

CD44 on LS174T Colon Carcinoma Cells Possesses E-Selectin Ligand Activity

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Abstract

Metastasis of circulating tumor cells requires a multistep cascade of events initiated by adhesion of tumor cells to the vascular endothelium of involved tissues. This process occurs under the forces of blood flow and is promoted by adhesion molecules specialized to interact under shear conditions. The endothelial molecule E-selectin is a major mediator of these adhesive events, and there is strong evidence that E-selectin receptor-ligand interactions contribute to the formation of metastasis. However, little is known about the identity of E-selectin ligand(s) expressed on cancer cells. To address this issue, we did SDS-PAGE analysis of membrane proteins, metabolic inhibition studies, and blot rolling assays of LS174T, a colon carcinoma cell line known to interact with E-selectin under physiologic flow conditions. Our studies show that LS174T cells express the hematopoietic cell E/L-selectin (HCELL) glycoform of CD44, which functions as a high-affinity E-selectin glycoprotein ligand on these cells. However, in contrast to the HCELL glycoform on human hematopoietic progenitor cells, which expresses carbohydrate-binding determinant(s) for E-selectin primarily on N-glycans of standard CD44, the relevant determinant(s) on LS174T cells is expressed on O-glycans and is predominantly found on variant isoforms of CD44 (CD44v). Our finding that tumor-associated CD44 splice variant(s) express E-selectin ligand activity provides novel perspectives on the biology of CD44 in cancer metastasis. (Cancer Res 2005; 65(13): 5812-7)

Introduction

Hematogenous metastasis is a highly regulated and dynamic process, whereby cells separate from a primary tumor, travel through the vascular system, and eventually colonize distant tissues. Tumor cells embark on this journey by using the same molecular adhesive pathways that mediate leukocyte-endothelial interactions in the inflammatory response. In this model, leukocytes first tether and roll on activated vascular endothelium under physiologic flow conditions and then become statically adherent and extravasate (1–3). Endothelial E-selectin is a critical regulator of the initial shear-resistant “rolling” phase of this process and, importantly, has also been shown to mediate

metastatic spread *in vivo* (4, 5) and tumor cell adhesion under dynamic flow conditions *in vitro* (6–8). E-selectin is known to bind sialofucosylated oligosaccharides, such as sialyl Lewis x (sLe^x) and sialyl Lewis a (sLe^a; ref. 9), which are recognized by the monoclonal antibody (mAb) HECA-452. The specificity of E-selectin adhesion to sialofucosylated carbohydrates has been confirmed by studies showing sensitivity to digestion with sialidase and fucosidase for E-selectin ligands expressed by human hematopoietic progenitor cells (HPCs; refs. 7, 10). Notably, expression of these sialofucosylated carbohydrate structures on cancer cells correlates with poor prognosis and tumor progression (11, 12). We and others have shown shear-dependent tumor cell adhesion to E-selectin (6–8). However, the relevant E-selectin ligand(s) of cancer cells has not been fully characterized or identified.

CD44 is a widely expressed cell adhesion glycoprotein that participates in cell-cell and cell-matrix interactions critical to the processes of hematopoiesis, inflammation, angiogenesis, and wound healing (13–15). The discovery of CD44 splice variants (CD44v) on tumor cells (16), coupled with evidence that up-regulation of CD44v confers metastatic potential *in vivo* (16–18) and results in poor prognosis (19), has focused attention on CD44v in the biology of human cancers. However, the direct role of CD44v in the metastatic process has remained obscure.

We report here that CD44v isoforms possessing sialofucosylated epitopes recognized by HECA-452 function as high-affinity glycoprotein E-selectin ligands on the LS174T colon carcinoma cell line. This finding was made possible by use of the blot rolling assay, a technique that detects shear-dependent selectin-ligand interactions on membrane proteins resolved by SDS-PAGE (20, 21). Prior work has shown that a HECA-452-reactive glycoform of CD44 is a high-affinity E-selectin ligand on human HPCs and that the E-selectin-binding determinant(s) is displayed on complex sialofucosylated N-linked glycans (i.e., glycosylations on the asparagine residue of the asparagine-X-serine/threonine consensus sequence; refs. 10, 15, 20, 22). However, studies using inhibitors of glycoconjugate synthesis show here that relevant carbohydrate structure(s) mediating CD44-dependent adhesion of LS174T cells to E-selectin are expressed on O-glycans (i.e., glycosylations extending from serine or threonine linkage). Our finding that O-glycosylated CD44v isoforms function as high-affinity E-selectin ligands expands our understanding of the structural biology of selectin ligands and provides novel insights on the apparent association of CD44v expression and increased metastatic potential of tumor cells.

Materials and Methods

Adhesion molecules, antibodies, and reagents. Anti-human CD44 (515), anti-human cutaneous lymphocyte antigen (CLA; HECA-452), function-blocking anti-E-selectin mAb (68-5H11), and secondary and isotype control antibodies were purchased from BD Biosciences PharMingen

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(San Jose, CA). Anti-human CD44 (2C5) was obtained from R&D Systems (Minneapolis, MN). Alkaline phosphatase (AP)-conjugated anti-mouse IgG and anti-rat IgM were obtained from Southern Biotech (Birmingham, AL). Deoxymannojirimycin was acquired from Calbiochem (San Diego, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Cell culture. LS174T human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in the recommended medium. Before LS174T membrane isolation (see below), cells were detached from culture flasks using 5 mmol/L EDTA in Dulbecco's PBS (D-PBS) for 15 minutes at 37°C. For flow cytometric analysis, LS174T cells were detached using 0.25% trypsin/EDTA for 2 minutes at 37°C and subsequently incubated (1×10^7 cells/mL) for 2 hours at 37°C to allow regeneration of surface glycoproteins (23). Chinese hamster ovary (CHO) cells stably transfected with cDNA encoding full-length E-selectin (CHO-E; kindly donated by Dr. Christine L. Martens, Affymax, Palo Alto, CA) and mock transfectants (CHO-M), were cultured in DMEM/F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum. CHO-E and CHO-M cells were harvested by nonenzymatic means (5 mmol/L EDTA at room temperature for 15 minutes) for use in blot rolling assays. Cell lines were routinely checked and confirmed to be negative for *Mycoplasma* infection and lipopolysaccharide contamination.

Cell treatments. Before metabolic inhibitor studies, LS174T cell suspensions (1×10^7 cells/mL) were pretreated with 0.1 unit/mL *Vibrio cholerae* neuraminidase (Roche Molecular Biochemicals, Indianapolis, IN) for 60 minutes at 37°C to remove terminal sialic acid residues and ensure *de novo* synthesis of newly generated HECA-452-reactive carbohydrate structures (20, 21). Complete removal of sialic acid was confirmed via flow cytometry using mAb HECA-452 that recognizes sialic-acid-bearing epitope(s). Subsequently, LS174T cells were cultured for 48 hours at 37°C in medium containing either 2 mmol/L benzyl-2-acetamido-2-deoxy- α -D-galactopyranoside (benzyl-GalNAc) to inhibit O-linked glycosylation (24) or 1 mmol/L deoxymannojirimycin to disrupt N-linked processing (22, 24); D-PBS diluent was used for control untreated cells. Cell viability was consistently >97% as detected by the trypan blue exclusion assay.

Flow cytometry. Expression levels of CD44 and HECA-452-reactive epitopes on LS174T cells before and after neuraminidase pretreatment and following metabolic inhibitor studies were quantified by using indirect single-color immunofluorescence and flow cytometry (FACSCalibur, BD Biosciences Pharmingen). Cell suspensions (1×10^6 cells/mL) were incubated with anti-CD44 (515) or HECA-452 mAb for 1 hour at 4°C followed by incubation with relevant phycoerythrin-conjugated secondary antibodies. Background levels were determined by incubating cell suspensions with properly matched isotype control antibodies.

Isolation of LS174T cell membrane proteins. LS174T cell membrane proteins were isolated by nitrogen cavitation followed by differential centrifugation (20, 22, 25). In brief, EDTA-detached LS174T cells were washed twice with D-PBS and resuspended (4×10^8 cells/mL) in lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 0.02% sodium azide, 20 mg/mL phenylmethylsulfonyl fluoride, and 1 Complete Protease Inhibitor Cocktail tablet/50 mL lysis buffer (Roche Molecular Biochemicals). The cell suspension was subjected to nitrogen cavitation (800 psi) using a high-pressure cell disruption bomb (Parr Instrument Co., Moline, IL). Ruptured cells were then centrifuged at $3,600 \times g$ for 15 minutes to pellet nuclear and mitochondrial debris and the supernatant was saved. The pellet was washed in lysis buffer and recentrifuged. The two supernatants were pooled and centrifuged at $22,000 \times g$ for 30 minutes to pellet membrane material. The membrane pellet was washed in lysis buffer and recentrifuged twice to obtain high-purity membrane material. The membranes were solubilized by resuspending in lysis buffer containing 2% NP-40 and rotating overnight at 4°C. Membrane lysate was aliquoted and stored at -20°C.

For lipid extractions, membrane preparations were mixed (1:5) with water-saturated chloroform/methanol (2:1) and vortexed for 3 minutes (22). The phases were separated by centrifugation at $16,000 \times g$ for

3 minutes. The organic phase was discarded, and the procedure was repeated once more to ensure complete lipid removal. The aqueous phase was then collected and used in native PAGE blot rolling analysis.

PAGE and Western blotting. Membrane proteins were diluted with Laemmli reducing sample buffer and separated using 4-20% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA). For native PAGE separations, membrane proteins and lipid-extracted membrane proteins were diluted with nonreducing, nondenaturing sample buffer [according to the manufacturer's (Bio-Rad Laboratories) recommendations] and separated using 4-20% PAGE gels. Resolved membrane proteins were transferred to Sequi-blot polyvinylidene difluoride membrane (Bio-Rad Laboratories) and blocked with TBS/0.1% Tween 20/20% fetal bovine serum for at least 1 hour. Immunoblots were stained with anti-CLA (HECA-452) or anti-CD44 (2C5) and then rinsed with TBS/0.1% Tween 20. In all cases, duplicate immunoblots were stained in parallel with relevant isotype control primary antibodies to assess nonspecific binding to protein bands. Subsequently, blots were incubated with appropriate AP-conjugated secondary antibodies. Western Blue AP substrate (Promega, Madison, WI) was used as developing reagent.

Blot rolling assay. The blot rolling assay has been described previously and used to detect selectin-binding activity of membrane proteins resolved by SDS-PAGE (20, 21). Western blots of LS174T membrane preparations representing 5×10^6 cells were stained with anti-CLA (HECA-452) or anti-CD44 (2C5) and rendered translucent by immersion in D-PBS with 10% glycerol. CHO-E cells were resuspended (5×10^6 /mL) in D-PBS containing $\text{Ca}^{2+}/\text{Mg}^{2+}$ and 10% glycerol. The blots were placed under a parallel plate flow chamber (250 μm channel depth, 5.0 mm channel width), and CHO-E cells were perfused at a physiologically relevant shear stress of 1.0 dyne/cm²; an adjustment in the volumetric flow rate was made to account for the increase in viscosity due to the presence of 10% glycerol in the flow medium (20, 21). Molecular weight markers were used as guides to aid placement of the flow chamber over stained bands of interest. The number of interacting cells per square millimeter was tabulated as a function of the molecular weight region and compiled into an adhesion histogram. Histograms are representative of at least three independent experiments. Nonspecific adhesion was assessed by perfusing CHO-E cell suspensions containing function-blocking anti-E-selectin mAb (68-5H11), using 5 mmol/L EDTA in the flow medium, or by perfusing CHO-M cells.

Immunoprecipitation of CD44. CD44 was immunoprecipitated from LS174T cells by incubating membrane lysates with anti-CD44 mAb (2C5) overnight at 4°C. The antibody-lysate mixture was then incubated with Protein G agarose beads (Invitrogen) under constant rotation for 4 hours at 4°C. Antigen-antibody-bound Protein G beads were washed six times with lysis buffer containing 2% NP-40/1% SDS/1% bovine serum albumin followed by three washes with lysis buffer containing 2% NP-40/1% SDS. Immunoprecipitates were then diluted with Laemmli reducing sample buffer (for SDS-PAGE) or native sample buffer (for native PAGE) and heated to 95°C for 5 minutes to release antigens. PAGE and Western blot analysis of immunoprecipitated CD44 was done as described above.

Results

Prior evidence supports the role of sialofucosylated glycoproteins expressed on colon carcinoma cells as important mediators of E-selectin-dependent adhesion to activated human umbilical vein endothelial cells (HUVEC; ref. 8). This finding is underscored by experiments that indicate that protease-treated colon carcinoma cells roll over E-selectin-expressing HUVECs at velocities noticeably faster than control cells (6.6 ± 0.8 versus 1.4 ± 0.9 $\mu\text{m}/\text{s}$ at 2 dyne/cm²; ref. 8). Consequently, we sought to identify and characterize the major E-selectin glycoprotein ligand(s) on LS174T colon carcinoma cells. To this end, we initially did Western blot analysis to survey expression of all HECA-452-reactive glycoproteins, as previous studies have shown a correlation between HECA-452 epitope expression and E-selectin ligand activity (7, 10, 21). As revealed in Fig. 1A, two major

HECA-452-reactive protein bands were identified, broadly distributed at ~150 and ~225 kDa in the 4-20% gradient gels.

We next examined the E-selectin-binding activity of the HECA-452-reactive protein bands using the blot rolling assay, a technique that identifies functional selectin-ligand adhesive interactions under physiologically relevant shear conditions (20, 21). Membrane-bound protein was assessed for its ability to interact with E-selectin under hydrodynamic shear by perfusing CHO-E cells at 1 dyne/cm². In agreement with our previous *in vitro* assays that showed extensive LS174T cell adhesion to E-selectin (8), CHO-E cells interacted and firmly adhered to the Western blot, indicating the retention of E-selectin ligand activity in the blotted membrane lysate. The number of interacting cells per square millimeter was tabulated as a function of the molecular weight region and compiled into an adhesion histogram (Fig. 1B). Whereas the HECA-452-stained blot indicated the presence of prominent sialofucosylated antigens distributed at ~150 and 225 kDa (Fig. 1A), CHO-E cells reproducibly rolled predominantly over the ~150 kDa region. Controls consisting of CHO-E cell suspensions containing function-blocking anti-E-selectin mAb, 5 mmol/L EDTA in the flow medium, or experiments using CHO-M cells had negligible cell adhesion to any region of the blot, confirming specificity for E-selectin ligand activity (data not shown).

Previously, a glycoform of standard CD44 (CD44s), termed hematopoietic cell E/L-selectin (HCELL), was determined to be a high-affinity E-selectin ligand on human HPCs (10). Accordingly,

we examined whether the ~150 kDa E-selectin-reactive glycoprotein on LS174T colon carcinoma cells represented a CD44v isoform. In agreement with previous findings (17), Western blots of whole LS174T membrane lysates stained with anti-CD44 (2C5) revealed the presence of a moderate level of CD44s at ~100 kDa, whereas a more prominent CD44 signal observed at ~150 kDa corresponded to CD44v isoforms (Fig. 1C, lane 1). We next immunoprecipitated CD44 from LS174T cells, did SDS-PAGE, and immunoblotted with 2C5 and HECA-452 in respective lanes of comigrated samples. The results are shown in Fig. 1C, revealing that although LS174T cells express both CD44s and CD44v the HECA-452 expression is prominent only on the CD44 variant isoforms. Blot rolling analysis showed that CHO-E cells interacted and firmly adhered to the higher molecular weight (CD44v) region at levels that were very similar to the whole membrane lysate (Fig. 1D). Interestingly, unlike CD44s expressed on human HPCs, no significant binding was observed at the ~100 kDa CD44s region.

To analyze the presence of potential non-CD44 E-selectin ligands, we depleted LS174T membrane lysate of CD44 by exhaustive immunoprecipitation and measured this depleted fraction for E-selectin ligand activity. After three rounds of immunoprecipitation with 2C5, no CD44 was detectable by Western blot analysis (Fig. 2A, lane 4). In addition, blot rolling assays showed that the number of interacting CHO-E cells over the ~150 kDa region of the CD44-depleted blot was markedly reduced (Fig. 2B), indicating that CD44v isoforms may serve as

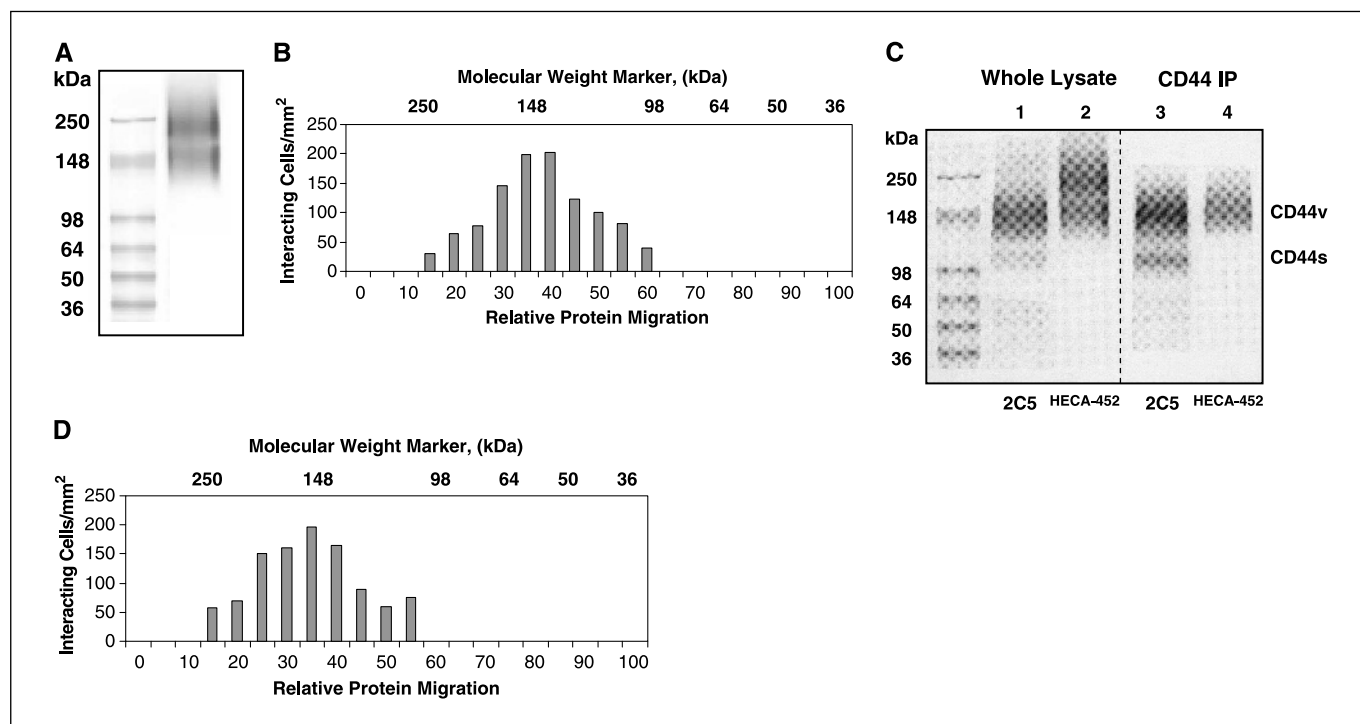


Figure 1. A, Western blots of LS174T whole membrane lysate. HECA-452 mAb staining of LS174T whole membrane lysate resolved by SDS-PAGE reveals two regions of reactivity broadly distributed at ~150 and 225 kDa. B, E-selectin-dependent adhesion to blotting membrane under physiologic flow conditions for whole LS174T membrane lysate. CHO-E cells were perfused over SDS-PAGE immunoblots of whole membrane lysate and the number of interacting cells per square millimeter was tabulated as a function of molecular weight to compile an adhesion histogram. C, Western blots of LS174T whole membrane lysate and immunoprecipitated CD44 isoforms (CD44 IP). Anti-CD44 mAb (2C5) and anti-human CLA mAb (HECA-452) were used to stain Western blots of whole membrane lysate (lanes 1 and 2) and immunoprecipitated CD44 isoforms from LS174T membrane proteins (lanes 3 and 4), respectively. In SDS-PAGE immunoblots using 2C5 (lanes 1 and 3), an intense band developed at ~150 kDa, indicating the presence of CD44v, whereas a much lighter band at ~100 kDa confirmed the presence of a relatively small amount of CD44s. Lane 4 shows that only CD44v was HECA-452 reactive. D, E-selectin-dependent adhesion to blotting membrane under physiologic flow conditions for immunoprecipitated CD44v. The degree of adhesion and shape of the adhesion histograms were very similar to whole membrane lysate in (B), providing direct evidence that CD44v possesses E-ligand activity.

major high-affinity glycoprotein E-selectin ligands on LS174T colon carcinoma cells. To examine whether other potential selectin ligands are rendered nonfunctional when reduced and subjected to SDS-PAGE, blot rolling assays were done using membrane proteins resolved by native PAGE (nonreducing and nondenaturing conditions). The extent of CHO-E cell adhesion was quantified over the entire blot, because protein migration is characteristically broadened under native conditions. CHO-E cells interacted and adhered over a broad area of the blot for the whole membrane lysate. Because lipid migration through polyacrylamide gel is retarded in the absence of SDS, we hypothesized that a major contributor to CHO-E interactions was sialofucosylated glycolipids,

which have been shown previously to play a role in LS174T cell adhesion to E-selectin (8). Accordingly, we depleted membrane preparations of lipids using biphasic solvent extraction and subjected lipid-extracted lysate to PAGE under native conditions. As shown in Fig. 2C, there was substantial residual E-selectin binding to lipid-extracted lysate. To assess the relative contribution of CD44, we then depleted the lipid-extracted lysate of CD44 by exhaustive immunoprecipitation. Lipid-extracted, CD44-depleted lysate displays profoundly decreased E-selectin ligand activity compared with whole lysate and lipid-extracted lysate, indicating that CD44 is the predominant glycoprotein E-selectin ligand on LS174T cells (Fig. 2C).

Next, we sought to characterize the structural linkage bearing E-selectin-binding determinants on CD44v using specific glycoconjugate biosynthesis inhibitors. To this end, CD44 isoforms were immunoprecipitated from LS174T cells that were first treated with neuraminidase and then cultured for 48 hours in medium containing deoxymannojirimycin, a metabolic inhibitor of N-linked glycan processing, or benzyl-GalNAc, an inhibitor of O-linked glycosylation. For each manipulation, medium containing D-PBS diluent was used as the control. In each case, neuraminidase pretreatment was done before culture in metabolic inhibitor or medium control to ensure *de novo* synthesis of newly generated HECA-452-reactive carbohydrate structures (20, 22). Neuraminidase efficacy was confirmed by loss of HECA-452 reactivity determined by flow cytometry (not shown) and by Western blot analysis (Fig. 3A, lane 5). Likewise, equivalent levels of CD44 expression on untreated and metabolically treated LS174T cells were also confirmed by flow cytometry (data not shown). Whereas immunoprecipitated CD44 from both untreated and metabolically treated cells contained equivalent CD44 protein levels (Fig. 3A, lanes 2-4), HECA-452-reactive epitopes were absent on CD44v immunopurified from benzyl-GalNAc-treated cells (Fig. 3A, lane 8). The effect of benzyl-GalNAc treatment was also apparent by the reduction in molecular weight of CD44v as evidenced by its faster migration in the gel compared with the control (Fig. 3A, lane 4), which is consistent with an effect on structural glycosylations. Also interesting was the apparent reduction in molecular weight of CD44s following benzyl-GalNAc treatment; thus, whereas CD44s is modified with O-linked glycan structures (in accord with previous studies; ref. 26), the critical HECA-452-reactive epitopes requisite for its selectin adhesion are not on O-glycans. In contrast, CD44v from deoxymannojirimycin-treated cells was modified with HECA-452-reactive epitopes (Fig. 3A, lane 7) similarly to the control (Fig. 3A, lane 6). CD44s from deoxymannojirimycin-treated cells (Fig. 3A, lane 3) also appeared to have a lower molecular weight as shown by its faster migration in the gel compared with the control (Fig. 3A, lanes 2 and 3), suggesting that it is modified with N-linked and O-linked glycans. Moreover, the efficacy of the deoxymannojirimycin treatment was verified in parallel studies showing the absence of HECA-452 reactivity on CD44s immunopurified from deoxymannojirimycin-treated KG1a cells (Fig. 3B), which is primarily on N-glycans (10, 20, 22). Collectively, these data show that E-selectin-binding determinants on CD44v on LS174T cells are presented predominantly, if not exclusively, on O-linked glycans.

Finally, we characterized the function of CD44v immunopurified from metabolically treated cell lysates by performing blot rolling analysis and compiling E-selectin adhesion histograms. Consistent with the profile of HECA-452 reactivity by Western blot analysis, CHO-E cells adhered to CD44v regions from control and deoxymannojirimycin-treated cells at equivalent levels (Figs. 1D

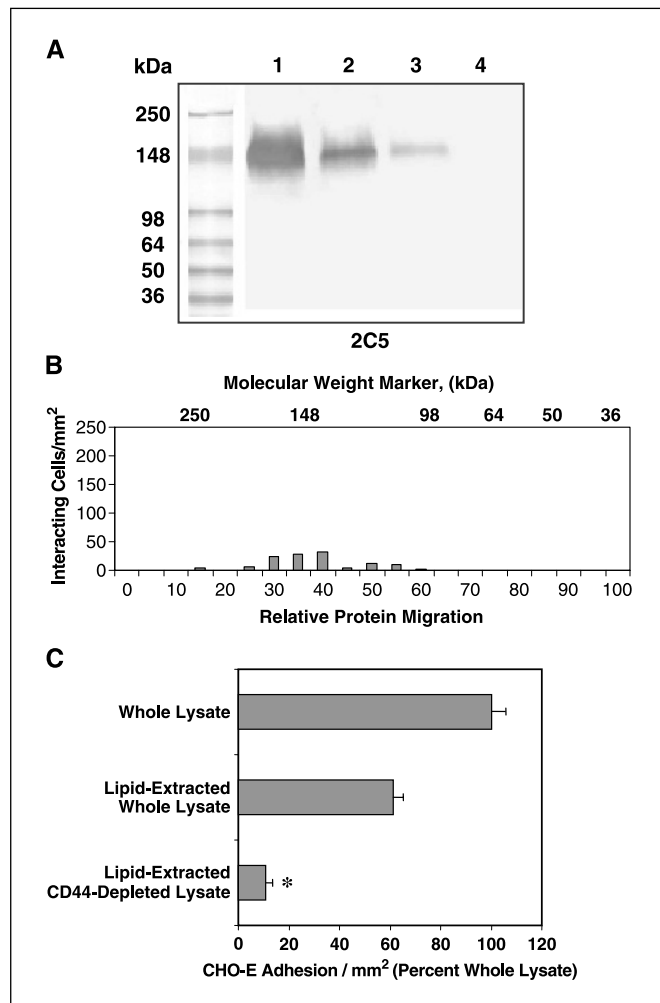


Figure 2. A, LS174T membrane lysate depleted of CD44 by sequential immunoprecipitation. Anti-CD44 mAb 2C5 was used to eliminate CD44 isoforms from LS174T membrane lysate by three rounds of immunoprecipitation. SDS-PAGE immunoblot for CD44: lane 1, whole lysate; lanes 2-4, sequential rounds of immunoprecipitation. Absence of signal in lane 4 confirms depletion. B, E-selectin-dependent adhesion to blotting membrane under physiologic flow conditions for CD44-depleted lysate. The number of interacting cells on the SDS-PAGE blot was dramatically lower for the CD44-depleted lysate, suggesting that CD44v isoforms may play an important role in E-selectin-mediated adhesion to LS174T cells. C, E-selectin-dependent adhesion to blotted membrane proteins separated by native PAGE. Blot rolling assays were done using membrane molecules resolved by native PAGE (nonreducing and nondenaturing conditions) and the extent of adhesion was quantified. The number of interacting CHO-E cells to lipid-extracted, CD44-depleted lysate was significantly lower than to lipid-extracted lysate and whole lysate (*, $P < 0.05$, Student's paired t test), indicating that CD44 is a predominant glycoprotein E-selectin ligand expressed by LS174T cells.

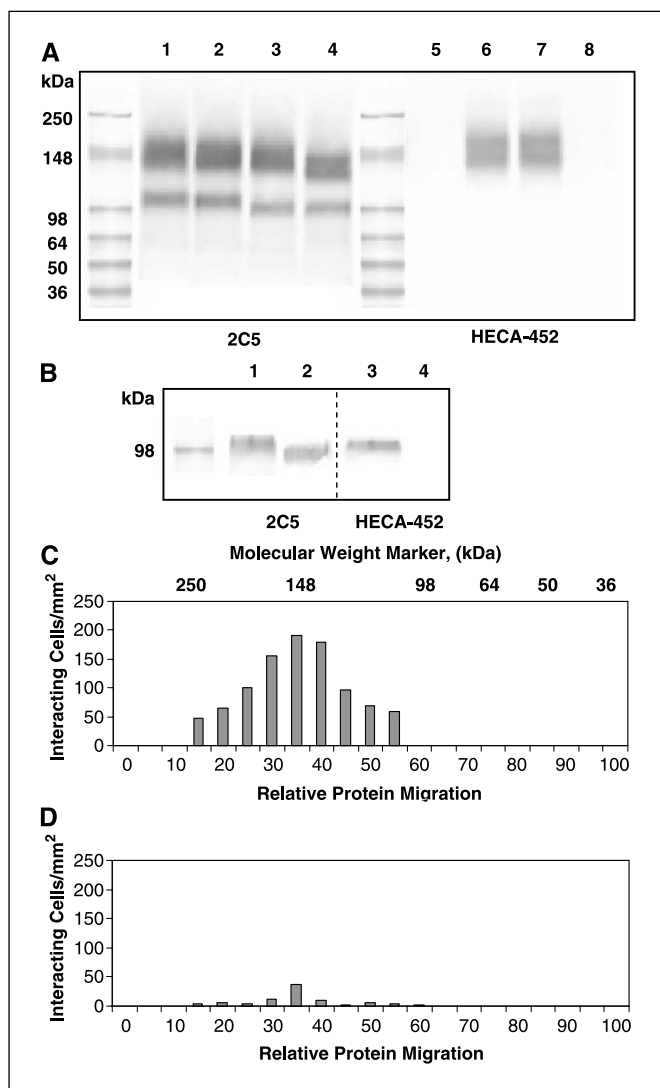


Figure 3. A, Western blots of LS174T cell lysates using cells pretreated with highly specific glycoconjugate biosynthesis inhibitors. CD44 was immunoprecipitated using mAb 2C5 from cells pretreated with 0.1 unit/mL neuraminidase to cleave sialic acid (*lanes 1 and 5*) and cells that were subsequently cultured for 48 hours in medium containing D-PBS (control; *lanes 2 and 6*), 1 mmol/L deoxymannojirimycin (to disrupt N-linked processing; *lanes 3 and 7*), or 2 mmol/L benzyl-GalNAc (to inhibit O-linked glycosylation; *lanes 4 and 8*). Immunoprecipitates were separated by SDS-PAGE before blotting and staining with 2C5 mAb (*lanes 1-4*) and HECA-452 mAb (*lanes 5-8*). Confirmation of sialic acid cleavage by neuraminidase is shown in *lane 5* by absence of HECA-452 reactivity; metabolic recovery of HECA-452 expression is seen in *lanes 6 and 7* but not in *lane 8* (following benzyl-GalNAc treatment). B, Western blot of KG1a cell lysates of cells treated with deoxymannojirimycin. The effectiveness of the deoxymannojirimycin treatment was verified by examining the CD44s expression of treated KG1a cells. Western blots were stained with 2C5 (*lanes 1 and 2*) and HECA-452 (*lanes 3 and 4*). *Lanes 1 and 3*, control; *lanes 2 and 4*, deoxymannojirimycin-treated cells. HECA-452-reactive epitopes on CD44s, known to be predominantly modified with N-linked glycans, were absent from deoxymannojirimycin-treated cell lysates (*lane 4*), indicating that deoxymannojirimycin was highly effective in preventing N-linked glycosylation. E-selectin-dependent adhesion to blotting membrane under shear flow conditions for immunoprecipitated CD44 isoforms from (C) deoxymannojirimycin-treated cells and (D) benzyl-GalNAc-treated cells. Adhesion histograms were compiled for immunoprecipitated CD44 from treated LS174T colon carcinoma cells. CHO-E cells were perfused at 1 dyne/cm² and the extent of adhesion was tabulated. Adhesion to blotted deoxymannojirimycin-treated LS174T CD44v was equivalent to the control (Fig. 1D), suggesting that N-linked carbohydrates do not play a major role in adhesion to E-selectin. Treatment of LS174T cells with benzyl-GalNAc abrogated adhesion to the blot, indicating that O-linked glycans on CD44v are essential for E-selectin-mediated adhesion.

and 3C), whereas CD44v from benzyl-GalNAc-treated cells was essentially incapable of supporting E-selectin adhesion (Fig. 3D). Thus, these analyses of immunoprecipitated CD44 corroborate findings of whole cell lysates showing that the E-selectin-binding determinants on LS174T CD44v are presented on O-linked carbohydrate structures.

Discussion

Recent *in vitro* (6–8) and *in vivo* (4, 5) studies have suggested that E-selectin-dependent receptor-ligand interactions may constitute a molecular mechanism responsible for hematogenous cancer metastasis. Although the “prototypical” target carbohydrates responsible for E-selectin adhesion are HECA-452-reactive sialofucosylated oligosaccharides, such as sLe^x, evidence suggests that expression of these moieties alone is correlated with but not sufficient for E-selectin adhesion (10). Indeed, incubation of cells, membrane lysates, or relevant purified E-selectin ligands with HECA-452 mAb does not interfere with E-selectin ligand activity (10, 21). Therefore, from a molecular perspective, elucidating the specific E-selectin counterreceptors is a critical step toward understanding and preventing metastasis. Prior work indicated that LS174T cells possess a protease-sensitive E-selectin ligand(s) (8). Accordingly, using a new technique, the blot rolling assay, we systematically analyzed LS174T colon carcinoma membrane proteins for E-selectin ligand activity. This approach allowed us to identify that variant isoforms of CD44 are high-affinity E-selectin glycoprotein ligands on LS174T colon carcinoma cells under physiologic flow conditions.

CD44 is encoded by a single gene, but its multiple isoforms are generated by alternative splicing of variant exons v1 to v10 at a single-membrane proximal site of the extracellular domain (27). Additional heterogeneity of CD44 originates from extensive post-translational modifications, including the addition of complex carbohydrate groups. To function as a high-affinity selectin ligand, CD44 must be properly and sufficiently glycosylated. Prior work has shown that glycoforms of CD44 expressed on human HPCs, called HCELL, were decorated with complex HECA-452-reactive N-linked glycans, which bind E-selectin (10, 15, 20, 22). Our work now shows that the LS174T cells express the HCELL glycoform of CD44 but prominently on O-glycosylations rather than N-glycosylations. Further, in contrast to human HPC, this glycoform is expressed on CD44v. Notably, HECA-452 identifies sLe^x and sLe^a carbohydrate structures, but the sLe^a epitope does not appear to be expressed on LS174T cells (8), indicating that the observed HECA-452 reactivity corresponds to sLe^x-type epitopes. Although the regulation of these altered glycosylation states is still under investigation, the incorporation of variant regions of CD44 may provide new sites for glycosylation, resulting in unique adhesion characteristics (28).

Clearance of CD44 from membrane lysates of LS174T cells by repeated immunoprecipitation results in markedly decreased E-selectin ligand activity as shown by blot rolling analysis. This finding indicates that CD44v glycoforms on these cells represent the major glycoprotein ligand(s) for E-selectin. Importantly, although the E-selectin ligand activity of CD44 was initially identified by blot rolling assay of LS174T membrane proteins separated by SDS-PAGE, CHO-E cells interacted and adhered similarly to blots of membrane molecules separated by native PAGE. This suggests that E-selectin-binding activity of structures on blots is not an artifact of denaturing and reducing conditions. Although previous studies have shown that the important

carbohydrate determinants requisite for selectin binding remain functional following SDS-PAGE and Western blotting (10, 15, 20, 22), it may be possible that non-CD44 selectin ligands are rendered nonfunctional when reduced and subjected to SDS-PAGE and Western blotting. To address this issue, blot rolling assays were done using native PAGE. Under these nonreducing and non-denaturing conditions, very little residual E-selectin activity was observed following lipid and CD44 depletion of membrane preparations. The results of native PAGE experiments are consistent with accumulating evidence that glycolipids may also play a role in mediating carcinoma cell attachment to E-selectin expressed on activated HUVECs (7, 8). Therefore, CD44v may cooperate with glycolipids to engage vascular E-selectin at sites of metastasis.

Previous studies have shown that CD44 helps to anchor cells to the extracellular matrix primarily through its interactions with hyaluronan (29), but emerging data suggest that CD44 also presents developmental growth factors (30), participates in signal transduction through interactions with the actin cytoskeleton (31), and acts as an agonist/antagonist in its soluble secreted form (32). It is the role of high molecular weight CD44v in the metastatic process, however, that has remained unclear. There is evidence that the up-regulation of CD44v correlates with metastatic potential of tumor

cells *in vivo* (16–18) and results in poor clinical prognosis (19). In addition, changes in the overall expression levels of CD44 as well the type of isoforms expressed have been linked to the metastatic behavior of tumor cells (17, 33, 34). Likewise, there have been numerous reports that link E-selectin-binding activity with metastatic spread *in vivo* (4, 5). Thus, our findings offer a unifying perspective on these associations and suggest that expression of the HCELL glycoform on CD44v promotes metastasis through adhesive interactions with expressed vascular E-selectin. Moreover, our findings expand the understanding of the structural biology of E-selectin ligands and support further research to investigate HCELL as a potential therapeutic target for the prevention of cancer metastasis.

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