Expression of L-selectin on human hematopoietic cells (HC) is associated with a higher proliferative activity and a more rapid engraftment after hematopoietic stem cell transplantation. Two L-selectin ligands are expressed on human HCs, P-selectin glycoprotein ligand-1 (PSGL-1) and a specialized glycoform of CD44 (hematopoietic cell E- and L-selectin ligand, HCELL). Although the structural biochemistry of HCELL and PSGL-1 is well characterized, the relative capacity of these molecules to mediate L-selectin-dependent adhesion has not been explored. In this study, we examined under shear stress conditions L-selectin-dependent leukocyte adhesive interactions mediated by HCELL and PSGL-1, both as naturally expressed on human HC membranes and as purified molecules. By utilizing both Stamper-Woodruff and parallel-plate flow chamber assays, we found that HCELL displayed a 5-fold greater capacity to support L-selectin-dependent leukocyte adhesion across a broad range of shear stresses compared with that of PSGL-1. Moreover, L-selectin-mediated leukocyte binding to immunopurified HCELL was consistently >5-fold higher than leukocyte binding to equivalent amounts of PSGL-1. Taken together, these data indicate that HCELL is a more avid L-selectin ligand than PSGL-1 and may be the preferential mediator of L-selectin-dependent adhesive interactions among human HCs in the bone marrow.

L-selectin, also known as the “peripheral lymph node homing receptor,” belongs to the selectin family of C∞-dependent adhesion molecules that includes E-selectin, a cytokine-inducible membrane protein found on activated endothelium, and P-selectin, which is found on activated platelets and endothelial cells. L-selectin is constitutively expressed on most mature leukocytes where it is best known for initiating lymphocyte “rolling” interactions on high endothelial venules in peripheral lymph nodes and, in combination with E- and P-selectin, leukocyte rolling on the post-capillary venules at sites of inflammation (1, 2). L-selectin is also characteristically expressed on human CD34+ hematopoietic cells (HC),1 and its expression on these cells correlates with higher clonogenic activity in vitro assays and faster hematologic recovery following bone marrow transplantation (3–6). These observations have raised interest in defining the nature of L-selectin/L-selectin ligand interactions among human HCs.

Human HCs express two distinct L-selectin ligands as follows: P-selectin glycoprotein ligand-1 (PSGL-1), a molecule that functions as a ligand for all three selectins; and a specialized glycoform of CD44 known as hematopoietic cell E- and L-selectin ligand (HCELL) (7, 8). PSGL-1 is expressed on various mature leukocytes (9), on several hematopoietic cell lines, as well as on primitive human hematopoietic progenitor cells (7, 10, 11). The L-selectin-binding determinant on PSGL-1 is localized within 17 amino acids of the mature N-terminal region and consists of critical tyrosine sulfation(s) combined with a sialyl Lewis X-bearing O-glycan (12, 13). Treatment of PSGL-1 with the enzymes O-sialoglycoprotein endopeptidase (OSGE) and moccarakhin (a cobra venom metalloprotease) eliminates the L-selectin ligand activity (11, 14–16). In contrast to PSGL-1, the L-selectin binding determinants of HCELL are resistant to OSGE and moccarakhin digestion, are sulfation-independent, and are presented on sialylated, fucosylated N-glycana (7, 17–19). Moreover, contrary to the broad expression of PSGL-1, HCELL expression is characteristic of only the earliest (CD34+) subsets of human HCs, of blasts in de novo human leukemias, and of the primitive human hematopoietic cell line KG1a (7, 8, 17–19).

Despite our knowledge of the biochemistry and cellular distribution of PSGL-1 and HCELL, there is no information on the relative L-selectin binding capabilities of these molecules. One of the unique aspects of the biochemistry/biophysics of the L-selectin receptor/ligand adhesive interactions is the requirement for a critical level of shear stress. Thus, typical static adherence assays cannot be employed to measure and study L-selectin/L-selectin ligand interactions. In this study, we utilized shear-based adherence assay systems to analyze the capacity of L-selectin to engage human HCELL and PSGL-1, as expressed on established hematopoietic cell lines and on a

1 The abbreviations used are: HC, hematopoietic cell; AML, acute myelogenous leukemia; CHO, Chinese hamster ovary; HCELL, hematopoietic cell E- and L-selectin ligand; OSGE, O-sialoglycoprotein endopeptidase; PSGL-1, P-selectin glycoprotein ligand-1; TDL, thoracic duct lymphocytes; RT, room temperature; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; Ab, antibody; mAb, monoclonal Ab; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RT-PCR, reverse transcriptase-polymerase chain reaction; kb, kilobase pairs; ST3Gal IV, a2,3-sialyltransferase; FucTIV and FucTVII, a1,3-fucosyltransferases; PBMC, peripheral blood mononuclear cell.
population of blasts isolated from a patient with myeloid leukemia, under a wide range of shear stress conditions. Results obtained from experiments examining the L-selectin ligand activities of PSGL-1 and HCELL, either natively expressed on human HC membrane or isolated from human HC membrane preparations, indicate that HCELL exhibits a higher capacity to support L-selectin binding compared with PSGL-1. Moreover, these data show that L-selectin ligands impart shear resistance on human HCs and that L-selectin-dependent adhesion interactions mediated by HCELL and by PSGL-1 on human HCs provide intrinsically distinct shear stress binding properties.

**EXPERIMENTAL PROCEDURES**

**Cell, Antibodies, and Enzymes—**Human hematopoietic cell lines KG1a (myelocytic leukemia, HCELL/PSGL-1), HL60 (promyelocytic leukemia, HCELL/PSGL-1), RPMI 8402 (lymphocytic leukemia, HCELL/PSGL-1), and K562 (erythroid leukemia, HCELL/PSGL-1), and circulating blasts from a de novo acute myeloid leukemia (AML) without maturation (M1) (HCELL/PSGL-1) were maintained in RPMI 1640, 10% FBS, 1% penicillin/streptomycin (Life Technologies, Inc.) Chinese hamster ovary (CHO) cells transfected with full-length CD44 (CHO-C), anti-CD44 (CHO-2), and CHO-empty vector (CHO-Mock) were obtained from Robert C. Fuhlbrigge (Harvard Medical School) and maintained in minimum Eagle’s medium, 10% FBS, 1% penicillin/streptomycin (Life Technologies, Inc.), and Ham’s F-2, 5 μM glutamine, 5% fetal calf serum, 1% penicillin/streptomycin. Human lymphocytes (PBMC) were prepared from whole blood as described previously (17). Rat thoracic duct lymphocytes (TDL) that express high levels of L-selectin, which functions identically to human lymphocyte L-selectin, were obtained by cannulation of the rat thoracic duct as lymphocytes (PBMC) were prepared from whole blood as described previously (7, 8) (1400, goat anti-CD45, anti-human L-selectin (LAM1) and CD44, fluorescein isothiocyanate anti-CD45, anti-human L-selectin (LAM1–3), and anti-CD34 QBEND Abs were purchased from Coulter-Immunotech, Marseilles, France. Anti-PSGL-1 monoclonal Ab PSL-275 was a gift from Dr. Ray Camphausen (Genetics Institute, Cambridge, MA). Anti-sialyl Lewis X monoclonal Ab (CSLEX-1), anti-CD44 (clone L178), and anti-CD43 antibodies were purchased from Becton Dickinson, San Jose, CA. Anti-CD44 mAb Hermes-1 (rat IgG2a) was originally characterized by Jalkanen et al. (20). Rat monoclonal Ab anti-human CLA (HECA-452), anti-rat L-selectin (HRL-1), ligand blocking antibody, and anti-human P-selectin (clone AK-4) were purchased from Pharmingen, San Diego, CA. All fluorochrome-conjugated secondary antibodies and isotype controls were obtained from Zymed Laboratories Inc., San Francisco, CA.

OGSE was purchased from Accurate Chemicals, Westbury, NY, and Vibrio cholerae neuraminidase and N-glycosidase F was obtained from Roche Molecular Biochemicals. Cobra venom metalloprotease, moccasin (11, 16), was a gift from Dr. Ray Camphausen (Genetics Institute, Cambridge, MA). The metabolic inhibitor, tunicamycin, and all other chemicals were purchased from Sigma.

**Flow Cytometric Analysis—** Flow cytometric analysis was performed on human HCs utilizing both direct and indirect immunofluorescence staining approaches. All cells utilized for these experiments were washed twice with cold PBS, 2% FBS and suspended at 10^6/ml PBS, 1% FBS. Primary antibodies, anti-CLA, -CD15s, -CD34, -CD43, -CD44, -CD62L, and -PSGL-1 (PSL-275 and PL-1), along with the appropriate isotype-matched control antibodies, were incubated with the cells for 30 min on ice. Cells were washed twice with PBS, 2% FBS, and resuspended in PBS, and flow cytometry was performed on a FACSScan apparatus equipped with an argon laser tuned at 488 nm (Becton Dickinson).

**Radiolabeling of Human HC Membrane Proteins, SDS-Polyacryl-
Hydrodynamic Parallel-Plate Flow Chamber Analysis

L-selectin-mediated Adhesive Interactions—By using the parallel-plate flow chamber under defined shear stress conditions, we studied L-selectin-mediated adhesive interactions between human HCs and L-selectin naturally expressed on leukocytes (21). Leukocyte tethering and rolling on human HC monolayers was visualized by video microscopy in real time using the parallel-plate flow chamber prepared in the following manner. Prior to experimentation, leukocytes were washed twice in HBSS and then suspended at 10^6/ml in HBSS, 10 mM HEPES, 2 mM CaCl_2 (H/H/Ca^2+). Negative control groups were prepared by treating cells with PMA (50 ng/ml H/H/Ca^2+ for 1 h at 37 °C) to induce the cleavage of L-selectin from the cell surface, by pretreating with mAb HRL-1 (10 μg/ml) to block L-selectin binding, or by incubating with 5 mM EDTA to chelate Ca^2+ required for L-selectin binding. To prepare human HC monolayers (100% confluent), suspensions of HCs (KC1a, HL60, RPMI 8402, and K562) at 2 × 10^6/ml RPMI 1640 without Na^+ bicarbonate, 2% FBS were seeded in 6-well plates at 5 × 10^5/well, centrifuged to layer cells, 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+. To assess the dependence of binding by sialic acid residues on L-selectin ligands, we pretreated cells with V. cholerae neuraminidase (0.1 units/ml H/H/Ca^2+ for 1 h at 37 °C). To examine the contribution of sialomucins (including PSGL-1) and PSGL-1 alone, OSE (60 μg/ml H/H/Ca^2+ for 1 h at 37 °C) or mocarhagin (10 μg/ml for 20 min at 37 °C) treatments were performed, respectively. Furthermore, since HCELL is expressed on KC1a cells and sialylated N-glycosylations on HCELL are critical for L-selectin ligand activity (7, 19), we distinguished the contribution of HCELL on KC1a cells by first cleaving all of the sialic acid residues from the cell surface with V. cholerae neuraminidase (0.1 units/ml for 1 h at 37 °C) and then incubating the cells with a metabolic inhibitor of N-glycosylation, tunicamycin (15 μg/ml for 24 h at 37 °C, 5% CO_2), to prevent de novo synthesis of N-glycans. Neuraminidase pretreatment removed all of the residual HCELL activity from the cell surface, and therefore, this treatment approach allowed for the assessment of newly synthesized HCELL on the cell surface (7, 19). HC cytopsin preparations were prepared in multwell plates as described above. The parallel-plate flow chamber was placed on top of the cell monolayers and leukocytes were perfused into the chamber. After allowing the leukocytes (human PBMC or neutrophils) to contact the cell monolayers at a shear stress of 0.5 dynes/cm^2 (at which they do not engage in adhesion events), we adjusted the flow rate accordingly to exert shear stress from 0.4 to >20 dynes/cm^2. We quantified the number of leukocytes rolling in one frame of five independent fields under ×100 magnification at shear stress of 0.4, 0.8, 2.2, 4.4, 8.8, 17.6, and 26.4 dynes/cm^2. A minimum of three experiments was performed over the entire range of shear stress, and results are expressed as the mean ± S.D.

P-selectin-mediated Adhesive Interactions—In these experiments, glutaraldehyde-fixed HC monolayers were prepared in 6-well plates as described above, and, where indicated, cells were pretreated with mocarhagin (10 μg/ml) for 30 min and washed extensively with RPMI 1640 without Na^+ bicarbonate, 2% FBS prior to fixation. To study P-selectin-adhesive interactions, confluent CHO cells stably expressing full-length P-selectin (CHO-P) or empty vector (CHO-Mock) were released from flasks with 5 mM EDTA, washed extensively in H/H/Ca^2+, and resuspended at 2 × 10^6/ml for utilization in the parallel-plate flow chamber. P-selectin expression on CHO-P cells was confirmed by flow cytometric analysis. Cell suspensions containing 5 mM EDTA or anti-P-selectin mAbs (10 μg/ml for 30 min on ice) were utilized as negative controls to confirm P-selectin-dependent binding. Cells were perfused into the chamber and allowed to fall onto cell monolayers before commencing the assessment of P-selectin adhesion at 0.2, 0.4, 0.8, and 2.2 dynes/cm^2. Cellular tethering and rolling was visualized at ×100 magnification and quantified and analyzed as described above.

Stamper-Woodruff Assay

L-selectin-mediated Lymphocyte Adherence to HCELL and to PSGL-1—Molar equivalents of immunoaffinity purified HCELL or PSGL-1 (1.5 μg of reduced HCELL (100 kDa) and 2 μg of fully reduced PSGL-1 (140 kDa)) were spotted onto glass slides and allowed to dry. These protein spots were then fixed in 3% glutaraldehyde, and reactive aldehyde groups were blocked in 0.2 M lysine, and slides were kept in RPMI 1640 without Na^+ bicarbonate, 2% FBS until ready for testing. Rat TDL or human PBMC (10^7/ml RPMI 1640 without Na^+ bicarbonate, 5% FBS) were overlaid onto these fixed immunoprecipitates and incubated on an orbital shaker at 80 rpm for 30 min at 4 °C. The number of adherent lymphocytes was quantified by light microscopy using an ocular grid under ×100 magnification (a minimum of five fields/slide, two slides/experiment, and three separate experiments). Data were presented as the mean number of bound lymphocytes ± S.D. KG1a CD34 and L-selectin control immunoprecipitates were also tested in this manner. To verify the dependence for sialic acid, glutaraldehyde-fixed HCELL monolayers were treated with V. cholerae neuraminidase (0.1 units/ml RPMI 1640 without Na^+ bicarbonate, 2% FBS for 1 h at 37 °C). In addition, to verify that cellular adhesion was dependent on L-selectin, all assays included negative controls, in which lymphocytes were treated with PMA (50 ng/ml for 30 min at 37 °C) or functional blocking antibodies (anti-rat-L-selectin mAb HRL-1 or anti-human L-selectin mAb LAM-1; 10 μg/ml), or lymphocyte suspensions contained 5 mM EDTA.

L-selectin-mediated Lymphocyte Adherence to Human HCs—For analysis of cellular L-selectin ligand activity of human HCs, cytospin preparations of KG1a, HL60, RPMI 8402, and K562 cells and of de novo leukemia blasts were fixed in 3% glutaraldehyde, blocked in 0.2 M lysine, and overlaid with lymphocytes (10^6 cells/ml RPMI 1640 without Na^+ bicarbonate, 5% FBS) on an orbital shaker at 80 rpm for 30 min at 4 °C. Slides were then carefully washed with PBS, and bound lymphocytes were fixed in 3% glutaraldehyde. All assays included negative controls as described above. Data were presented as the mean (± S.D.) number of bound lymphocytes at ×100 magnification from a minimum of five fields/slide in duplicate slides from three separate experiments. 

RESULTS

HCELL Is Capable of Engaging with L-selectin Over a Wide Range of Shear Stress—A major goal of this study was to assess the capability of HCELL and PSGL-1 on human HCs to support shear-dependent L-selectin binding activity over a range of shear stress. We analyzed the L-selectin binding characteristics of each of these molecules by performing shear-based adhesion assay systems using human HCs that expressed HCELL and/or PSGL-1 as follows: KG1a (HCELL/PSGL-1), HL60 (HCELL/PSGL-1), RPMI 8402 (HCELL/PSGL-1), and K562 (HCELL/PSGL-1) and a de novo AML without maturation (M1) (HCELL/PSGL-1) (Table I) (7, 17–19).

By using the parallel-plate flow chamber under defined hydrodynamic shear stress, we observed L-selectin-mediated tethering and rolling of leukocytes over glutaraldehyde-fixed human HC monolayers. All experiments included negative controls to verify the sole contribution of L-selectin in mediating cell-cell adherence. A shear stress threshold of ~0.5 dynes/cm^2 was required for L-selectin-mediated adhesive interactions in this system as demonstrated previously (25, 24) (Fig. 1A). After reaching this level of shear stress, we enumerated leukocyte tethering and rolling with V. cholerae neuraminidase treatment as described above (7, 20).

To examine the capability of HCELL and PSGL-1 on human HCs to support L-selectin-mediated adhesive interactions, confluent CHO cells stably expressing full-length P-selectin (CHO-P) or empty vector (CHO-Mock) were released from flasks with 5 mM EDTA, washed extensively in H/H/Ca^2+, and resuspended at 2 × 10^6/ml for utilization in the parallel-plate flow chamber.
lymphocyte/TDL rolling was absent past 17 dynes/cm² (Fig. 1A). In addition, the frequency of rolling lymphocytes on KG1a cells was up to a 5-fold greater over the entire range of shear stress that supported L-selectin-mediated rolling on HL60 cells (Fig. 1A). The disparity between the high L-selectin ligand activity on KG1a cells and low activity on HL60 cells was also observed by using human neutrophils, which expressed equivalent levels of L-selectin to that of lymphocytes by flow cytometric analysis; KG1a cells supported 4-fold greater L-selectin-mediated neutrophil rolling than that on HL60 cells (Fig. 1B). These data show that KG1a cells possess greater capacity to support L-selectin-mediated leukocyte adherence over a broader range of shear stress and that L-selectin natively expressed on lymphocytes or on neutrophils exhibits comparable binding activity to HCELL or to PSGL-1 expressed on human HCs. Moreover, human PBMC and rat TDL had equivalent capacity for L-selectin-mediated binding; due to the ability to obtain large quantities of lymphocytes by rat thoracic duct cannulation, all further parallel-plate flow chamber studies of hematopoietic cell L-selectin ligand activities were performed using rat lymphocytes.

To distinguish the contribution of PSGL-1 activity from HCELL activity on KG1a cells, we performed enzymatic digestion of cells with OSGE or moccarhagin, or we incubated cells with a functional blocking Ab PL-1 that renders PSGL-1 incapable of binding to L-selectin (11, 14–16). Alternatively, to distinguish the contribution of HCELL activity on KG1a cells (which is expressed exclusively on sialylated N-glycans (19)), KG1a cells and blasts from the de novo leukemia were pretreated with neuraminidase and then incubated in tunicamycin, a procedure previously shown not to affect PSGL-1 L-selectin ligand activity but to prevent expression of HCELL-mediated binding (7, 19). Accordingly, L-selectin ligand activity of KG1a cells (via HCELL) was resistant to enzymatic digestion with OSGE or moccarhagin and PL-1 antibody treatments (Table II). However, KG1a L-selectin ligand activity was eliminated following neuraminidase digestion; as shown previously (19), re-expression of ligand activity was markedly reduced following tunicamycin treatment, whereas ligand activity of cells treated with Me₃SO alone (control) returned to base-line levels (p < 0.001) (Table II). These data were consistent with prior data (7, 19) and helped show that N-glycan-dependent HCELL is the primary mediator of L-selectin binding on KG1a cells (7, 19). In contrast, L-selectin ligand activity of HL60 cells was completely eliminated by digestion with OSGE (p < 0.001) (Table II) and significantly inhibited following moccarhagin digestion (p < 0.001) and by treatment with functional blocking anti-PSGL-1 PL-1 monoclonal antibody (p < 0.002) (Table II). The effectiveness of OSGE and moccarhagin treatments was confirmed by flow cytometric analysis of the sensitive epitopes on CD34 and PSGL-1 with mAb QBEND-10 and mAb PSL-275, respectively (data not shown). Interestingly, the fact that L-selectin ligand activity on HL60 cells was completely eliminated following OSGE digestion, but not by PL-1 mAb or moccarhagin treatments, suggests that HL60 cells express other non-PSGL-1 O-sialoglycoprotein L-selectin ligands. These data are consistent with previous studies (25) demonstrating the expression of OSGE-sensitive, non-PSGL-1 L-selectin ligands on HL60 cells.

To investigate further the L-selectin ligand activities of HCELL and PSGL-1 expressed on human HCs, we performed Stamper-Woodruff assays of L-selectin-mediated lymphocyte adherence to glutaraldehyde-fixed HC monolayers under a range of revolutions/min. KG1a cells possessed HCELL ligand activity from 40 to 120 rpm, which was maximal at 80 rpm, whereas HL60 cells exhibited L-selectin ligand activity predominantly at 80 rpm (Fig. 1C). In addition, L-selectin-mediated lymphocyte adherence to KG1a cells was 10-fold higher than that of HL60 cells at 80 rpm, and there was no evidence of lymphocyte binding to K562 and RPMI 8402 cells (Fig. 1C). Because the primary L-selectin ligand on HL60 cells is PSGL-1 and its expression is equivalent on KG1a and HL60 cells (19), our data further suggest that, on a per cell basis, KG1a HCELL activity possesses a higher capacity to function as a ligand over a wider range of shear stress.

Although the level of expression of PSGL-1 is equivalent on KG1a and HL60 cells, we sought to examine whether PSGL-1 on KG1a cells was functioning equivalently to that of PSGL-1 on HL60 cells. Because the critical N-terminal binding determinant of PSGL-1 for P-selectin overlaps with the structural binding determinant(s) for L-selectin (13, 16), we reasoned that P-selectin binding capabilities of KG1a and HL60 cells correlate with the efficiency of PSGL-1 binding to L-selectin. Thus, we performed flow chamber assays of P-selectin ligand activity utilizing Chinese hamster ovary cells transfected with cDNA encoding full-length human P-selectin (CHO-P) (16). Both HL60 and KG1a cells supported equivalent PSGL-1-mediated CHO-P cell rolling, and K562 and RPMI 8402 cells did not possess any activity (Fig. 2A). P-selectin ligand activity on KG1a and HL60 cells was prevented following moccarhagin treatment (Fig. 2B). Unlike the differential capability to support L-selectin ligand activity between KG1a and HL60 cells, these data suggested that native PSGL-1 as expressed in the cell membrane was similar both structurally and functionally in these cell lines. Of note, RPMI 8402 PSGL-1 was non-functional as both an L- or P-selectin ligand, consistent with a finding that PSGL-1 on certain lymphoid cells is non-functional due to a lack of activity of α,1,3-fucosyltransferases and core 2 β1,6-N-acetylgalactosaminyltransferases required for creation of a bioactive ligand (26).

**HCELL Is the Preferred L-selectin Ligand on Human HCs**—The distinction in L-selectin ligand activity between HCELL and PSGL-1 on whole cells may reflect differences in surface
Adhesion of L-selectin Ligands on Human Hematopoietic Cells

By using the parallel-plate flow chamber, thoracic duct lymphocytes (10^7/ml H/H with Ca^2+ were perfused over glutaraldehyde-fixed monolayers of cells treated with either mocarhagin (10 μg/ml; 1 h at 37 °C), OSGE (60 μg/ml; 1 h at 37 °C), PL-1 (10 μg/ml; 30 min on ice) at a defined shear stress of 4.4 dynes/cm².

Table II

| Cells and treatments | Control mean lymphocyte binding (%) |
|----------------------|-------------------------------------|
| KG1a cells + mocarhagin | 11.0 ± 10.5 |
| + OSGE (60 μg/ml) | 104.2 ± 17.1 |
| + PL-1 (anti-PSGL-1; 10 μg/ml) | 104.8 ± 18.0 |
| + Neuraminidase (0.1 units/ml) | 0.3 ± 0.8 |
| + Neuraminidase + Me2SO | 103.3 ± 12.3 |
| + Neuraminidase + tunicamycin (15 μg/ml) | 34.3 ± 9.8 |
| HL60 cells + mocarhagin | 50.0 ± 20.9 |
| + OSGE | 12.5 ± 0.2 |
| + PL-1 | 62.5 ± 1.6 |
| Negative controls | <0.5 ± 0.3 |

* Mean lymphocyte binding from five fields of view from triplicate samples and a minimum of three experiments was divided by the mean lymphocyte binding of the untreated control cells for each respective treatment group.

† Mocarhagin digestion was verified by the inability of anti-PSGL-1 mAb PSGL-275 to recognize the P- and L-selectin binding determinant or mocarhagin-sensitive epitope on PSGL-1 by flow cytometry.

‡ OSGE activity was confirmed by the inability of anti-CD34 mAb QBE10-10 to recognize its OSGE-sensitive epitope on CD34 by flow cytometry.

§ Statistically significant difference in lymphocyte binding compared with untreated control cells; Student’s paired t test, p < 0.001.

¶ Statistically significant difference in lymphocyte binding compared with untreated control cells; Student’s paired t test, p < 0.002.

‖ Negative control groups consisted of 5 mM EDTA-containing assay medium and anti-L-selectin antibody-treated (HRL-1; 10 μg/ml) rat lymphocytes.

adherence to molar equivalents of either HCELL or PSGL-1 immunoaffinity purified from KG1a and HL60 cells, and from a de novo AML (M1).

To isolate HCELL and PSGL-1 for cell binding experiments, we immunoaffinity-purified CD44 (Hermes-1 rat IgG) and PSGL-1 (PL-2 mouse IgG) from cell membrane protein preparations. Autoradiography of Hermes-1 and PL-2 immunoprecipitates obtained from whole cell lysates of [35S]-metabolically radiolabeled KG1a cells showed the specificity of Hermes-1 and PL-2 for their respective antigens (Fig. 3A), revealing that the 100-kDa form of CD44 was principally isolated and that both dimer (220 kDa) and monomer (100-kDa form of CD44) was principally isolated and that both dimer (220 kDa) and monomer (100 kDa) were isolated (Fig. 3A). There was a minor contaminant protein of 30 kDa immunoprecipitated by Hermes-1, which was removed by subsequent passage of immunoprecipitates through a 50-kDa cut-off filter. To normalize for molar equivalency of purified protein utilized in Stamper-Woodruff assays, we compared the densitometric optical density of [35S]methionine-labeled CD44 and PSGL-1 (each passed through a 50-kDa cut-off filter) on autoradiograms of immunoaffinity-purified material spotted onto glass. We found that the optical density of 2 μg of PSGL-1 was 2-fold greater than the optical density of 1.5 μg of CD44. Because monomer PSGL-1 (140 kDa) is ~1.4-fold higher mass than CD44 (100 kDa) and since PSGL-1 has twice as many methionine residues than CD44 (13 kera plus 6), the 2-fold higher signal of PSGL-1 indicated that 1.5 μg of CD44 and 2 μg of PSGL-1 represent equimolar amounts of the respective proteins. By using these equimolar amounts, CD44 supported up to 10-fold greater lymphocyte binding compared with PSGL-1 at 80 rpm and supported a 5-10-fold higher lymphocyte adherence compared with PSGL-1 from 40 to 100 rpm (Fig. 3B). CD34 and L-selectin immunoprecipitated from KG1a cells (negative molecular con-

FIG. 1. L-selectin-mediated leukocyte adherence to human hematopoietic cell lines over a range of shear stress. By using the parallel-plate flow chamber, we measured lymphocyte rolling on glutaraldehyde-fixed hematopoietic cell lines KG1a, HL60, K562, and RPMI 8402 at ~0.4 dynes/cm² (A). Note that the mean number of lymphocytes rolling on KG1a cells was up to 5-fold greater than on HL60 cells over a 2-fold higher shear stress range (Student’s paired t test; p < 0.001). There was no evidence of lymphocyte rolling on K562 and RPMI-8402 cells. B, alternatively, we measured neutrophil rolling on glutaraldehyde-fixed hematopoietic cell lines KG1a, HL60, K562, and RPMI 8402 at ~0.4 dynes/cm². Similarly, neutrophil rolling on KG1a cells was up to 4-fold greater than on HL60 cells over a higher shear stress range (Student’s paired t test; p < 0.001). There was no evidence of neutrophil rolling on K562 and RPMI-8402 at ~0.4 dynes/cm². C, using the shear-based Stamper-Woodruff assay, we evaluated the ability of KG1a, HL60, K562, and RPMI 8402 cell lines to support L-selectin-mediated lymphocyte binding over a range of revolutions/min. Mean lymphocyte adherence to KG1a cells was 10-fold greater than on HL60 cells (Student’s paired t test; p < 0.001). All L-selectin-mediated lymphocyte adherence was prevented by pretreating lymphocytes with anti-L-selectin monoclonal antibodies (10 μg/ml), by using PMA-treated lymphocytes, or by using assay medium containing 5 mM EDTA.
rolling interactions, confirming the requirement for P-selectin. Functional blocking anti-P-selectin and CHO-Mock cells did not exhibit PSGL-1 binding to P-selectin. In addition, CHO-P cells treated with mepacrin (10 μg/ml), which helped substantiate the sole contribution of PSGL-1 binding to P-selectin. In addition, CHO-P cells treated with functional blocking anti-P-selectin and CHO-Mock cells did not exhibit rolling interactions, confirming the requirement for P-selectin.

trolls) did not support any L-selectin-mediated lymphocyte adhesion (Fig. 3B). These data directly comparing the relative L-selectin binding efficiencies of purified CD44 and PSGL-1 were similar to results obtained from whole cell analysis.

To explore whether disparate HCELL and PSGL-1 L-selectin ligand activities were also present in native human hematopoietic cells, we investigated the L-selectin ligand activity of HCELL and PSGL-1 on circulating blasts from a patient with an AML without maturation (M1). In preliminary Stamper-Woodruff assays of whole AML (M1) cell activities, AML (M1) cells showed comparable L-selectin ligand activity to that of KG1a cells, and the expression of CD44 and PSGL-1 (measured by flow cytometry) was also similar to that of KG1a (data not shown). We then immunoprecipitated CD44 and PSGL-1 from these cells and examined their binding capacity by Stamper-Woodruff assay (80 rpm). In parallel, L-selectin ligand activities of HCELL and PSGL-1 from KG1a and HL60 cells were also assessed, and digestions with N-glycosidase F and OSGE were performed for comparative analysis. CD44 from both KG1a and AML (M1) supported 10-fold greater mean number of bound lymphocytes than on KG1a PSGL-1, whereas cell adhesion molecule negative controls, KG1a and CD44 and L-selectin, did display any L-selectin ligand activity. L-selectin-mediated lymphocyte adherence was confirmed in all experiments. No lymphocyte binding was observed by using PMA-treated lymphocytes, anti-L-selectin-treated lymphocytes, and assay medium containing 5 mM EDTA (data not shown).

Blasts from KG1a cells (Table III) possessed any L-selectin ligand activity. Similar to N-glycosidase F-treated CD44 from KG1a cells, the L-selectin binding activity of N-glycosidase F-treated AML M1 CD44 was markedly reduced (to background levels) (Table III). Interestingly, PSGL-1 isolated from KG1a cells and AML (M1) blasts possessed a greater capacity to engage with L-selectin than PSGL-1 from HL60 cells (Table III), even though P-selectin-mediated binding to native PSGL-1 was identical between KG1a and HL60 cells (Fig. 2, A and B). Photomicrographs of lymphocytes bound to CD44 or PSGL-1 in Stamper-Woodruff assays illustrated the distinctive differences in the range of L-selectin ligand activity of KG1a CD44 and AML (M1) CD44 (Fig. 4, A and D, respectively) compared with KG1a PSGL-1 (Fig. 4G); even at 3-fold molar excess of KG1a PSGL-1 (Fig. 4H), L-selectin ligand activity of CD44 (Fig. 4A) was still greater than that of PSGL-1. N-Glycosidase F (Fig. 4, B and E) and OSGE (Fig. 4I) treatments markedly diminished lymphocyte binding comparable with isotype control treatments (Fig. 4, C and F) confirming the relevant contributions of N-glycans and O-glycans on HCELL and PSGL-1, respectively.

To gain insight into the higher capacity of HCELL to bind L-selectin compared with that of PSGL-1, we examined the expression of the sialyl-fucosylated structures (recognized by rat mAb HECA-452) on CD44 and on PSGL-1, which correlates with L-selectin binding capacity (7, 27, 28). SDS-PAGE and
HECA-452 immunoblot analysis of equimolar amounts of CD44 and PSGL-1 immunoprecipitated from KG1a membrane proteins revealed that CD44 was distinctly more HECA-452-reactive than PSGL-1 (Fig. 5), suggesting that HCELL contains a greater number of HECA-452 epitope(s) than PSGL-1, which could account for its higher avidity toward L-selectin.

HECA-452 expression is dependent on critical sialofucosylation on core poly-N-acetyllactosaminyl chains. We thus investigated whether the relative difference in HECA-452 epitope expression and L-selectin binding activity was a consequence of up-regulated α2,3-sialyltransferase (ST3Gal IV) and leukocyte α1,3-fucosyltransferases (FucTIV and FucTVII), which are required for biosynthesis of HECA-452 epitope (7, 22, 27, 29–31). RT-PCR analysis of FucTIV and FucTVII and of ST3Gal IV gene expression in KG1a, HL60, K562, and RPMI 8402 cells showed that FucT IV expression was relatively similar in all cell lines (Fig. 6, A and B), but the FucTVII expression was highest in HL60 and KG1a cells (Fig. 6A, lane 1, FucTV, and lane 2, FucTVI). Interestingly, ST3Gal IV (Fig. 6B, lane 1) was expressed at a high level in KG1a cells and at a very low level in all other cell lines, suggesting that the inherent level of ST3Gal IV may help regulate the expression of relevant HECA-452-reactive structures and critical L-selectin binding determinants on CD44 and/or PSGL-1.

**TABLE III**

| L-selectin ligand       | Mean no. bound lymphocytes* |
|-------------------------|-----------------------------|
| KG1a CD44               | 357.8 ± 36.6                |
| N-Glycosidase-F CD44   | 30.7 ± 18.4                 |
| Isotype control         | 23.3 ± 7.1                  |
| PSGL-1                  | 44.8 ± 6.7                  |
| Isotype control         | 11.0 ± 1.6                  |
| CD34 (molecular control)| 4.0 ± 2.0                   |
| L-selectin (molecular control)| 0.5 ± 0.6                |
| Isotype control         | 8.5 ± 3.5                   |
| HL60 CD44               | 6.5 ± 3.6                   |
| Isotype control         | 5.4 ± 2.3                   |
| PSGL-1                  | 11.0 ± 2.1                  |
| Isotype control         | 3.1 ± 2.9                   |
| AML (M1) CD44           | 425.3 ± 9.5                 |
| N-Glycosidase-F CD44   | 23.4 ± 6.1                  |
| Isotype control         | 34.3 ± 4.1                  |
| PSGL-1                  | 141.5 ± 22.6                |
| Isotype control         | 19.5 ± 6.2                  |

*The average number of bound lymphocytes from five fields of view at × 100 magnification from duplicate slides and a minimum of three experiments. Each experiment included anti-L-selectin Ab-treated (HRL-1; 10 μg/ml) or PMA-treated lymphocytes and 5 mM EDTA-containing assay medium groups, or V. cholerae neuraminidase-treated spots (ligand), which all completely eliminated lymphocyte binding (<0.8 mean number of bound lymphocytes).

**DISCUSSION**

One of the unique aspects of selectin receptor/ligand adhesive interactions is the dependence on shear stress for adhesion (23). This shear dependence is most critical for L-selectin recep-
capacities and biophysical features of HCELL and of PSGL-1. We analyzed the L-selectin ligand activity of HCELL and PSGL-1 natively expressed on human HCs, and we examined the individual ligand activities of immunoaffinity-isolated HCELL and PSGL-1 from these cells. Our results from all experimental approaches consistently reveal that HCELL possesses greater L-selectin ligand activity than PSGL-1.

We first performed analysis of L-selectin binding and the relative shear resistance of HCELL and of PSGL-1 on whole cells to examine the relative functional capacity of these molecules as expressed in their native state on the cell surface. In parallel with previous findings (7, 17–19), L-selectin-mediated leukocyte rolling on KG1a cells persisted after enzymatic digestions that inactivate L-selectin ligand activity of PSGL-1; KG1a cells treated with either OSGE or mocarhagin maintained a high level of leukocyte rolling similar to that of untreated cells, separating the contribution of PSGL-1 from HCELL (7, 19). HCELL exhibited a greater capacity to support L-selectin-mediated cell-cell adhesive interactions compared with that of PSGL-1, as evidenced by 5-fold greater leukocyte rolling on KG1a cells than on HL60 cells over a greater shear stress range (2-fold higher on KG1a cells than on HL60 cells). The high shear resistance feature was also characteristic of HCELL expressed on the de novo AML (M1) leukemic blasts.

Because the binding capabilities of cellular HCELL may have been attributable to its density as it is presented in its native state on the cell surface, we performed conventional Stamper-Woodruff assays of lymphocyte binding to immunoaffinity purified CD44 and PSGL-1 normalized for molar equivalents. These experiments show that CD44 is a more avid L-selectin ligand than PSGL-1 (5-fold higher). Both AML (M1) CD44 and KG1a CD44 possessed a greater shear resistance than PSGL-1 as evidenced by the ability to sustain L-selectin-mediated lymphocyte adherence at a high shear stress range (≥100 rpm). Inhibition of lymphocyte binding to N-glycosidase F-treated CD44 and OSGE-treated PSGL-1 verified the critical contribution of N- and O-glycosylations on HCELL and PSGL-1, respectively. There was no evidence of lymphocyte adherence to neuraminidase-treated CD44 or PSGL-1, indicating that terminal sialic acid on the N- and O-glycan binding determinant(s) is a critical component of L-selectin binding determinants on HCELL and PSGL-1, respectively. These data indicate that the sialylated N-glycosylations on HCELL (six potential N-glycosylation sites on this standard hematopoietic isoform) comprise a higher binding capacity for L-selectin than the sialylated O-glycosylation site(s) on PSGL-1 (one potential O-glycosylation site in the L-selectin binding determinant of the reduced isoform (9)) (7, 37). The fact that KG1a CD44 is strikingly more HECA-452-reactive than KG1a PSGL-1 further supports this contention.

Previous studies from our laboratory show that HL60 cells express HECA-452-reactive PSGL-1 (monomer isoforms at 140 kDa), but not CD44 (7, 8), and HL60 CD44 does not function as an L-selectin ligand (7). Because KG1a cells express both CD44 and PSGL-1 decorated with HECA-452 epitope(s) and HL60 cells possess only HECA-452-reactive PSGL-1, we examined whether inherent differences in α2,3-sialyltransferase and α1,3-fucosyltransferase gene expression, which are involved in the synthesis of HECA-452 epitopes and critical L-selectin binding determinants (7, 22, 27, 29–31), might regulate the differential HECA-452 decoration of CD44 and PSGL-1. Interestingly, KG1a cells express higher levels of α2,3-sialyltransferase, ST3Gal IV, in comparison with HL60 cells, whereas both cell lines express similar levels of α1,3-fucosyltransferases, FucTIV and FucTVII. In addition, gene expression of ST3Gal IV in RPMI 8402 and K562 cells, which possess neither L-selectin ligand activity nor HECA-452-reactive CD44 and PSGL-1 (7), is minimal, whereas FucTIV and FucTVII are expressed at a lower level than HL60 and KG1a cells. These findings indicate that although α1,3-fucosyltransferases are important for HECA-452 epitope synthesis and L-selectin ligand activity, ST3Gal IV expression may be one of the critical regulator(s) for generating high avidity L-selectin binding determinants on CD44 (HCELL). Given the broad expression of CD44 on hematopoietic cells, the restricted expression of HCELL on the CD34+ subset of normal BM mononuclear cells and on certain acute leukemias, but not on erythrocytes, platelets, or on more mature leukocytes (7, 8, 19), suggests that regulated expression of glycosyltransferases (such as ST3Gal IV) directs the expression of this L-selectin ligand at different stages of hematopoietic cell differentiation.

Although L-selectin/L-selectin ligand adhesive interactions under shear are typically observed in the vasculature, these interactions would also occur in the bone marrow where fluid flow conditions exist. Indeed, shear stress probably contributes to the creation of appropriate microenvironmental niches for hematopoiesis, and numerous studies have shown that ex vivo expansion of BM mononuclear cells in the presence of cytokines is enhanced under continuous perfusion conditions (32–36). In these bioreactors, the constant perfusion or flow of culture medium generates a hydrodynamic shear stress that augments the expansion of self-renewing stem cells and of progenitor cells. The data presented here demonstrate that HCELL expressed on human HCs is capable of supporting L-selectin binding over a broad shear stress range, and compared with HCELL, PSGL-1 appears to play a modest role in human HC adherence to L-selectin (37). In fact, L-selectin and CD44 expression on human hematopoietic stem cells, independently, have been correlated with enhanced clonogenic activity (3–6), whereas ligation of PSGL-1 appears to suppress the clonal expansion of hematopoietic progenitors (38). These findings, together with the results of our studies, offer new insights into the preferential engagement of HCELL with L-selectin in the biology of hematopoiesis.

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