A distinct glycoform of CD44 is an L-selectin ligand on human hematopoietic cells

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We previously have obtained operational evidence of a hematopoietic cell L-selectin ligand expressed on normal human hematopoietic cells and on leukemic blasts. Using a technique developed in our laboratory for analyzing and identifying adhesion molecules, we show here that hematopoietic cell L-selectin ligand is a specialized glycoform of CD44. This L-selectin ligand activity of CD44 requires sialofucosylated N-linked glycans and is sulfation-independent. These data provide important insights on the structural biology of CD44 and reveal a role for this protein as an L-selectin ligand on human hematopoietic cells.

The specialized cytoarchitecture of the hematopoietic microenvironment is created by discrete, cell–cell and cell–matrix adhesive interactions that are tightly regulated by lineage-specific expression of adhesion molecules. The earliest human hematopoietic progenitor cells (HPCs) are characterized by the absence of lineage-specific cell surface markers and by expression of CD34. A variety of adhesion molecules are expressed on HPCs, including CD44 (the "hyaluronic acid receptor" or H-CAM), members of the integrin (e.g., lymphocyte function-associated antigen-1, very late antigen-4) and Ig (e.g., intercellular adhesion molecule-1) superfamilies, and L-selectin (1, 2). L-selectin (CD62L) is a calcium-dependent, carbohydrate-binding protein in a family of adhesion molecules that also includes E-selectin (CD62E), expressed on activated vascular endothelium, and P-selectin (CD62P), found on both activated platelets and endothelial cells. L-selectin is best known for its function in the "rolling" phase of leukocyte–endothelial adhesive interactions (3). On lymphocytes, L-selectin mediates trafficking to lymph nodes through its interaction with mAb MECA 79-reactive sialomucin ligands expressed constitutively on high endothelial venules (HEV; refs. 4 and 5).

Whereas L-selectin expression is characteristic of mature leukocytes, its expression among hematopoietic cells is conspicuously regulated: only the earliest HPCs express the protein, and intermediate stages of leukocyte development and all erythroid and megakaryocytic lineage cells are devoid of L-selectin expression (6–8). This highly ordered expression of L-selectin among bone marrow cells suggests that there is a role for L-selectin/L-selectin ligand adhesive interactions in the creation and/or perpetuation of discrete hematopoietic microenvironmental niches. To date, only two naturally expressed L-selectin ligands have been identified among bone marrow cells: P-selectin glycoprotein ligand-1 (PSGL-1) and hematopoietic cell L-selectin ligand (HCLL). Notably, although CD34 functions as an L-selectin ligand in lymph node HEV (9), the CD34 glycoform expressed on human HPCs is not an L-selectin ligand (8, 10). PSGL-1, a mucin-like protein that is expressed on the earliest human HPCs (11), serves as a ligand for all three selectins, and its L-selectin and P-selectin-binding domain requires tyrosine sulfation for ligand activity (12, 13). HCLL is an integral membrane glycoprotein that heretofore has been identified only by functional studies using a conventional Stamper–Woodruff assay technique (10, 14, 15). HCLL is expressed on the human leukemia cell line KG1a, on blasts from *de novo* human leukemias, and, distinctly, on normal human $CD34^+$/$lineage^-$ HPCs but not on more committed ($lineage^+$) HPCs (8, 10, 14). In contrast to all other glycoprotein L-selectin ligands described to date, HCLL activity is *O*-sialoglycoprotein endopeptidase-resistant and sulfation-independent (10, 15). Moreover, HCLL lacks MECA-79 antigen or antigens (15) and its L-selectin binding determinants on HCLL are displayed on N-linked glycans (14).

We recently have developed a technique to analyze and identify molecules directing adhesive interactions under shear conditions. We report here the first application of this approach to identify an unknown/unassigned adhesion molecule. We show that HCLL is a previously unrecognized function of a well characterized adhesion molecule, CD44. Consistent with our previous findings and in contrast to all other glycoprotein L-selectin ligands, HCLL-CD44 isolated from human hematopoietic cells shows sulfation-independent L-selectin ligand activity, and the L-selectin-binding determinants of isolated HCLL-CD44 are displayed on sialylated, fucosylated, N-linked glycans. This specialized glycoform implicates a dual function of CD44, depending on variations in posttranslational modifications, as an adhesive receptor for extracellular matrix elements or as a ligand for an integral membrane glycoprotein, L-selectin.

Materials and Methods

Hematopoietic Cell Lines and Isolation of Human Hematopoietic Cells and Reagents. All hematopoietic cell lines (KG1a, HL60, K562, RPMI 8402) were propagated in RPMI 1640/10% FBS/1% penicillin-streptomycin (GIBCO/BRL). Mononuclear cells were isolated from whole blood of leukemic patients and marrow harvest material by Ficoll/Hypaque (Sigma) density gradient centrifugation. Cell membrane proteins were isolated as described previously (14). Bone marrow cells were sorted on a MoFlo apparatus (Cytomation, Fort Collins, CO) by using fluorochrome-conjugated anti-CD34 mAb (HPCA-2) and anti-CD44 mAb (L178) (Becton Dickinson).

Anti-CD44 mouse mAb A3D8 was from Sigma. Rat IgG mAbs against CD44 (Hermes-1 and IM7) were gifts from Brenda Sandmaier (Fred Hutchinson Cancer Research Center, Seattle). Mouse IgG anti-CD34 mAb QBEND-10 and anti-human L-selectin mAb LAM1–3 were from Coulter–Immunotech. Rat IgM mAb HECA-452 and anti-rat L-selectin mAb HRL-1 were from PharMingen. All fluorochrome- and alkaline phosphatase (AP)-conjugated secondary Abs and isotype controls were from Zymed. Protein G-agarose was from GIBCO/BRL.

O-sialoglycoprotein endopeptidase (OSGE) was from Accurate Chemicals. *Vibrio cholera* neuraminidase and N-glycosidase

Abbreviations: HPC, hematopoietic progenitor cell; PSGL-1, P-selectin glycoprotein ligand-1; HCLL, hematopoietic cell L-selectin ligand; OSGE, *O*-sialoglycoprotein endopeptidase; PVDF, poly(vinylidene difluoride).

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F were from Roche (Gipf-Oberfrick, Switzerland). Sodium chlorate and tunicamycin were from Sigma.

Radiolabeling of KG1a Cellular Proteins. Where indicated, KG1a cultures were incubated with 200 μCi/ml 2-[^{3}H]D-mannose (NEN) for 6 h in glucose-free DMEM growth medium (GIBCO/BRL) or with [^{35}S]Na_2SO_4 (150 μCi/ml) or [^{35}S]EasyTag-L-methionine (150 μCi/ml) with or without sodium chlorate (10 mM) for 8 h. Radiolabeled membrane proteins were isolated as described below.

Preparation of Cellular Membrane Proteins for SDS/PAGE and Immunoblot Analysis of HECA-452-Reactive Proteins and CD44. Cellular membrane proteins were isolated and quantitated as described previously (14) and stored in aliquots at −20°C. For SDS/PAGE and Western blotting, membrane preparations (30 μg/sample) were diluted in reducing sample buffer and separated on 6% or 9% SDS/PAGE gels. Resolved membrane proteins were transferred to Sequi-blot poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad) and blocked with PBS/Tween-20/20% (vol/vol) FBS. Blots were incubated with HECA-452 (1.2 μg/ml PBS), Hermes-1 (2.0 μg/ml PBS), or A3D8. Isotype control immunoblots were performed by using either rat IgM, mouse IgG, or rat IgG, respectively. After washing with PBS/0.1% Tween-20, blots were incubated with either AP-conjugated rabbit anti-rat IgM Abs (1:400), AP-conjugated goat anti-rat IgG (1:200), or AP-conjugated goat anti-mouse IgG (1:8,000). Blots were developed by using Western Blue (Promega), an AP substrate.

To determine whether HECA-452 epitopes on KG1a membrane proteins were dependent on sialic acid and/or O-linked sialoglycoproteins, we treated membrane preparations with either neuraminidase (0.1 unit/ml for 1 h at 37°C) or OSGE (60 μg/ml for 1 h at 37°C) before SDS/PAGE and Western blotting. Alternatively, to assess HECA-452-reactive oligosaccharide(s) produced in the presence of an N-glycosylation inhibitor, we treated KG1a cultures with neuraminidase (0.1 unit/ml), washed the cells two times in PBS, and incubated in tunicamycin (15 μg/ml) or DMSO (diluent control) for 24 h at 37°C (14). Because neuraminidase completely abrogates HECA-452 reactivity, pretreating KG1a cells in this manner allowed for the direct assessment of *de novo* synthesized HECA-452-reactive oligosaccharide(s) expressed on N-glycans (14).

Purification and Mass Spectrometry Analysis of 98-kDa, HECA-452-Reactive KG1a Protein. To isolate the HECA-452-reactive 98-kDa protein(s), we excised the 98-kDa band and then resolved the component protein(s) again by SDS/PAGE by using different polyacrylamide concentrations and reducing conditions. To guide the localization of the relevant band, a gel was run in parallel and subjected to Western blotting with HECA-452 to confirm retention of the reactive protein(s). After three rounds of purification, the 98-kDa band was excised and submitted for analysis by matrix-assisted laser desorption ionization–time of flight mass spectrometry of trypsin-digested gel fragments. Using the University of California at San Francisco Mass Spectrometry Facility MS-FIT Program, the NCBInr database was searched for possible peptide matches.

Immunoprecipitation of CD44. Membrane proteins of hematopoietic cell lines or of leukemia blasts from patients prepared as described above were solubilized in 2% Nonidet P-40 and precleared with Protein G-agarose. Precleared samples (100 μg) were incubated with anti-CD44 mAb Hermes-1 (3 μg) or rat IgG (3 μg) isotype control and then incubated with Protein G-agarose. Immunoprecipitates were washed three times with lysis buffer/2% Nonidet P-40/1% BSA and subjected to reducing 6% or 9% SDS/PAGE, transferred to PVDF membrane, and immunoblotted with mAb HECA-452, A3D8, or Hermes-1. For analysis in the Stamper–Woodruff adherence assay (14), immunoprecipitates were diluted in lysis buffer/2% Nonidet P-40, boiled for 5 min at 100°C, cooled on ice, and spotted onto glass slides.

Hydrodynamic Flow Analysis of L-Selectin Ligands on Blotting Membrane. Using a parallel-plate flow chamber, we studied L-selectin-mediated adhesive interactions, under defined shear stress conditions, between KG1a membrane proteins immobilized on PVDF blotting membrane and human peripheral blood lymphocytes or rat thoracic duct lymphocytes (10, 14, 15), both expressing high levels of L-selectin. Lymphocytes were washed twice in Hanks' balanced salt solution and suspended at 2×10^7/ml in Hanks' balanced salt solution/10 mM Hepes/2 mM $CaCl_2$ (H/H/Ca^{2+})/10% glycerol. Western blots of KG1a membrane preparations stained with HECA-452, A3D8 or Hermes-1 were rendered transparent by immersion in H/H/Ca^{2+}/10% glycerol. To study L-selectin-mediated adhesive interactions, the blots were placed in the parallel-plate flow chamber and lymphocytes were perfused into the chamber at a shear stress of 2.3 dynes/cm^2 (16). We quantified the number of lymphocytes rolling on each region multiplied by five fields. A minimum of three experiments was performed, and results were expressed as the mean of cells rolling per field. Negative controls consisted of assays in the presence of 5 mM EDTA, lymphocytes treated with PMA [50 ng/ml, which induces the cleavage of L-selectin (10)], and lymphocytes treated with functional blocking anti-L-selectin mAbs (10 μg/ml).

Results

Identification of KG1a Membrane Glycoproteins Displaying Sialylated, Fucosylated Structures on N-Glycans. mAb HECA-452 recognizes sialylated, fucosylated epitopes related to sialyl Lewis X structures (16, 17). To compare the relative expression of HECA-452 immunoreactivity on KG1a with that of other hematopoietic cell lines that do not express HCLL activity, we analyzed membrane protein preparations from the HCLL-negative human leukemia cell lines, K562 (erythroid), RPMI-8402 (lymphocytic), and HL-60 (promyelocytic), by Western blot analysis. HECA-452-reactive epitopes were widely distributed on KG1a membrane proteins, revealing several distinct bands whereas only a single, broad band (120–140 kDa) was detected on HL-60 membrane proteins (Fig. 1*A*). K562 and RPMI-8402 membrane proteins were devoid of HECA-452 reactivity. The HECA-452-reactive epitopes of KG1a membrane preparations were completely removed after *Vibrio cholerae* neuraminidase treatment (Fig. 1*A*). Pretreatment of KG1a membrane proteins with OSGE, however, did not affect the HECA-452 staining of several proteins (Fig. 1*A*), suggesting that these structures contained HECA-452-reactive sialylated epitopes displayed on N-linked glycans. Because HCLL activity is also unaffected by OSGE digestion (10), we pursued the OSGE-resistant, HECA-452-reactive proteins as candidates for HCLL.

To confirm expression of HCLL on N-linked glycans, we first digested KG1a cells with neuraminidase to remove HECA-452-reactive epitopes, and then incubated in media with tunicamycin (a metabolic inhibitor of N-linked glycosylation) or with DMSO (diluent control). Membrane preparations were resolved on a reduced 6% SDS/PAGE gel and immunoblotted with HECA-452. As seen in Fig. 1*B*, HECA-452-reactive bands were markedly reduced by treatment with neuraminidase. Culturing in DMSO diluent control resulted in the reexpression of all major HECA-452-reactive bands whereas culture in tunicamycin resulted in the selective absence of a 98-kDa band, variably seen as a doublet on 6% gels (Fig. 1*A*), and a significant reduction in the expression of bands at 60 and 190 kDa (Fig. 1*B*). To evaluate further the presence of N-glycans on HECA-452-reactive proteins and uncover candidate L-selectin ligands, we performed metabolic radiolabeling of KG1a cells with 2-[^{3}H]mannose and studied the [^{3}H]mannose-labeled membrane proteins by SDS/PAGE. Autoradiography of SDS/PAGE-separated 2-[^{3}H]man-

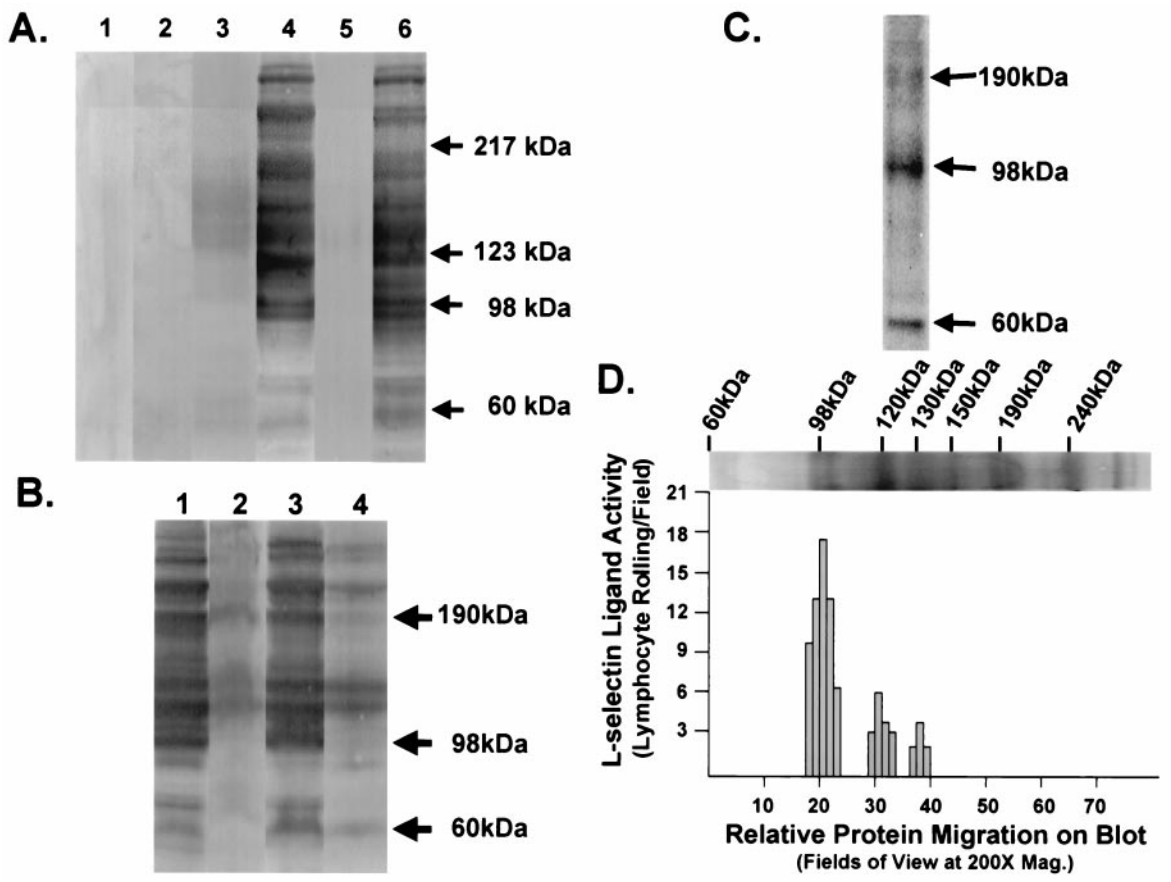

Fig. 1. Identification of potential L-selectin ligands on KG1a cells. (*A*) SDS/PAGE (6% reducing gel) and Western blot analysis of HECA-452-reactive membrane proteins (30 μg/lane) from human leukemia cell lines K562 (lane 1), RPMI-8402 (lane 2), HL-60 (lane 3), KG1a (lane 4), neuraminidase-treated KG1a (lane 5), and OSGE-treated KG1a (lane 6). (*B*) Reexpression of HECA-452-reactive proteins on KG1a whole cells after neuraminidase and then tunicamycin treatment. Lanes: 1, untreated; 2, neuraminidase; 3, neuraminidase then 24-h DMSO recovery; and 4, neuraminidase then 24-h tunicamycin recovery. (*C*) Autoradiography of KG1a membrane proteins metabolically radiolabeled with 2-[^{3}H]mannose resolved on reducing 6% SDS/PAGE. (*D*) L-selectin-dependent, lymphocyte tethering and rolling on blotting membrane under hydrodynamic flow conditions (2.3 dynes/cm^2).

nose-labeled KG1a membrane proteins revealed that the major radiolabeled proteins were 190, 98, and 60 kDa under reducing conditions (Fig. 1*C*). Taken together, these results showed that the 190-, 98-, and 60-kDa proteins identified were the major carriers of sialylated N-linked glycans on KG1a cells.

Application of Novel Blot-Rolling Assay to Identify L-Selectin Ligand Activity of KG1a Membrane Glycoproteins Resolved by SDS/PAGE. To study the HCLL activity of specific HECA-452-stained KG1a membrane glycoproteins, we used a novel cell-adhesion assay system by using a parallel-plate flow chamber that allows for the rapid and reproducible identification of adhesive interactions between cells in flow and molecules immobilized on blotting membranes (R.C.F., S. King, C.J.D., T. S. Kupper, and R.S., unpublished results). In this system, cell lysate material is separated by SDS/PAGE and transferred to PVDF under standard blotting conditions. Blotting membranes are rendered transparent and are incorporated directly into the flow chamber apparatus for analysis of binding characteristics. Relevant cells are introduced into the chamber under defined flow conditions, and cellular adhesive interactions are observed by video microscopy and analyzed in real time. We applied this system to Western blots of KG1a membrane proteins that were immunostained with HECA-452. L-selectin-expressing lymphocytes (10, 15) were perfused over the blots to assess for L-selectin ligand activity on resolved immunostained bands. We observed shear-dependent lymphocyte tethering and rolling (at a shear stress of 2.3 dynes/cm^2) on HECA-452-stained bands at 98, 120, and 130 kDa. Specificity was verified in each case by conducting the assay in the presence of 5 mM EDTA and by pretreating the lymphocytes with either anti-L-selectin-blocking mAbs or PMA (Fig. 1*D*). There was no L-selectin ligand activity observed on any non-HECA-452-stained areas of the blot (Fig. 1*D*). The 98-kDa HECA-452-stained band displayed the greatest L-selectin ligand activity (as much as 6-fold higher compared with other bands) and is also the major *N*-glycan-bearing protein expressed on KG1a cells (Fig. 1*C*). Several of the HECA-452-reactive KG1a bands did not possess L-selectin ligand activity, suggesting that the structural modification(s) associated with these HECA-452-reactive proteins was not sufficient for L-selectin ligand activity. L-selectin ligand activity was absent on Western blots of HL60, K562, and RPMI 8402 membrane proteins, despite evidence of HECA-452-reactive bands (data not shown). HECA-452 staining did not interfere with L-selectin-mediated lymphocyte adherence to the relevant KG1a proteins in hydrodynamic flow assays of Western blots, as parallel blots of KG1a proteins without HECA-452 staining showed equivalent lymphocyte tethering and rolling.

The 98-kDa KG1a Membrane Protein is CD44. The 98-kDa HECA-452-reactive KG1a membrane protein was purified by sequential excision and resolution on SDS/PAGE gels of varying acrylamide percentage. At each stage, retention of HCLL activity was confirmed in the blot-based flow assay. After three rounds of SDS/

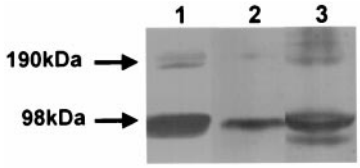

Fig. 2. Western blot analysis of KG1a CD44 with HECA-452 or anti-CD44 mAbs. SDS/PAGE (6% reducing gel) resolution of the 98-kDa gel fragment from the third round of SDS/PAGE purification immunoblotted with either HECA-452 (lane 1) or anti-CD44 mAbs A3D8 (lane 2) and Hermes-1 (lane 3).

PAGE purification, a strong, HECA-452-stained band at 98 kDa was accompanied by a faint band at ≈190 kDa, suggesting that some aggregation of the 98-kDa protein may have occurred. The 98-kDa Coomassie blue-stained gel fragment was then submitted for mass spectrometry analysis of trypsin-digested peptide fragments. The primary peptide map matched that of the standard form of CD44 previously shown to be expressed on KG1a cells (18). To verify this relationship, we immunoblotted purified material with mAbs against CD44 (mouse IgG A3D8 or rat IgG Hermes-1) along with HECA-452 (Fig. 2). Each antibody detected the 98-kDa species as well as the faint band at ≈190 kDa, thought to represent aggregated protein.

L-Selectin Ligand Activity of Immunoprecipitated CD44 and Dependence of N-Glycosylation for L-Selectin Ligand Activity and for Immunodetection by HECA-452. CD44 (Hermes-1) immunoblots of total KG1a cell lysate showed a 98-kDa band, as well as 120- and 130-kDa bands, which may reflect isoforms previously designated as CD44R2 and CD44R1, respectively (Fig. 3; ref. 18). We also observed a faint signal at 190 kDa that was detected by Hermes-1 and A3D8, which may reflect a chondroitin sulfate-modified form of CD44 (Fig. 3; ref. 19). To directly analyze whether CD44 from KG1a cells exhibited L-selectin ligand activity, we immunoprecipitated CD44 with Hermes-1 mAb and performed blot-rolling assays on immunoblots stained with either Hermes-1 or HECA-452. Surprisingly, immunoblots of Hermes-1-immunoprecipitated CD44 that were stained with Hermes-1 displayed only the 98- and 190-kDa species, whereas immunoblots of the same material stained with HECA-452 illustrated not only the 98- and 190-kDa species, but also 120- and 130-kDa species (Fig. 3). In all cases, however, only the

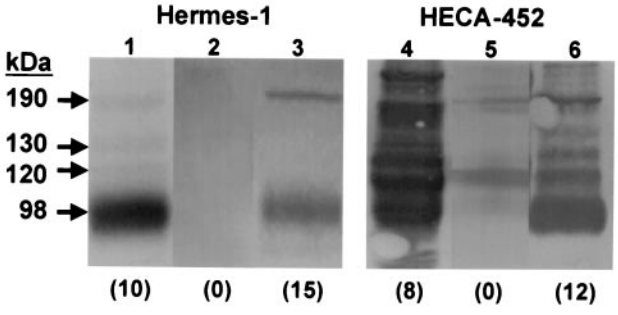

Fig. 3. L-selectin ligand activity of immunoprecipitated CD44. KG1a CD44 immunoprecipitates (Hermes-1 mAb; lanes 3 and 6) resolved on a reducing 9% SDS/PAGE gel, transferred to PVDF membrane, and immunostained with either Hermes-1 or HECA-452. Immunoblots included total cell lysate (lanes 1 and 4) and rat IgG isotype control immunoprecipitate (lanes 2 and 5) groups. L-selectin-dependent lymphocyte rolling was observed in the parallel-plate flow chamber (2.3 dynes/cm^2) only over the 98-kDa protein (lymphocyte-binding data presented in parentheses as the mean number of lymphocytes rolling per field at ×200 magnification from more than five different fields).

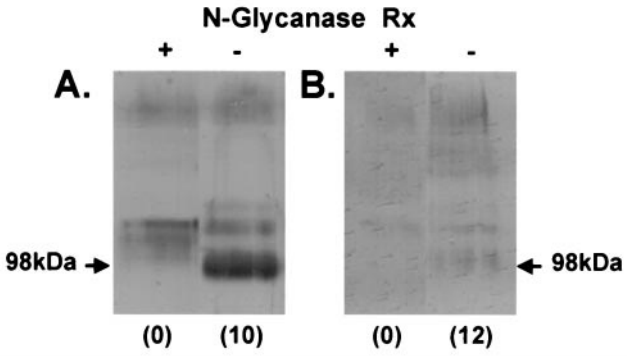

Fig. 4. Effect of N-glycosidase-F on L-selectin ligand activity of immunoprecipitated CD44 from KG1a cells and from AML (M5) blasts. Immunoprecipitated CD44 from KG1a cells (*A*) or from AML (M5) blasts (*B*) were untreated (−) or treated (+) with N-glycosidase-F, resolved on reduced 9% SDS/PAGE, and immunoblotted with HECA-452. L-selectin ligand activity of the 98-kDa band in each case was observed in the parallel-plate flow chamber (2.3 dynes/cm^2), and lymphocyte-binding data are presented in parentheses as the mean number of lymphocytes rolling at ×200 magnification; five fields counted.

HECA-452-reactive, Hermes-1-reactive, 98-kDa protein supported functional L-selectin ligand activity (Fig. 3).

To examine the dependence of L-selectin ligand activity on N-glycosylation, immunoprecipitation of CD44 with Hermes-1 was also performed on KG1a membrane proteins pretreated with N-glycosidase-F. As seen in Fig. 4*A*, N-glycosidase-F treatment completely eliminated HECA-452 staining of the 98-kDa species and abolished all L-selectin-mediated lymphocyte tethering and rolling over the entire blot (ligand activity was analyzed over all molecular mass ranges in the N-glycosidase-F-treated sample, because we expected some change in molecular mass with de-N-glycosylation of the glycoprotein).

To analyze further the L-selectin ligand activity of CD44, we performed Stamper–Woodruff assays by using immunoprecipitated CD44 or isotype control (rat IgG) immunoprecipitates of KG1a cells that was spotted onto glass slides as previously described (14). Assays using *Vibrio cholerae* neuraminidase-treated CD44, anti-L-selectin mAb (HRL-1 for rat lymphocytes; LAM1–3 for human lymphocytes)-treated lymphocytes, or 5 mM EDTA coincubation served as negative controls. We found that immunoprecipitated CD44 supported L-selectin-mediated lymphocyte adherence (362 ± 15 bound cells per field, 5 fields counted, 3 slides per experiments, 2 experiments), whereas no binding was observed with isotype control immunoprecipitate or with neuraminidase-treated immunoprecipitated CD44 or EDTA/anti-L-selectin Ab treatments (<10 bound cells per field). Of note, HECA-452 did not block lymphocyte adherence to isolated CD44, intact KG1a cells, or KG1a membrane proteins, despite input concentrations as high as 100 μg/ml. These results corroborated and confirmed the data from the parallel-plate flow chamber studies described above.

Evidence That CD44 Functions as an L-Selectin Ligand in Freshly Isolated Human Hematopoietic Cells. HCLL activity within normal marrow mononuclear cells was examined by Stamper–Woodruff assays of sorted cell populations of $CD34^+/CD44^+$, $CD34^+/CD44^-$, $CD34^-CD44^+$, and $CD34^-CD44^-$ cells. HCLL activity was absent on all $CD44^-$ subsets, but was present on >80% of $CD34^+/CD44^+$ cells and only ≈1% of $CD34^-CD44^+$ cells. Because biochemical studies of CD44 on normal human $CD34^+$ bone marrow cells were limited by the difficulties in acquiring sufficient quantities of cells for such analysis, we examined the HCLL activity of CD44 isolated from blasts known either to express HCLL or lack HCLL activity (14). To date, we have

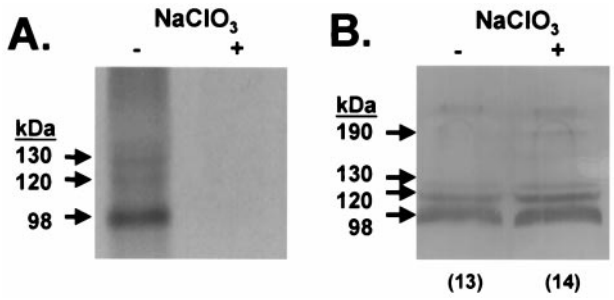

Fig. 5. Effect of chlorate treatment on sulfation and L-selectin ligand activity of KG1a CD44. (*A*) SDS/PAGE (6%, reduced) autoradiogram of immunoprecipitated CD44 from non-chlorate- and chlorate-treated KG1a cells radiolabeled with $[^{35}S]SO_4$. (*B*) HECA-452 immunoblots of immunoprecipitated CD44 from chlorate-treated (+) and non-chlorate-treated (−) KG1a cells. L-selectin ligand activity of the 98-kDa band observed in the parallel-plate flow chamber (2.3 dynes/cm^2) was equivalent in sulfated and nonsulfated (chlorate-treated) CD44 (binding data presented in parentheses as the mean number of lymphocytes at ×200 magnification per field; five fields counted).

surveyed blasts from 11 leukemias: 9 myelocytic [2 undifferentiated (M0), 2 without maturation (M1), 1 with maturation (M2), 2 myelomonocytic (M4), and 2 monocytic (M5)], 1 acute lymphocytic (pre-B), and 1 biphenotypic. With exception of one M0, all leukemic blasts displayed HCLL activity. We analyzed the L-selectin ligand activity of CD44 isolated from blasts of five leukemias described above: one M0 shown to lack HCLL activity and the others with HCLL activity (the biphenotypic leukemia, the other M0, an M4, and an M5). In blot-rolling assays of total membrane protein, lymphocyte tethering and rolling was observed over a 98-kDa band in all leukemias expressing HCLL, but no rolling was observed on any membrane protein from the M0 lacking HCLL activity. CD44 was immunoprecipitated from each of these cells, and, similar to CD44 from KG1a cells, the predominant isoform was a 98-kDa species. A representative example of these data, from an M4 leukemia, is shown in Fig. 4*B*. The requirement of N-glycosylated structures on CD44 for HECA-452 reactivity and L-selectin ligand activity was verified by pretreating the leukemia membrane proteins with N-glycosidase-F (Fig. 4*B*). Conversely, although the HCLL(−) M0 blasts expressed CD44 at levels equivalent to that of the HCLL(+) leukemia specimens (as determined by flow cytometry and by Western blotting using Hermes-1 Ab; data not shown), CD44 from these cells was not HECA-452-reactive and did not exhibit L-selectin ligand activity. Taken together, these observations indicated that the CD44 glycoform exhibiting HCLL activity was not a unique feature of the KG1a cell line, but represented a modification of CD44 present in native blasts of some subsets of human leukemias.

L-Selectin Ligand Activity of CD44 Is Independent of Sulfation. To determine first whether CD44 on KG1a cells is sulfated, we metabolically labeled KG1a cell cultures with $[^{35}S]SO_4$ and found that CD44 immunoprecipitated from these cells was radiolabeled (Fig. 5*A*). To test whether sulfation was critical for L-selectin ligand activity of CD44, we first pretreated KG1a cells with 0.1% bromelain, a protease that eliminates all KG1a HCLL activity (14) and also removes CD44 from the cell surface (20). Our previous studies have shown that after bromelain digestion of KG1a, reexpression of HCLL requires *de novo* protein synthesis and that all protein synthesized in the presence of chlorate (a metabolic inhibitor of both protein and carbohydrate sulfation) is nonsulfated (15). Therefore, we treated KG1a with bromelain and confirmed removal of CD44 by flow cytometry. We then cultured the KG1a cells in the absence or presence of 10 mM sodium chlorate for 24 h and metabolically radiolabeled the cells for the last 8 h of incubation with $[^{35}S]SO_4$ in sulfate-deficient CRCM-30 medium. As illustrated in Fig. 5*A*, the incorporation of $[^{35}S]SO_4$ into immunoprecipitable CD44 (Hermes-1) was completely inhibited in chlorate-treated cells. This effect of chlorate was specific for sulfate incorporation and not a general inhibition of CD44 protein synthesis, because [^{35}S]methionine/cysteine metabolic radiolabeling of CD44 was identical in chlorate- and non-chlorate-treated cell populations (data not shown).

Blot-rolling assays then were performed on CD44 immunoprecipitated from control and chlorate-treated cells. The L-selectin ligand activity of sulfated and nonsulfated CD44 was equivalent (Fig. 5*B*). These experimental data confirmed the results of our previous studies that demonstrate the sulfation independence of HCLL activity (15). Interestingly, recognition of sulfate-free CD44 with HECA-452 was not prevented (Fig. 5*B*). These data show that sulfation was not a critical feature of the epitope on CD44 recognized by the HECA-452 mAb.

Discussion

Using a cell adhesion assay system to detect adhesive interactions under shear conditions, we show here that HCLL, a heretofore operationally defined hematopoietic cell L-selectin ligand, is a distinct glycoform of CD44 containing HECA-452-reactive sialylated, fucosylated N-glycans. Before this report, the identity of HCLL had been defined by a number of discrete biochemical and functional properties. Accordingly, to confirm that HCLL was a glycoform of CD44, we investigated the relevant properties of isolated CD44 obtained from HPCs with and without HCLL activity. Our data showed that only CD44 isolated from HPCs displaying HCLL activity had L-selectin-binding activity, whereas CD44 obtained from cells devoid of HCLL activity did not support L-selectin binding. CD44 isolated from HPCs with HCLL activity ("HCLL-CD44") displayed L-selectin binding with biochemical properties identical to those observed in prior functional studies of HCLL. The observed calcium dependence of L-selectin and HCLL-CD44 interactions is notable in that a cation-dependent adhesive interaction has not been described previously for any CD44-mediated adhesive function. Moreover, although sulfation of CD44 is critical for binding to hyaluronate (21), our data show that the L-selectin ligand activity of HCLL-CD44 is sulfation-independent.

Although several human hematopoietic cell lines (e.g., HL-60 and RPMI-8402) express high levels of CD44, we show that these cells are deficient in HCLL activity (10) and they lack critical, sialylated, fucosylated structure(s) recognized by the rat mAb HECA-452. Even though HECA-452 expression is closely associated with the HCLL phenotype, it alone does not appear to confer HCLL activity: (*i*) HECA-452 antibody binding to blots did not interfere with the binding of L-selectin; (*ii*) in Stamper–Woodruff assays, incubation of KG1a cells with HECA-452 did not prevent HCLL activity; and (*iii*) multiple HECA-452-reactive membrane proteins are present on KG1a cells and *de novo* leukemias that did not display L-selectin ligand activity.

CD44 is a polymorphic integral membrane protein that is widely distributed on the cell surface of not only HPCs, but also on lymphocytes and nonhematopoietic cells (22, 23). Two potential O-linked and six potential N-linked glycosylation sites are found within the hematopoietic or "standard," 37-kDa CD44 core protein isoform, which is the principal isoform on KG1a cells (18). Numerous isoforms of CD44 can result from alternative splicing of the CD44 gene generated by the addition of 1–10 encoding exons (24). Additional polymorphisms may occur through posttranslational modifications of these variant exons. This structural diversity of CD44 translates into a highly pleiotropic adhesion molecule capable of interacting with numerous extracellular matrix proteins (25). However, to date, no integral membrane protein counterreceptor for CD44 had been identified.

One primary function of CD44 is binding to hyaluronic acid (26), and glycosylation of CD44 has a pronounced effect on this inter-

action. The role of N-glycosylation on CD44 apparently has a dual role as a negative (27) and positive regulator (28) of hyaluronic acid binding; this discrepancy apparently is due to the expression of terminal α2,3-sialic acid residues on the N-glycans as sialylation negatively regulates CD44 binding to hyaluronan (29, 30). In contrast, the capability of the standard hematopoietic isoform of CD44 (98 kDa) to function as HCLL is critically dependent on N-glycan sialylation. Thus, the discrete balance between sialyltransferase and sialidase activities in cells bearing CD44 may be pivotal in directing the biological activity of this molecule and of the cells and stromal elements that interact with it.

The role of CD44 in the biology of hematopoiesis and in the regulation of HPC homing and seeding within the bone marrow microenvironment is complex and relatively undefined at present. Mice deficient in all isoforms of CD44 show no alteration in peripheral blood counts but exhibit increased numbers of colony-forming unit/granulocyte–macrophage progenitors in the bone marrow and lower numbers of splenic and peripheral blood progenitors (31). However, the specific contributions of both positive and negative effects on hematopoiesis of discrete CD44–ligand interactions and the temporal sequence of such interactions would be eliminated in aggregate by knock-out of CD44, confounding interpretation of the specific role(s) of this highly polymorphic and pleiotropic molecule in the hematopoietic process. Indeed, antibodies directed against a specific functional epitope of CD44 can result in disruption or enhancement of hematopoiesis, with effects occurring at different stages of hematopoietic differentiation. For example, certain mAbs to CD44, particularly to the N-terminal globular head region, have been shown to inhibit both lympho- and myelopoiesis (32, 33) and interfere with HPC homing and seeding to the bone marrow (34), whereas antibodies to other variably spliced exons within CD44 inhibit only more terminally differentiated progenitors (33). Treatment of mice with anti-CD44 antibodies after syngeneic or allogeneic bone marrow transplantation results in delayed recovery of hematopoiesis (35), whereas other studies using different antibodies to CD44 in a canine model actually have shown an enhancement of bone marrow engraftment after allogeneic transplant (36) and increased hematopoiesis in both autologous and allogeneic long-term marrow cultures (37). Collectively, results of these studies underscore the need to define the stages of differentiation of hematopoietic cells in which relevant CD44 isoforms with differing functional determinants are expressed. Our results showing that HCLL activity is present on $CD34^+/CD44^+$ marrow cells and not present on $CD34^+/CD44^-$ cells suggest that CD44 may serve as the scaffold for ligand-binding determinants and acquisition of the HCLL phenotype on normal HPCs, as it is on leukemic blasts and cell lines. Moreover, these data indicate that the distinct posttranslational modification(s) that confer this unique phenotype are tightly regulated in hematopoietic cell differentiation.

There is abundant evidence, obtained from both clinical studies and *in vitro* clonogenic assays, that L-selectin expression on human $CD34^+$ cells is associated with higher proliferative activity and faster recovery after hematopoietic stem cell transplantation (1, 38–40). Other than HCLL-CD44, the only other potential L-selectin ligand currently known to be expressed on hematopoietic cells is PSGL-1, a molecule that functions as an inhibitor of hematopoiesis (41). In our studies, no significant L-selectin ligand activity was observed in blots at molecular masses corresponding to PSGL-1 [140 kDa (monomer) or 220–240 kDa (dimer)] on any preparation of human hematopoietic cells, despite HECA-452-reactive proteins evident at these regions. Interestingly, consistent with our data, a recent study has shown that normal human $CD34^+$ progenitor cells roll on immobilized L-selectin under shear flow: these interactions did not involve PSGL-1 but, instead, were mediated by an unidentified L-selectin ligand (42).

Besides L-selectin, CD44 expression on $CD34^+$ cells has been associated independently with faster hematopoietic recovery after stem cell transplantation (39). This observation further underscores the importance of understanding the biology of L-selectin and of CD44 in hematopoiesis and, in particular, the role of receptor–ligand interactions mediated by these proteins. The discovery that a distinct glycoform of CD44 on human hematopoietic cells is a novel L-selectin ligand highlights the importance of posttranslational modifications in CD44 function, provides new information on the structural biology of the selectin ligands, and expands our understanding of the molecular basis of adhesive interactions in the hematopoietic microenvironment.

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