin mAb (clone AK-4) (10 μg/ml; BD PharMingen), CHO-E cells treated with function-blocking anti–E-selectin Abs (clone 68-5H11; 10 μg/ml), or mock transfectants were perfused into the chamber (2×10^6/ml H/H/Ca^{2+}) at a flow rate of 0.2 ml/min until the cells were in contact with the substrate. The flow rate was then increased to achieve a shear stress of 2.8 dyns/cm^2. The frequency of cells rolling per 100× magnification field was determined, and data were expressed as the mean ± SD of eight fields visualized from a minimum of three experiments.

Blot Rolling Assay

This blot rolling assay has been described previously (Dimitroff et al., 2000). In brief, CHO-E, CHO-P, or CHO-mock cells were isolated as described above, washed twice in HBSS, and suspended at 10^7/ml in HBSS/10 mM Hepes/2 mM $CaCl_2$ (H/H/Ca^{2+})/10% glycerol. Western blots of HPC membrane preparations stained with HECA-452 were rendered transparent by incubating them in H/H/Ca^{2+}/10% glycerol. These blots were then placed in the parallel plate flow chamber, and CHO transfectants (2×10^6/ml) were perfused into the chamber. After allowing the cells to come in contact with the blotting membrane, the flow rate was adjusted to exert a shear stress of 3.8 dyn/cm^2. The viscosity of 10% glycerol adhesion assay medium was considered in our calculation of shear stress values. The number of cells rolling on and between each immunostained banding region was quantified under 100× magnification within each field of view on the video monitor using Kaleidoscope Molecular Weight Markers (Bio-Rad Laboratories) and See Blue® (Novex, Inc.) as guides to help align and visualize the apparent molecular weights of the proteins of interest. A minimum of three experiments was performed, and results were expressed as the mean ± SD of cell rolling per field at 100 × magnification. Negative controls were prepared by either adding 5 mM EDTA to the CHO-E H/H assay buffer to chelate Ca^{2+} required for binding, pretreating CHO-E cells with anti–E-selectin Abs (clone 68-5H11; 10 μg/ml), or by assessing the ability of CHO-mock cells to interact with the immobilized proteins.

Results

SDS-PAGE and Western Blot Analysis of HECA-452–reactive Membrane Glycoproteins on Human Hematopoietic Cells

SDS-PAGE and Western blot analysis of HECA-452–reactive epitope(s) were performed on membrane proteins from various human hematopoietic cell lines (KG1a, HL60, RPMI-8402, and K562). As illustrated in Fig. 1 A, numerous and distinct HECA-452–reactive bands were detected on SDS-PAGE of membrane protein isolated from KG1a cells. Despite 10-fold less KG1a membrane protein loaded for analysis in these blots compared with that of HL60, RPMI-8402, or K562 cellular membrane protein, KG1a cells contained markedly more HECA-452 staining displayed on several component protein bands. Only one broad band of ~140 kD was detected on HL60 cells, which corresponded to the monomer species of PSGL-1 by immunoblot (Fig. 1 A) (Fuhlbrigge et al., 1997). There were no HECA-452–reactive membrane proteins from RPMI-8402 or K562 cells, even though PSGL-1 was detected on Western blots of these cells by using the anti–PSGL-1 antibody, 4H10 (Dimitroff et al., 2000). This finding suggested that these cells lacked the appropriate HECA-452–binding epitope and, at minimum, the E-selectin–binding species of PSGL-1.

Parallel Plate Flow Analysis of E-Selectin Ligand Activity of Human Hematopoietic Cells

To examine whether HECA-452 expression by human hematopoietic cell lines correlated with E-selectin ligand activity, parallel plate flow chamber studies were performed to assess E-selectin binding under defined shear flow conditions. Tethering and rolling of CHO-E cells was observed on glutaraldehyde-fixed monolayers of KG1a and HL60 cells at 2.8 dynes/cm^2 (Fig. 1 B). CHO-mock cells displayed no rolling. CHO-E cell rolling was twofold higher on KG1a cells than on HL60 cells, and was completely inhibited by adding 5 mM EDTA to the assay medium, by preincubating CHO-E cells with anti–E-selectin Abs, or by pretreating KG1a or HL60 cells with *Vibrio cholerae* neuraminidase (which cleaves terminal sialic acids). There was no E-selectin ligand activity on cell lines RPMI-8402 and K562, whose membrane proteins did not contain HECA-452 epitopes. These findings confirm previous reports that E-selectin ligand activity of PSGL-1 requires the expression of HECA-452–reactive posttranslational modifications (Fuhlbrigge et al., 1997). Interestingly, the E-selectin ligand activity of KG1a and HL60 cells was not inhibited by incubating cells with HECA-452 at 50 μg/ml, and was not abrogated by pretreatment with OSGE (Fig. 1 B), though OSGE bioactivity was confirmed by the disappearance of the OSGE-sensitive epitope, QBEND-10, on KG1a CD34 as measured by flow cytometry.

To analyze the interaction between human HPCs and naturally expressed E-selectin on cells of physiologic importance, we used freshly isolated human BMEC for parallel plate flow chamber studies. Under physiologic shear flow conditions, we observed KG1a and HL60 cell rolling

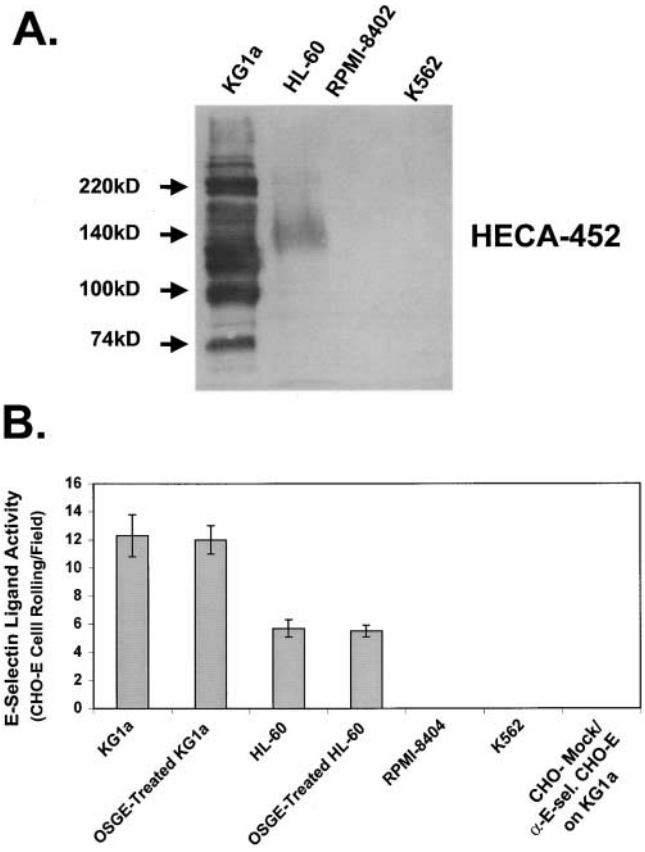

Figure 1. Expression of HECA-452–reactive glycoproteins on human hematopoietic cells and E-selectin ligand activity of human hematopoietic cell lines. (A) Membrane preparations of human hematopoietic cell lines, KG1a (10 μg), HL60 (100 μg), RPMI-8402 (100 μg), and K562 (100 μg) were resolved on a reducing 6% SDS-PAGE gel and immunoblotted with HECA-452. (B) Parallel plate flow chamber analysis of CHO-E cell tethering and rolling on glutaraldehyde-fixed monolayers of hematopoietic cell lines (shear stress of 2.8 dynes/cm^2). Twofold more CHO-E cell tethering and rolling was observed on KG1a than on HL60 cell monolayers, which was not affected by OSGE pretreatment. There was no CHO-E cell rolling on RPMI-8402 or K562 cell monolayers. Negative controls consisted of CHO-mock transfectants and CHO-E cells treated with anti–E-selectin Abs (10 μg/ml). Data are presented as mean ± SD CHO-E cell rolling per field × 5 fields, minimum of three experiments.

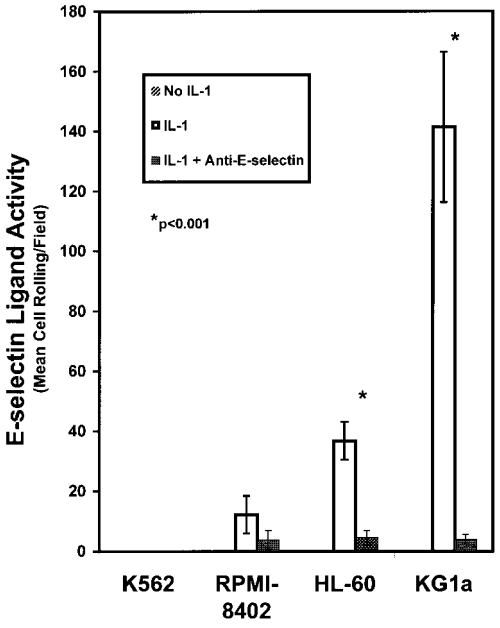

Figure 2. Human hematopoietic cell rolling on freshly isolated human BMEC. KG1a, HL60, RPMI-8402, and K562 were perfused over live IL-1α–treated primary BMEC cultures at 2.8 dynes/cm^2 in the parallel plate flow chamber, and cell rolling was observed and recorded for video analysis. Controls consisted of untreated BMEC and IL-1α–treated BMEC in the presence of anti–E-selectin mAb. Untreated BMEC showed no E-selectin ligand activity, and rolling on IL-1α–treated BMEC was eliminated by incubation with function-blocking anti–E-selectin mAb 68-5H11. Data represent mean ± SD cell rolling frequency per 100 × magnified field × 5 fields of view, minimum of three experiments. Note that KG1a cell rolling was 3.5-fold greater than that of HL60 cells.

on live monolayers of IL-1α–activated BMECs. Consistent with results from experiments using CHO-E cells to assess HPC E-selectin ligand activity, KG1a cells possessed a fourfold greater capacity to roll on E-selectin on IL-1α–activated BMECs than HL60 cells, while RPMI-8402 and K562 cells possessed minimal E-selectin ligand activity (Fig. 2). KG1a and HL60 cellular E-selectin ligand activities were not observed on non–IL-1α–activated BMECs, or on IL-1α–activated BMECs treated with a functional-blocking anti–E-selectin mAb.

Blot Rolling Assay of E-Selectin Glycoprotein Ligands from Human Hematopoietic Cells

To examine the E-selectin ligand activity of all HECA-452–reactive KG1a membrane proteins, we used a new method for assessing the adhesive interactions under shear flow between selectin-expressing whole cells and proteins immobilized on Western blots (Dimitroff et al., 2000). Membrane proteins were separated on 6% SDS-PAGE gel, transferred onto PVDF membrane, and then stained with HECA-452. Component membrane proteins were analyzed for their ability to support CHO-E cell rolling under defined hydrodynamic flow conditions. We reproducibly observed E-selectin ligand activity on HECA-452–stained bands at 100, 120, 140, 190, and 220 kD, but not at 74 kD (Fig. 3 A). CHO-mock cells showed no interactions with any bands. Specificity for E-selectin was demonstrated by the abrogation of CHO-E cell rolling in the presence of either 5 mM EDTA or anti–E-selectin functional blocking Abs. On HL60 cells, CHO-E cell rolling was observed only over the broad 140-kD HECA-452–immunostained band (i.e., PSGL-1/CLA) (data not shown). To determine whether E-selectin–binding determinants reside on *N*-glycans, we treated KG1a membrane protein with *N*-glycosidase F. De–*N*-glycosylated proteins were resolved by SDS-PAGE and analyzed for HECA-452 reactivity and E-selectin ligand activity. *N*-glycosidase F treatment markedly diminished HECA-452 staining (Fig. 3 B) and completely abolished CHO-E cell rolling on all proteins on the blot, indicating that all glycoprotein E-selectin binding determinants on KG1a cells are displayed exclusively on *N*-glycans.

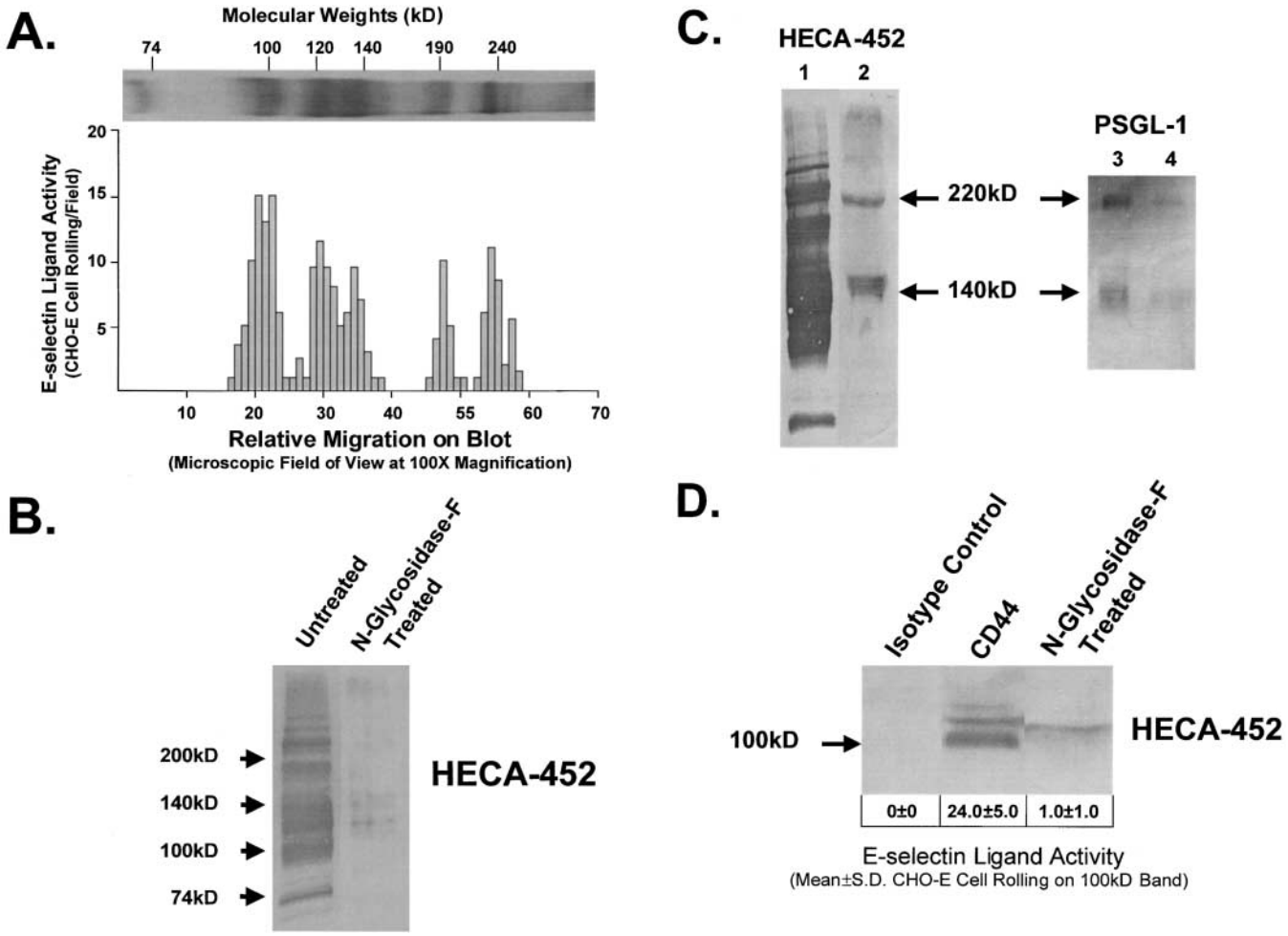

Figure 3. HECA-452–reactive CD44 functions as an E-selectin ligand. (A) KG1a membrane protein (10 μg) was resolved on a reducing 6% SDS-PAGE gel, blotted onto PVDF membrane, stained with HECA-452, and HECA-452 immunoblots blots were rendered transparent with 10% glycerol. CHO-E cells (2×10^6/ml) were then perfused over these blots at a defined shear stress of 3.8 dynes/cm^2. Several HECA-452–stained bands from KG1a membrane protein–supported, E-selectin–dependent CHO-E cell rolling. (B) KG1a membrane proteins (10 μg) were treated with *N*-glycosidase F, separated on a reducing 6% SDS-PAGE gel, and immunostained with HECA-452. (C) Immunoprecipitated PSGL-1 was resolved on a reducing 6% SDS-PAGE gel and Western blotted with either HECA-452 (left) or anti–PSGL-1 antibody 4H10 (right). (lane 1) 10 μg of total KG1a membrane protein; (lane 2) immunoprecipitated PSGL-1 from 100 μg of KG1a membrane protein; (lane 3) 100 μg of total KG1a membrane protein, and (lane 4) immunoprecipitated PSGL-1, from 100 μg of KG1a membrane protein. Note that HECA-452–stained bands at 140 and 220 kD correspond to PSGL-1. (D) Isotype control or Hermes-1 immunoprecipitated CD44 from KG1a membrane protein (50 μg) was resolved on a reducing 9% SDS-PAGE gel and immunoblotted with HECA-452. Immunoprecipitated CD44 from KG1a membrane proteins (50 μg) treated with *N*-glycosidase F was also immunoblotted with HECA-452. Though CHO-E cell rolling frequencies are presented as the mean ± SD of E-selectin–mediated cell rolling at 3.8 dynes/cm^2 measured on the 100-kD isoform of CD44, no CHO-E rolling was observed along the entire length of the *N*-glycosidase F–treated lane.

To distinguish KG1a cellular PSGL-1/CLA from the other HECA-452–reactive bands, we immunostained blots of immunoaffinity-purified PSGL-1 and of total KG1a membrane protein with either HECA-452 or anti-PSGL-1 (Fig. 3 C). We found that KG1a cells express both the monomer and dimer isoforms of PSGL-1, which represented the 140- and 220-kD HECA-452–reactive proteins (Fig. 3 C). Thus, the HECA-452–reactive bands at 100, 120, and 190 kD, which support CHO-E cell rolling (Fig. 3 A), corresponded to non–PSGL-1 proteins. Recent data from our laboratory show that HECA-452–reactive membrane proteins at 100, 120, and 190 kD from Western blots of KG1a cells represent isoforms of CD44 (Dimitroff et al., 2000). Accordingly, to directly analyze whether *N*-glycan–specific modifications of CD44 confer E-selectin ligand activity, we immunoaffinity-purified CD44 from KG1a membrane proteins and tested its capacity to serve as an E-selectin ligand in blot rolling assays. As shown in Fig. 3 D, KG1a CD44 showed HECA-452 reactivity and possessed E-selectin ligand activity. Treatment of CD44 with *N*-glycosidase F markedly reduced HECA-452 reactivity and completely abrogated CHO-E cell rolling (Fig. 3 D). Exhaustive immunoprecipitation of CD44 (three rounds) resulted in the disappearance of stainable CD44 molecule at 100 kD (Hermes-1 immunoblot and HECA-452 immunoblot) and of all E-selectin ligand activity at the

100-kD and 190-kD bands (Fig. 4, A and B). Moreover, there was a 55% decrement in E-selectin ligand activity at the 120-kD band after three rounds of immunoprecipitation (Fig. 4 B). Notably, there was no difference in HECA-452 staining or in E-selectin ligand activity of the 140-kD monomer species of PSGL-1 after removal of CD44.

Analysis of E-Selectin Ligand Activities of PSGL-1 and CD44 Isolated from Human Hematopoietic Cells

To further explore the E-selectin ligand activity of CD44, we directly immunoprecipitated CD44 from human hematopoietic cell lines and analyzed CHO-E cell binding in the parallel-plate flow chamber. While CD44 isolated from human hematopoietic cell lines HL60, K562, and RPMI-8402 did not support any CHO-E cell rolling, KG1a CD44 exhibited E-selectin ligand activity over a range of shear stress. Significantly greater CHO-E cell rolling was observed on untreated KG1a CD44 than on *N*-glycosidase F, on an α-L-fucosidase–treated KG1a CD44, on *V. cholerae* neuraminidase–treated KG1a membrane protein or on isotype Ab immunoprecipitates (control) (Fig. 5 A). In addition, compared with a molecular equivalent amount of immunoprecipitated KG1a PSGL-1, CD44 showed markedly greater E-selectin ligand activity at 2.8 dynes/cm^2 ($P <$ 0.001) (Fig. 5 A). However, at a lower shear stress of 0.6 dynes/cm^2, CHO-E cell rolling on PSGL-1 was equivalent to that of CD44. These data suggest that PSGL-1 and CD44 have overlapping contributions to E-selectin binding at low shear stress, but that CD44 engagement with E-selectin predominates at higher physiologic shear stress. Notably, CHO cells transfected with P-selectin rolled on KG1a PSGL-1, but not on KG1a CD44 (Fig. 5 B), indicating that CD44 is not a P-selectin ligand (Fig. 5 B). In all experiments, negative control CHO cells (CHO-mock cells and CHO-E or CHO-P cells treated with function-blocking anti–E- or anti–P-selectin mAbs, respectively) did not tether and roll on any proteins.

Analysis of E-Selectin Ligand Activity of CD44 from Native Human Hematopoietic Progenitor Cells

To determine whether CD44 naturally expressed on normal human HPCs functions as an E-selectin ligand, we investigated the distribution of HECA-452 reactivity and E-selectin ligand activity of CD44 expressed on early CD34+ cells and more mature (CD34−/lineage+) human BM cells (including populations enriched for monocytes [CD14+]), granulocytes [CD15+], and lymphocytes (B cells [CD19+] and T cells [CD3+]). Notably, though SDS-PAGE of Hermes-1 immunoprecipitates of KG1a cells reveals three bands (100, 120, and 190 kD), only a single 100-kD CD44 was immunoprecipitated from both CD34+ and lineage+/CD34− cells, and only CD44 from CD34+ cells stained with HECA-452 and functioned as an E-selectin ligand (Fig. 6 A). Identical results were obtained whether CD34+ cells were enriched by negative or positive selection. Indeed, even when tenfold excess lineage+ cell membrane protein was used for CD44 immunoprecipitation, there was still neither HECA-452 staining of CD44, nor E-selectin ligand activity of CD44 (Fig. 6 A). Moreover, immobilized on plastic, CD44 immunoprecipitated only from CD34+/CD44+ cells supported CHO-E cell rolling,

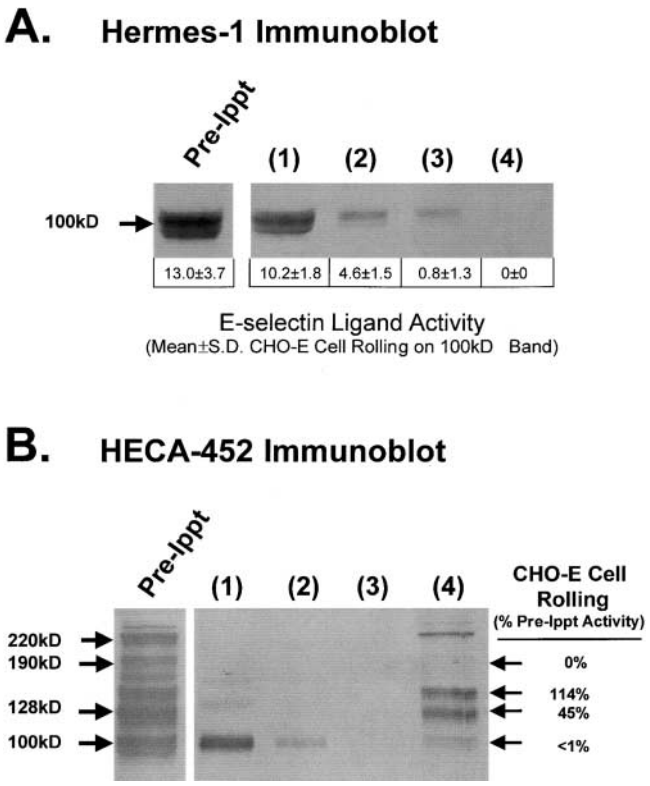

Figure 4. Exhaustive immunoprecipitation of CD44 (Hermes-1) and blot rolling assay of residual E-selectin ligand activity. Hermes-1 immunoblot (A) or HECA-452 immunoblot (B) of KG1a lysate (10 μg) subjected to three rounds of immunoprecipitation with Hermes-1 mAb. (Pre-Ippt) Total KG1a lysate 10 μg; (lane 1) first round Hermes-1 immunoprecipitate; (lane 2) second round Hermes-1 immunoprecipitate; (lane 3) third round Hermes-1 immunoprecipitate; (lane 4) residual lysate after three rounds of Hermes-1 immunoprecipitation. E-selectin ligand activity (CHO-E cell rolling) correlates with intensity of the Hermes-1 staining and of HECA-452 staining of 100-kD band, is reduced after each round of Hermes-1 immunoprecipitation (A), and was completely absent in the residual lysate material after the third round of immunoprecipitation (lane 4, A and B). CHO-E cell rolling was also eliminated over the 190-kD band by exhaustive Hermes-1 immunoprecipitation, whereas CHO-E cell rolling on 120-kD stained band was markedly reduced, and the 140-kD band (PSGL-1) retained full activity (B, lane 4).

whereas immunoprecipitated CD44 from CD34− cells did not possess any E-selectin ligand activity.

To further analyze the expression and structure of HECA-452–reactive CD44 on human hematopoietic cells, we evaluated whether native leukemic blasts displayed this CD44 glycoform. Four major HECA-452 stained bands were detected (74, 100, 140, and 190 kD) from leukemic blasts of an acute myelogenous leukemia (AML) (subtype M5) (Fig. 6 B). HECA-452 staining was completely eliminated in the 100-kD region following *N*-glycosidase F treatment, while the 74- and 140-kD bands had persistent staining and the 190-kD band stained at an apparently reduced molecular weight (Fig. 6 B). When immunoprecipitated CD44 from AML (M5) membrane protein was treated with *N*-glycosidase F, the HECA-452 reactivity as well as the E-selectin ligand activity was com-

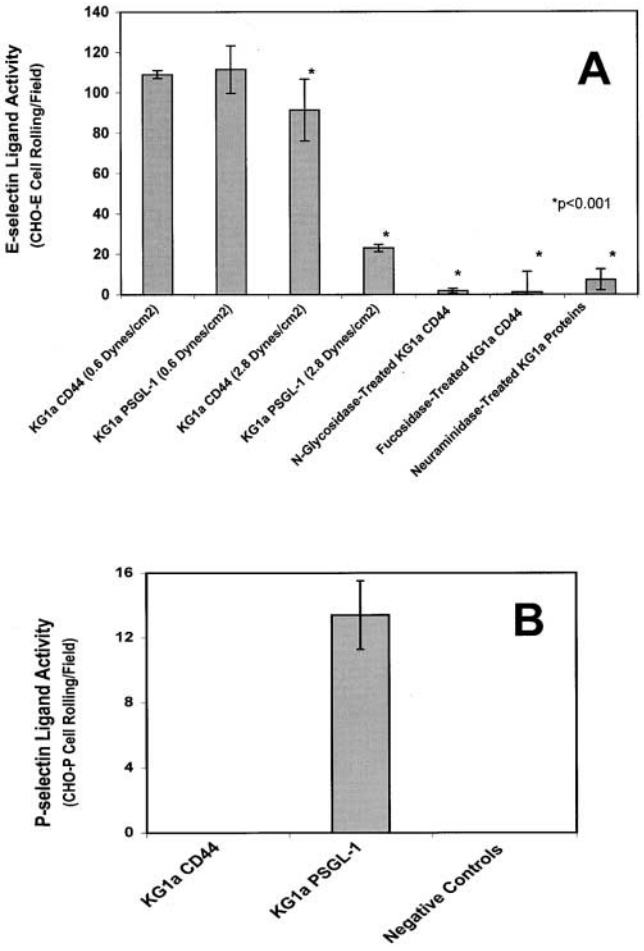

Figure 5. HECA-452–reactive CD44 is a more avid E-Selectin ligand than PSGL-1. Equivalent amounts (1 μg) of either immunoprecipitated CD44 or PSGL-1 were analyzed for E-selectin and P-selectin ligand activity in the parallel plate flow chamber. (A) E-selectin–mediated CHO-E cell rolling was observed at 2.8 dynes/cm^2 on KG1a CD44, but was significantly lower on KG1a PSGL-1 at 2.8 dynes/cm^2 ($P < 0.001$). *N*-glycosidase F- and α-L-fucosidase-treated KG1a CD44, and *Vibrio cholerae* neuraminidase treatment of KG1a membrane protein abrogated CHO-E cell rolling ($P < 0.001$), and no CHO-E cell rolling was observed on isotype control rat IgG– or mouse IgG–immunoprecipitated KG1a protein (data not shown). (B) CHO-P cell rolling was observed on KG1a PSGL-1 but not on KG1a CD44 (2.8 dyn/cm^2). No rolling was observed on negative controls (CHO-Mock cells and CHO-P cells pretreated with function-blocking anti–P-selectin mAb AK-4 [10 μg/ml]).

pletely abolished (Fig. 6 C). Similar to immunoprecipitated KG1a CD44, AML (M5) CD44 displayed a minor isoform at 120 kD detected by HECA-452, but the major band and biologically active protein was the 100-kD CD44 isoform. CD44 was also immunoprecipitated from blasts of an undifferentiated AML (M0), an AML without maturation (M1), and an atypical chronic myelogenous leukemia (bcr/abl$^-$). Notably, expression of the HECA-452–reactive epitopes on immunoprecipitated CD44 directly correlated with the ability to support CHO-E cell rolling (Fig. 6 D). The expression of CD44 (Hermes-1 mAb) on these leukemias was equivalent (>90% positive cell staining by flow cytometry), further indicating that the ability to interact with E-selectin was dependent on the elaboration of HECA-452–reactive glycosylations. Moreover, since CD44 is also expressed on nonhematopoietic cells, we analyzed CD44 expressed on the human BM endothelial cell line BMEC-1. Though BMEC-1 expressed high levels of CD44 (Fig. 6 E), CD44 from these cells was not HECA-452 reactive and did not possess any E-selectin ligand activity (Fig. 6 D).

Discussion

In contrast to its usual pattern of inducible expression, E-selectin is constitutively expressed on dermal and BM microvascular endothelium, indicating that selective tropism(s) of circulating cells to these tissues is regulated by expression of cell-specific E-selectin ligands. The goal of this study was to identify E-selectin ligands expressed on human HPCs that could function in mediating HPC homing to BM. Earlier studies of E-selectin ligands expressed on mature human leukocytes showed that expression of the HECA-452 epitope(s) on lymphocyte PSGL-1 and on neutrophil L-selectin correlates with E-selectin binding capability (Picker et al., 1991b; Fuhlbrigge et al., 1997; Zöllner et al., 1997). Similarly, in the mouse, HECA-452 decoration of the neutrophil E-selectin ligand, ESL-1, was found to predict E-selectin ligand activity (Zöllner and Vestweber, 1996). Since expression of HECA-452 epitope(s) is functionally correlated with E-selectin ligand activity of glycoproteins expressed on mature leukocytes, we sought to determine whether HECA-452–reactive glycoproteins on human HPCs could function as E-selectin ligands.

To analyze the E-selectin ligand activity of human hematopoietic cells, we performed parallel-plate flow chamber studies of CHO-E cell tethering and rolling on hematopoietic cells lines and of hematopoietic cell rolling on naturally expressed E-selectin on IL-1α–stimulated primary BM endothelial cultures. The data presented here show that KG1a and HL60 cells, and not RPMI-8402 or K562 cells, possess E-selectin ligand activity. The E-selectin–mediated adhesive interactions between these hematopoietic cell lines and CHO-E cells parallel results of binding studies using primary BM endothelial cells, demonstrating the utility and fidelity of CHO-E as a cellular reagent to detect and analyze E-selectin ligands. Consistent with results from these binding studies, Western blot analysis of HECA-452 epitope(s) revealed that KG1a and HL60 membrane proteins display HECA-452–reactive glycoproteins, whereas RPMI-8402 and K562 membrane proteins do not. The major HECA-452–reactive membrane glycoprotein(s) of HL60 cells consist of the monomer (140 kD) form of PSGL-1, however, KG1a cells possess multiple HECA-452–reactive glycoproteins at MW of 74, 100, 120, and 190 kD in addition to the 140-kD and 220-kD (dimer) PSGL-1 bands.

Using the hydrodynamic shear-based blot rolling assay to investigate the E-selectin ligand activity of HL60 and KG1a membrane glycoproteins, we show here that the HECA-452–reactive PSGL-1 expressed on these cells functions as an E-selectin ligand. Additionally, with the exception of the 74-kD glycoprotein, HECA-452–reactive glycoproteins each exhibit E-selectin ligand activity on KG1a cells at 100, 120, and 190 kD. The absence of CHO-E–bind-

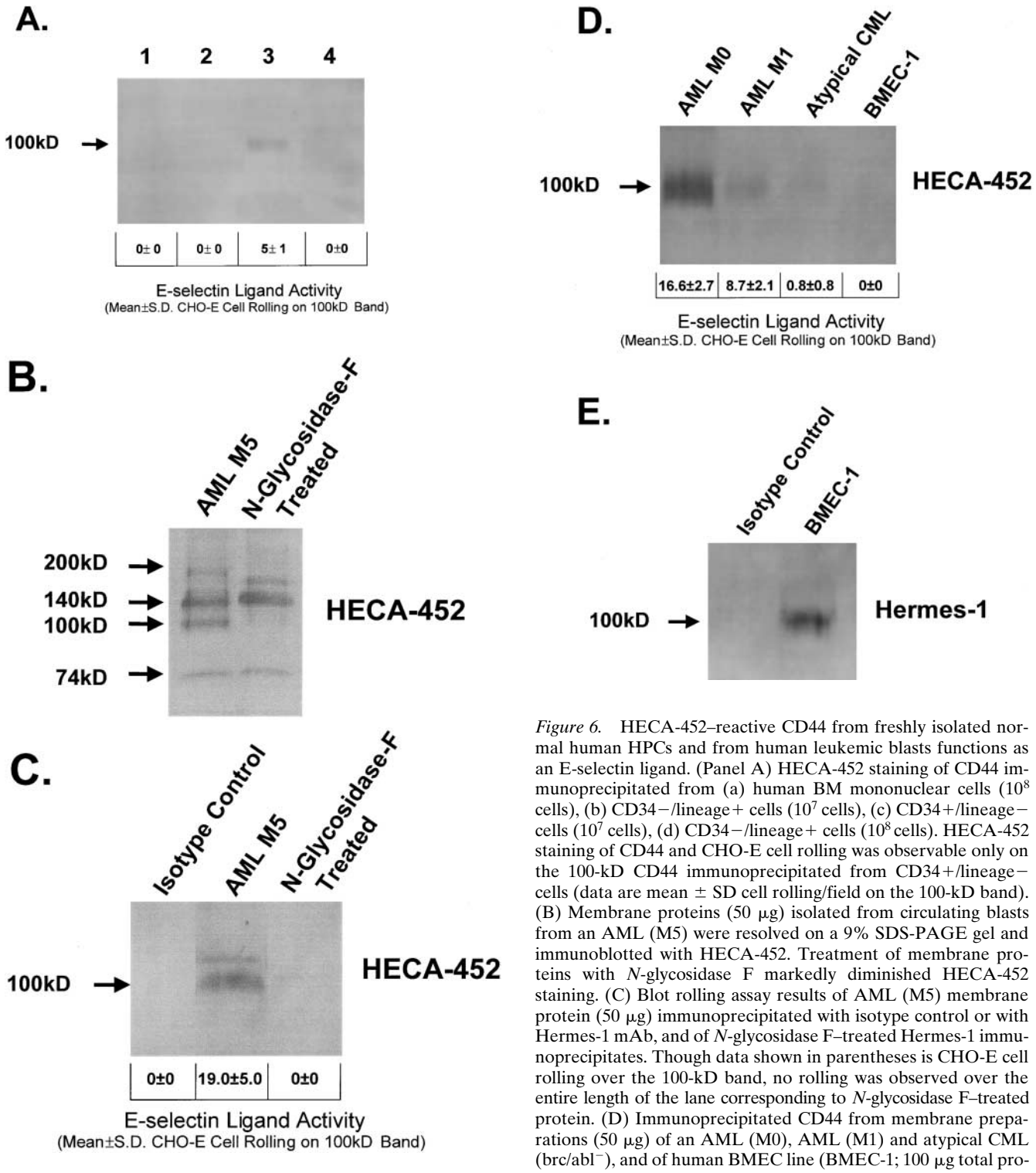

Figure 6. HECA-452–reactive CD44 from freshly isolated normal human HPCs and from human leukemic blasts functions as an E-selectin ligand. (Panel A) HECA-452 staining of CD44 immunoprecipitated from (a) human BM mononuclear cells (10^8 cells), (b) CD34−/lineage+ cells (10^7 cells), (c) CD34+/lineage− cells (10^7 cells), (d) CD34−/lineage+ cells (10^8 cells). HECA-452 staining of CD44 and CHO-E cell rolling was observable only on the 100-kD CD44 immunoprecipitated from CD34+/lineage− cells (data are mean ± SD cell rolling/field on the 100-kD band). (B) Membrane proteins (50 μg) isolated from circulating blasts from an AML (M5) were resolved on a 9% SDS-PAGE gel and immunoblotted with HECA-452. Treatment of membrane proteins with *N*-glycosidase F markedly diminished HECA-452 staining. (C) Blot rolling assay results of AML (M5) membrane protein (50 μg) immunoprecipitated with isotype control or with Hermes-1 mAb, and of *N*-glycosidase F–treated Hermes-1 immunoprecipitates. Though data shown in parentheses is CHO-E cell rolling over the 100-kD band, no rolling was observed over the entire length of the lane corresponding to *N*-glycosidase F–treated protein. (D) Immunoprecipitated CD44 from membrane preparations (50 μg) of an AML (M0), AML (M1) and atypical CML (brc/abl$^-$), and of human BMEC line (BMEC-1; 100 μg total protein), was separated on a 6% SDS-PAGE gel, immunostained with HECA-452, and evaluated for E-selectin ligand activity. E-selectin ligand activity correlates with intensity of HECA-452 staining of 100-kD band. (E) Western blot of immunoprecipitated CD44 from BMEC-1 stained with Hermes-1 mAb.

ing interactions by the 74-kD HECA-452 reactive protein indicates that the presence of the HECA-452 epitope does not, in itself, confer E-selectin ligand activity. Moreover, the observations that HECA-452 staining of immunoblots did not block CHO-E cell binding to relevant glycoproteins, and that HECA-452 incubation of KG1a cells and HL60 cells did not inhibit rolling on BMEC or CHO-E binding to the cells, indicate that the HECA-452 epitope is not the direct binding determinant for E-selectin.

The data obtained from blot rolling assays of Hermes-1–immunoprecipitated KG1a membrane proteins demonstrate that the E-selectin ligand activity at 100 and 190 kD

is solely due to CD44, and that CD44 is the major contributor of the E-selectin ligand activity at 120 kD. The residual E-selectin ligand activity observed at 120 kD following exhaustive Hermes-1 immunoprecipitation of KG1a lysates may reflect the minor contribution of a non–CD44 E-selectin ligand, or, less likely, of a CD44 isoform lacking the Hermes-1 epitope. The E-selectin–binding, HECA-452–reactive glycoform of CD44 is also expressed on the CD34+ progenitor cell subset of normal human BM mononuclear cells and on blasts from some de novo human leukemias, but is not present on more mature (lineage+) BM cells, including those enriched for subpopulations consisting of monocytes, granulocytes, and lymphocytes.

Blot rolling assays of HECA-452 immunoblots of KG1a membrane protein show that the cumulative E-selectin ligand activity of HECA-452–reactive CD44 (100-, 120-, and 190-kD bands) was markedly greater than that represented by PSGL-1 (140 and 220 kD), and reveal that the predominant E-selectin ligand of human hematopoietic cells is the 100-kD CD44 band. This 100-kD form of CD44 corresponds to the "standard" hematopoietic form of CD44 (Stamenkovic et al., 1991), and it is the only form immunoprecipitated from normal human BM cells (Fig. 6 A). Since this molecule is also an L-selectin ligand (Dimitroff et al., 2000), we now designate this structure as hematopoietic cell E-/L-selectin ligand (HCELL). However, unlike PSGL-1, which binds all three selectins, HCELL is not a ligand for P-selectin. Compared with PSGL-1, in flow chamber assays (a) HCELL/CD44 E-selectin binding was significantly higher at 2.8 dynes/cm^2 and functioned over a wider range of shear stress, and (b) HCELL/CD44 expression conferred a greater capacity of human hematopoietic cells to roll on E-selectin naturally expressed on BM microvasculature under physiologic shear stress conditions. These data show that HCELL is a major glycoprotein ligand on human HPCs and suggest that engagement of HPCs with E-selectin at a physiologic postcapillary venule shear stress level is mediated preferentially by HCELL.

Sialidase and fucosidase treatment each abrogated E-selectin–binding activity of CD44, highlighting the importance of sialic acid and fucose modifications for creation of the selectin binding determinant. Elimination of ligand activity following *N*-glycanase digestion of KG1a membrane protein indicates that these critical sialofucosylated modifications are displayed on *N*-glycosylations, whereas the insensitivity to OSGE digestion suggests that the relevant glycosylations for E-selectin binding of HCELL are not clustered on extensively *O*-glycosylated peptide regions (Steininger et al., 1992). Our data showing that CD44 lacking HECA-452 did not possess E-selectin ligand activity is consistent with results from prior studies showing that HECA-452–nonreactive CD44 from human tonsils does not exhibit E-selectin ligand activity (Berg et al., 1991). Thus, CD44, a molecule principally known for its capacity to bind extracellular matrix molecules such as hyaluronic acid (HA) (Aruffo et al., 1990), requires critical sialylated and fucosylated carbohydrate modifications to function as HCELL. Of interest, sialylation of CD44 has been shown to inhibit binding to HA (Skelton et al., 1998; Katoh et al., 1999), which may explain the finding that though human CD34+ BM cells characteristically express high levels of CD44 (Kansas et al., 1990; Peled et al., 1999), an unexpectedly small fraction of these cells bind to HA (Peled et al., 1999).

Previous studies have shown that human HPCs possess E-selectin ligand(s) (Naiyer et al., 1999; Peled et al., 1999; Greenberg et al., 2000) and that human HPC interactions with human BMECs under shear conditions are mediated principally by E-selectin receptor/ligand interactions (Schweitzer et al., 1996; Rood et al., 2000), but the human HPC E-selectin ligand(s) have not been identified heretofore. In the mouse, intravital microscopy studies have demonstrated that E-selectin, which is constitutively expressed on murine BM microvasculature in vivo, can mediate HPC rolling interactions and that adhesive interactions between very late antigen (VLA)-4 expressed on HPCs and BM endothelium vascular cell adhesion molecule (VCAM)-1 also mediate rolling interactions (Mazo et al., 1998). However, even though IL-1α stimulation of primary BMEC cultures markedly upregulates the expression of VCAM-1 (Rafii et al., 1994) and despite high VLA-4 expression on KG1a and HL60 cells (Oxley and Sackstein, 1994), rolling of KG1a or HL60 cells on primary BMECs did not utilize the VLA-4/VCAM-1 receptor–ligand pair, as evidenced by the complete abrogation of rolling interactions by anti–E-selectin mAb treatment alone. This finding is consistent with prior reports showing that VCAM-1 in the absence of SDF-1 is incapable of supporting CD34+ adhesive interactions on BM endothelium under flow (Peled et al., 1999; Schweitzer et al., 1996; Rood et al., 2000), further highlighting the importance of E-selectin in this process.

The broad expression of the triselectin ligand PSGL-1 (and of VLA-4) among both immature and mature nucleated hematopoietic cells raises the possibility that selectivity in human HPC homing to BM may involve expression of non–PSGL-1 E-selectin ligands, such as HCELL, on human HPC. In this regard, the fact that HCELL expression is uniquely restricted to CD34+ hematopoietic cells indicates that this CD44 glycoform may impart specificity for progenitor cell rolling on BM microvasculature. Interestingly, though binding to HA could contribute to CD34+ rolling interactions on BM endothelium (DeGrendele et al., 1996; Peled et al., 1999), the rather extensive distribution of CD44 among both immature and mature human BM cells (Kansas et al., 1990) and the fact that human CD34+ cell rolling on endothelium is not inhibited by antibodies that block binding to HA and is inhibited by chelation of divalent ions (which does not affect CD44–HA binding) (Peled et al., 1999), indicates that CD44–HA interactions are neither unique nor critical to HPC rolling on BM endothelium. Other studies have provided various independent lines of evidence showing that CD44 may play a role in HPC homing to BM (Khaldoyanidi et al., 1996; Zoller et al., 1998), in tissue distribution of myeloid progenitors (Schmits et al., 1997), and in engraftment of transplanted hematopoietic progenitor cells (Sandmaier et al., 1990; Watanabe et al., 1998). Thus, whereas L-selectin is operationally known as the "lymph node homing receptor" and CLA is known as a "skin homing receptor," the data reported here are consistent with a role for HCELL as a "bone marrow homing receptor." Our findings expand the current knowledge of the structural biology of E-selectin ligands, bestow a new perspective on ligands facilitating HPC trafficking to E-selectin-expressing tissue

microvessel beds, and have broad implications exploiting HCELL as a biological surrogate for positive selection of HPCs with improved engraftment potential.

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