HCELL Is the Major E- and L-selectin Ligand Expressed on LS174T Colon Carcinoma Cells*

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Engagement of vascular E-selectin and leukocyte L-selectin with relevant counter-receptors expressed on tumor cells contributes to the hematogenous spread of colon carcinoma. We recently demonstrated that the LS174T colon carcinoma cell line expresses the CD44 glycoform known as hematopoietic cell E-/L-selectin ligand (HCELL), which functions as a high affinity E- and L-selectin ligand on these cells. To define the contribution of HCELL to selectin-mediated adhesion on intact tumor cells, we measured the binding of LS174T cells transduced with CD44 short interfering RNA (siRNA) or with vector alone to 6-h interleukin-1α-stimulated human umbilical vein endothelial cells (HUVEC) and to human peripheral blood mononuclear cells (PBMC) under physiological flow conditions. LS174T cell attachment to HUVEC was entirely E-selectin-dependent, and PBMC tethering to tumor cell monolayers was completely L-selectin-dependent. At physiological shear stress, CD44 siRNA transduction led to an ~50% decrease in the number of LS174T cells binding to stimulated HUVEC relative to vector-alone-transduced cells. CD44 siRNA-transduced cells also rolled significantly faster than vector-transduced cells on HUVEC, indicating prominent HCELL participation in stabilizing tumor cell-endothelial adhesive interactions against fluid shear. Furthermore, HCELL was identified as the principal L-selectin ligand on LS174T cells, as PBMC binding to CD44 siRNA-transduced tumor cells was reduced ~80% relative to vector-transduced cells. These data indicate that expression of HCELL confers robust and predominant tumor cell binding to E- and L-selectin, highlighting a central role for HCELL in promoting shear-resistant adhesive interactions essential for hematogenous cancer dissemination.

When confined to a primary site (early stage disease), the five-year survival rate from colon cancer is ~90%. However, when disseminated from primary sites (metastatic), survival drops precipitously to 8%. These dramatic survival statistics underscore the need to define the pathobiology of the metastatic process to devise novel interventions to prevent or inhibit colon cancer spread. Hematogenous metastasis occurs as a highly regulated cascade of events, initiated by the escape of tumor cells from the primary site into the blood stream and culminating in the formation of secondary colonies in distant organs. This process critically involves the binding of circulating colon cancer cells to the endothelium of target tissue(s) under fluid shear conditions, as well as the dynamic formation of leukocyte/cancer cell emboli, each of which are directed by selectin-selectin ligand interactions.

The selectin family of adhesion molecules, E-, P-, and L-selectin, are Ca2+-dependent lectins that bind sialofucosylated carbohydrate structures, the prototypes of which are sia1y Lewis X (sLea) and sia1y Lewis A (sLeb) (1, 2). E- and P-selectin are typically inducible endothelial molecules (P-selectin is also expressed on activated platelets), and L-selectin expression is restricted to leukocytes (3). There are multiple reports that tumor cell expression of E-selectin ligand(s) promotes the metastatic spread of numerous cancer types in vivo, including colon cancer (4–10). Colon carcinoma cells also tether and roll under dynamic flow conditions on E-selectin purified and immobilized on plastic (11, 12), as well as E-selectin presented by cytokine-stimulated human umbilical vein endothelial cells (HUVEC) (11–13). Independently, L-selectin has been shown to promote colon cancer metastasis in vivo (14), presumably mediated by the physical association of leukocytes with tumor cells resulting in leukocyte-colon cancer cell emboli (15, 16). Although expression of E-selectin ligand(s) on tumor cells itself promotes metastasis, one model holds that selectins work cooperatively to promote the spread of cancer; leukocyte L-selectin engagement of relevant tumor ligand(s) mediates the formation of leukocyte-tumor aggregates, which possess heightened binding capacity to endothelium in an E-selectin-dependent manner (16).

Colon carcinoma cells express selectin ligands, and there is abundant evidence that selectin-dependent adhesive events are central to the metastatic process (4–10,14). However, these selectin ligands have yet to be fully characterized or identified other than by general classifications (e.g. mucins). We recently identified the sialofucosylated hematopoietic cell E- and L-selectin ligand (HCELL) glycoform of CD44 on the LS174T colon carcinoma cell line and demonstrated its function as a high affinity E- and L-selectin ligand using the blot rolling assay (17, 18). However, these studies did not specifically address the relative contribution(s) of HCELL to the observed potent E- and L-selectin ligand activity of intact LS174T cells. In the present study, using short interfering RNA (siRNA) to specifically inhibit CD44 (i.e. HCELL) expression in LS174T cells, we directly assessed how HCELL expression mediates binding to E- and L-selectin under physiologic flow conditions. We observed that CD44 siRNA treatment resulted in an ~50% decrease in E-selectin ligand activity accompanied by a significant increase in rolling velocity (loss in

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2 The abbreviations used are: HUVEC, human umbilical vein endothelial cell(s); HCELL, hematopoietic cell E-/L-selectin ligand; siRNA, short interfering RNA; PBMC, peripheral blood mononuclear cell(s); mAb, monoclonal antibody; CHO, Chinese hamster ovary; sCD44, standard isoform CD44; vCD44, variant isoform CD44; FBS, fetal bovine serum; IL, interleukin; CMV, cytomegalovirus; HA, hyaluronic acid.
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binding strength) and a nearly complete (>80%) loss in E-selectin ligand activity over a physiologically relevant shear stress range. These findings provide compelling evidence that HCELL is the predominant E- and L-selectin glycoprotein ligand expressed on LS174T colon carcinoma cells and suggest that therapeutic strategies to inhibit HCELL expression/function on cancer cells bearing this CD44 glycoform may be beneficial in the prevention of metastasis.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—LS174T human colon adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in the recommended medium (minimal essential medium containing 10% FBS, 1% sodium-pyruvate, 1% non-essential amino acids, and 1% penicillin/streptomycin). HUVEC were obtained from the tissue culture core facility at the Pathology Department of Brigham and Women’s Hospital (BWH) and were maintained in M199 supplemented with 15% FBS, 5 units/ml heparin, 50 μg/ml endothelial growth factor, and 1% penicillin/streptomycin. For adhesion assays, the HUVEC were cultured at the center of 100-mm tissue culture dishes coated with 10 μg/ml human plasma fibronectin (Sigma). All experiments were performed with confluent HUVEC monolayers. To stimulate expression of vascular adhesion molecules, HUVEC were treated with 1 ng/ml of recombinant human IL-1β.

To stimulate expression of vascular adhesion molecules, HUVEC were treated with 1 ng/ml of recombinant human IL-1β (endotoxin < 0.1 ng/μg IL-1β; Research Diagnostics, Inc., Concord, MA) for 6 h prior to use in the adhesion studies. Chinese hamster ovary cells transfected with full-length E-selectin (CHO-E) or mock-transfected CHO (CHO-mock) cells, provided by Dr. Robert Fuhlbrigge (BWH, Boston, MA), were maintained in minimal essential medium containing 10% FBS, 1% sodium-pyruvate, and 1% penicillin/streptomycin. Human peripheral blood mononuclear cells (PBMC) were prepared from whole blood as described previously (19).

Anti-human CD44 monoclonal antibodies (mAbs) 2C5 and A3D8 were purchased from R & D Systems (Minneapolis, MN) and Sigma, respectively, whereas Hermes-1 was kindly provided by Dr. Brenda Sandmaier (Fred Hutchison Cancer Research Center, Seattle, WA). Anti-human CD29 (mAb 13), function-blocking anti-human E-selectin (68–5H11), and isotype control mAb and fluorescein isothiocyanate-conjugated secondary antibodies were purchased from BD Biosciences. Anti-human CD29 (mAb 13), function-blocking anti-human E-selectin (68–5H11), and isotype control mAb and fluorescein isothiocyanate-conjugated secondary antibodies were purchased from BD Biosciences. Blocking anti-human L-selectin mAb (LAM1-116) was kindly donated by Dr. Thomas F. Tedder (Duke University, Durham, NC). Alkaline phosphatase-conjugated anti-mouse IgG was obtained from Southern Biotech (Birmingham, AL).

Lentiviral CD44 siRNA Construct Design—The pLKO.1 puro vector was sequentially digested with AgeI and EcoRI and ligated with a 5’-mer oligonucleotide containing (i) a 21-nucleotide sense strand and a 21-nucleotide antisense strand separated by a six-nucleotide loop (CTCGAG), (ii) a 5’ end containing an AgeI restriction site (ACCGGT) and a 3’ end containing an EcoRI site (GAAATT), and (iii) a stretch of five adenines (TTTTTT) as a template for the Pol III promoter termination signal (20). The following oligonucleotides were used (sense and antisense strands in bold, loop in underlined letters, restriction enzyme sites in italics, and the Pol III termination signal in lowercase letters): CD44–35 (5’-ACCGGTATGCAATGCTACTGATGTCTCGAGCAATCAGTGACATTTGCAATTtttG-3’) and CD44–3AS (5’-GAAATTGAaaaATGCAATGCTACTGATGTCTCGAGCAATCAGTAATGCAATTGCAATT-3’).

Lentiviral Production, Transduction, and Analysis of CD44 siRNA—The recombinant lentiviruses were produced by co-transfection of 293T cells in Dulbecco’s modified Eagle’s medium containing 10% FBS and 1% penicillin/streptomycin with a mixture of pLKO.1-CD44 siRNA (or pLKO.1 puro), pCMV-VSVG, and pHIR’CMV8.2AR at a ratio of 10:1:9 using FuGene-6 (Roche Applied Science). Infectious lentiviruses were harvested at 48 h post-transfection, and a final concentration of 10 mM HEPES was added to the viral supernatant. The harvested viral supernatant was filtered through a syringe-driven 0.45-μm pore membrane filter (Millipore).

Transductions of LS174T cells with recombinant lentiviruses were conducted in the presence of 8 μg/ml polybrene for 6 h. Following lentiviral infection, the cells were selected with 2.5 μg/ml puromycin. CD44 expression in untreated, vector alone-transduced, and CD44 siRNA-transduced cells was routinely verified by flow cytometry and Western blot analysis and was found to be unchanged within each of the cell treatment groups through serial cell passages.

Flow Cytometry—Surface molecule expression on LS174T cells was assessed using indirect single-color immunofluorescence and flow cytometry. Cell suspensions (10^7 cells/ml) were incubated with primary mAb anti-CD44 (2C5 or A3D8), anti-CD29, or the appropriately matched isotype control antibodies for 30 min on ice, washed twice, and incubated with relevant fluorochrome isothiocyanate-conjugated secondary antibodies. Cells were washed twice, resuspended in phosphate-buffered saline, and analyzed using the Cytomics FC 500 MPL flow cytometer (Beckman Coulter, Miami, FL).

SDS-PAGE and Western Blotting—LS174T cell lysates were prepared by incubation with 2% Nonidet P-40. The cell lysates were diluted with Laemmli reducing sample buffer and separated using 7.5% SDS-PAGE gels (Bio-Rad). Resolved membrane proteins were transferred to Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) and blocked with 100% PBS for at least 1 h at 4°C. Immunoblots were stained with anti-CD44 mAb (2C5) and then washed with Tris-buffered saline/0.1% Tween 20. 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 alkaline phosphatase-conjugated secondary antibodies. Western Blue alkaline phosphatase substrate (Promega, Madison, WI) was used as the developing reagent.

Hyaluronic Acid Binding Assay—To assess the effect of siRNA transduction on CD44 function, binding to hyaluronic acid (HA) was measured. Adhesion of LS174T cells to immobilized hyaluronic acid (from rooster comb; Sigma) was performed in multiwell plates as described previously (21). Briefly, tumor cells (10^6/ml in RPMI 1640 medium containing 10 mM HEPES, 0.2% bovine serum albumin, and 1 mM sodium-pyruvate) were centrifuged to contact the HA-coated substrate (previously incubated with 3% bovine serum albumin/RPMI 1640 medium to block nonspecific interactions), and binding was performed under shear conditions on a rocker platform (5 cycles/min). Nonadherent cells were removed by vigorous washing with assay medium. Bound cells were quantified by light microscopy. Controls consisted of pretreatment of input LS174T cells with Hermes-1 mAb (10 μg/ml), treatment of HA-coated wells with Streptomyces hyalolactylicus hyaluronidase (20 units/ml, Sigma), and omission of HA in the wells.

Parallel Plate Flow Chamber Adhesion Assays—Dynamic flow adhesion assays were performed using a parallel plate flow chamber (250 μm channel depth x 5.0 mm channel width) and visualized in real time using videomicroscopy. For LS174T cell interactions with HUVEC, attachment assays were performed by perfusing cells (10^6/ml in Hank’s balanced salt solution containing 10 mM HEPES, 2 mM CaCl2 (H/H/ Ca^2+), and 5% FBS) at the appropriate flow rates to obtain wall shear stresses of 1.0–2.0 dynes/cm^2, thereby mimicking the fluid mechanical environment of the post-capillary venules (22). The total number of interacting cells in a single 4× field of view (fixed in the middle of the
flow channel, 1.4 mm²) during the 5-min perfusion period was evaluated. Interacting cells were defined as those that bound to HUVEC (both cells initially tethering in the field of view and cells that rolled into the field of view after tethering upstream) and then remained in contact with the monolayer for at least 2 s. To assess binding strength between molecules mediating adhesion between cells in flow and monolayer cells, wall shear stress was increased stepwise every 30 s after the initial attachment period. Average rolling velocity, a quantitative measure of binding strength, was computed as the displacement by the centroid of the cell divided by the time interval of observation, 5 s (13).

To analyze L-selectin-dependent adhesive interactions between PBMC and LS174T cells under flow conditions, 5 × 10⁶ LS174T cells were centrifuged per well of a 6-well plate (Costar) and fixed with 3% glutaraldehyde as previously described (23). Reactive aldehyde groups were blocked with 0.2 M lysine before final equilibration of wells with H/H/Ca²⁺. PBMC in 5% FBS/H/H/Ca²⁺ were perfused at 2 × 10⁶/ml at shear stresses from 0.2 to 2.0 dynes/cm², and the number of PBMC interacting with LS174T monolayers in four independent fields of view after a 2-min attachment period was evaluated.

For some experiments, perfused cells (10⁶/ml) were pretreated for 30 min on ice with function-blocking mAbs (10 µg/ml) prior to use in adhesion assays. For other experiments, HUVEC monolayers were pretreated with function-blocking anti-E-selectin mAb (10 µg/ml) for 30 min at 37 °C. The addition of 5 mM EDTA in the flow medium was used to assess the role of divalent cations in cell binding.

**Blot Rolling Assay**—The blot rolling assay has been previously described and utilized to detect the selectin binding activity of cell lysates resolved by SDS-PAGE (17, 24, 25). Western blots of LS174T...
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TABLE 1
CD44 siRNA transduction of LS174T colon cancer cells specifically reduces expression of CD44

| CD44  | CD29  |
|-------|-------|
| Untreated | 7.0 ± 2.1 (97.2 ± 1.02) |
| Vector  | 8.6 ± 2.2 (96.7 ± 0.9) |
| CD44 siRNA | 1.1 ± 2.0 (27.8 ± 13.5) |

*a p < 0.05 with respect to untreated and vector control.

RESULTS

Transduction of LS174T Cells with CD44 siRNA Leads to Specific Reduction of CD44 Expression and Abrogation of Adhesion to Hyaluronic Acid—The HCELL glycoform of CD44 mediates adhesive interactions with three structures: E-selectin, L-selectin, and HA. More generally, CD44 is recognized for its role as an HA ligand. Accordingly, we measured binding to HA in combination with flow cytometry as indicators of the level of CD44 expression following targeted silencing using siRNA. As shown in Fig. 1, A–C, and Table 1, CD44 siRNA transduction of LS174T cells led to a significant reduction (~90% decrease in mean fluorescence intensity) in surface expression of CD44 relative to vector-alone-transduced cells or untreated cells, as detected by flow cytometry using anti-CD44 mAb 2C5. Similar results were obtained with another anti-CD44 mAb, A3D8 (data not shown). CD44 siRNA treatment was specific, as expression of another cell surface molecule CD29, which mediates adhesion to extracellular matrix elements and not E-selectin, was unchanged after all manipulations (Fig. 1, D–F, and Table 1). Moreover, LS174T cell CD44 silencing did not preferentially target sCD44 or vCD44 isoforms but instead resulted in overall loss of CD44 expression, as shown in a representative Western blot of SDS-PAGE-resolved cell lysates stained with anti-CD44 mAb (Fig. 1G). For both untreated and vector-transduced cell lysates, an intense band developed at ~150 kDa, indicating the presence of vCD44 isoforms, whereas a lighter band at ~100 kDa confirmed the presence of a lesser amount of sCD44. No staining was observed on CD44 siRNA-transduced cell lysate, confirming the silencing of all forms of CD44 by siRNA. The growth rate of LS174T cells under normal culture conditions was not affected by siRNA or vector transduction (data not shown) in agreement with a prior study using antisense to target CD44 expression (26).

In the HA binding assay, untreated LS174T cells bound successfully to substrate (Fig. 2A). Binding was HA-specific, because cells failed to adhere to hyaluronidase-digested HA (data not shown) or non-HA-coated substrate (data not shown). All LS174T cell adhesion to HA was inhibited in the presence of function-blocking anti-CD44 mAb.

lysates representing 3 × 10⁶ cells were stained with anti-CD44 mAb (2C5) and rendered translucent by immersion in H/H/Ca²⁺ with 10% glycerol. CHO-E cells were resuspended (5 × 10⁶ cells/ml) in H/H/Ca²⁺ and 10% glycerol. The blots were placed within a parallel plate flow chamber, 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 because of the presence of 10% glycerol in the flow medium (17, 24, 25). Molecular weight markers were used as guides to aid the placement of the flow chamber over stained bands of interest. The number of interacting cells/mm² was tabulated as a function of the molecular weight region and compiled into an adhesion histogram. Nonspecific adhesion was assessed by perfusing CHO-E cell suspensions containing function-blocking anti-E-selectin mAb, using 5 mM EDTA in the flow medium or by perfusing mock transfectants (CHO-mock).

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FIGURE 2. Micrographs of LS174T-HA binding assay. Untreated (A) or vector-transduced LS174T (C) cells adhered to HA, but adhesion was abrogated in the presence of function-blocking anti-CD44 mAb (B) (Hermes-1, with untreated cells) or with transduction with CD44 siRNA (D). Field = 0.2 mm².

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resolved vector-transduced LS174T cell lysate. Maximum cell binding to membrane occurred at ~150 kDa, which corresponds to the molecular weight of vCD44 on LS174T cells and is consistent with our previous work, including blots of gels run under non-denaturing conditions (17). Adhesive interactions were E-selectin-specific, as no CHO-E cell binding was observed in the presence of anti-E-selectin mAb or EDTA or when CHO-mock cells were perfused. When CHO-E cells were perfused over resolved CD44 siRNA-transduced cell lysate, no binding was observed. Therefore, HCELL is the only E-selectin glycoprotein ligand on LS174T cells, and the residual E-selectin binding observed in HUVEC experiments is attributable to glycolipids.

**HCELL Is the Predominant L-selectin Ligand on Intact LS174T Cells**—PBMC were perfused over monolayers of untreated, vector alone-transduced, or CD44 siRNA-transduced LS174T cells to determine colon cancer HCELL contribution to L-selectin-mediated binding. As shown in Fig. 5, PBMC bound to untreated and vector alone-transduced LS174T cells in a shear stress-dependent manner. PBMC failed to attach at 0.2 dyne/cm², consistent with the shear threshold requirement for L-selectin engagement to its ligands. The upper limit of binding was reached at shear stress levels ≥2.0 dyne/cm². All PBMC attachment to LS174T cells was L-selectin-dependent, as binding was completely abrogated in the presence of function-blocking anti-L-selectin mAb and blockade in the presence of EDTA (data not shown). Transduction of LS174T cells with CD44 siRNA led to nearly complete inhibition of binding of PBMC; L-selectin ligand activity present in CD44-silenced cells likely reflects the minor contribution of residual (unsilenced) HCELL. Values are mean ± S.E., n = 4, *p < 0.05 relative to untreated and vector control.

**Figures**

**FIGURE 3.** LS174T cells transduced with CD44 siRNA bind less efficiently to stimulated HUVEC under physiologic flow conditions. A, native LS174T cells were perfused over 6 h IL-1β-stimulated HUVEC in the parallel plate flow chamber, and the number of cancer cells interacting with the monolayer was counted. All binding was E-selectin-dependent, confirmed by inhibition in the presence of function-blocking anti-E-selectin mAb and by treatment with EDTA (data not shown). E-selectin binding was most evident at shear stress levels ≤2.0 dyne/cm². Values are mean ± S.E., n = 4, B, similar perfusion of vector-transduced or CD44 siRNA-transduced cells revealed that the HCELL contribution to LS174T-HUVEC binding was shear stress-dependent, prominently mediating binding within 1.2–1.8 dyne/cm². Values are mean ± S.E., n = 4, *p < 0.05 relative to vector control. dyn, dyne(s).

**TABLE 2**

CD44 siRNA transduction of LS174T colon cancer cells increases cell rolling velocity on 6-h IL-1β-stimulated HUVEC

| Shear Stress (dyne/cm²) | Untreated | Vector | CD44 siRNA |
|-------------------------|-----------|--------|------------|
| 2 dyne/cm²              | 2.73 ± 0.69 | 2.18 ± 0.68 | 6.59 ± 1.29 | * |
| 4 dyne/cm²              | 4.87 ± 0.79 | 4.25 ± 0.59 | 7.03 ± 0.91 | ** |

*p < 0.05 relative to untreated and vector control.

**FIGURE 4.** Blot rolling assays. HCELL is the primary E-selectin glycoprotein ligand on LS174T cells. CHO-E cells were perfused at 1.0 dyne/cm² over Western blots of vector-transduced (A) or CD44 siRNA-transduced LS174T cell lysates (B) (equivalent to 3 × 10⁶ cells, resolved on a 7.5% SDS-PAGE gel under reducing conditions), and the number of interacting cells/mm² was tabulated as a function of molecular weight to compile an adhesion histogram. The number of interacting cells on the CD44 siRNA blot was dramatically lower than on the vector control blot, indicating that HCELL is the predominant E-selectin glycoprotein ligand on LS174T cells. Data are representative of n = 3 experiments.

**FIGURE 5.** HCELL is the major L-selectin ligand on LS174T cells mediating binding of PBMC. Human PBMC expressing L-selectin were perfused over monolayers of untreated, vector-transduced, or CD44 siRNA-transduced LS174T cells at the indicated shear stresses, and the number of rolling cells were counted. All interactions were wholly L-selectin-dependent, as demonstrated by abrogation in the presence of function-blocking anti-L-selectin mAb and blockade in the presence of EDTA (data not shown). Transduction of LS174T cells with CD44 siRNA led to nearly complete inhibition of binding of PBMC; L-selectin ligand activity present in CD44-silenced cells likely reflects the minor contribution of residual (unsilenced) HCELL. Values are mean ± S.E., n = 4, *p < 0.05 relative to untreated and vector control. dyn, dyne(s).
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DISCUSSION

We recently identified and characterized the sialofucosylated HCELL modification on primarily high molecular weight vCD44 isoform(s) of the LS174T colon carcinoma cell line and demonstrated its function as a high affinity E- and L-selectin ligand using the blot rolling assay (17, 18). However, these studies did not measure the functional contribution of HCELL to the E- and L-selectin ligand activity of intact LS174T cells. Accordingly, we implemented siRNA targeted against CD44 to silence the expression of CD44 on LS174T cells, allowing the study of HCELL-mediated adhesion in a whole cell system. The results herein show that HCELL is the predominant glycoprotein ligand mediating LS174T colon cancer cell adhesion to E- and L-selectin.

Selectin binding capability provides an advantage for tumor cells to extravasate and metastasize to distant sites. In the present study, the optimal operational shear stress of HCELL in the binding of E- and L-selectin is ~1.0–2.0 dynes/cm². Within this range, it is possible that both selectins work synergistically to promote successful leukocyte-tumor cell-endothelium adhesion in the metastatic cascade. The role of E-selectin in promoting adhesion of tumor cells to endothelium has been well described (9, 11–13), but the contribution of L-selectin and leukocytes to the adhesion of cancer cells is less developed. In a mouse model, L-selectin on monocytes acting synergistically with P-selectin on platelets has been implicated in promoting colon cancer metastasis (14). It has also been shown that L-selectin expressed on human neutrophils is necessary to bind LS174T cells (15), but this molecule is apparently not involved in the neutrophil-mediated recruitment of melanoma cells (lacking L-selectin ligands) to stimulated endothelium (27).

Whereas the contribution of HCELL is nearly total for L-selectin-mediated binding (Fig. 5), HCELL contribution to E-selectin binding is ~50%, as determined using CD44 siRNA to silence expression on LS174T cells. The data obtained from blot rolling assays indicate that CD44 is essentially the only glycoprotein ligand for E-selectin, as blots of CD44 siRNA-transduced LS174T cell lysates failed to support CHO-E (i.e. E-selectin) binding, whereas blots of vector-transduced lysates supported robust attachment on CD44 (Fig. 4). Collectively, the results of binding studies of untreated, vector-transduced, and CD44 siRNA-treated cells, together with our previous data revealing that CD44-immunoepitope LS174T cell lysate could not support E-selectin-mediated binding in the blot rolling assay (17), indicate that residual LS174T binding to E-selectin is reflective of glycolipids. It has been previously reported that colon cancer cell glycolipids can mediate binding to E-selectin under flow conditions (13). Although it is difficult to parse the individual versus overlapping adhesive contributions of HCELL and glycolipid ligand(s) to E-selectin-mediated adhesion, E-selectin binding determinant(s) presented on highly extended protein scaffolds (i.e. vCD44 isoforms) have a greater probability of contacting the endothelial surface compared with E-selectin binding determinants on relatively shorter glycolipids, which are shielded by the glycocalyx (28). More importantly, as is the case with relative “unmasking” of glycolipids following protease digestions of cells (29–31), CD44 siRNA transduction results in a loss of CD44 surface expression, thereby accentuating the relevant glycolipid activities on the LS174T cells by allowing otherwise “hidden” glycolipid ligands to engage E-selectin. Thus, on intact cells, it is likely that the HCELL contribution to E-selectin engagement is even more dominant than suggested by the results seen with CD44 gene silencing.

Malignant transformation is frequently associated with alterations in sCD44 and vCD44 expression and aberrant glycosylation. Numerous published reports have shown that CD44 plays a critical role(s) in the metastatic potential of a variety of cancers, including colon, breast, lung, and ovarian cancers (26, 32–35). Moreover, it has been reported that decreasing overall CD44 (standard and variant isoforms) expression (26) or increasing the expression of sCD44 in colon cancer cells blunts metastasis to liver and tumor growth in vivo (36, 37). It is well established that CD44 can engage HA and thus contribute to the metastatic process through extracellular matrix adhesion, cancer cell motility, tumor growth, and signaling (35, 38). However, it has not been shown until presently that CD44 on solid cancer cells will bind to E-selectin on vascular endothelium and L-selectin on leukocytes in a manner similar to that of HCELL originally described on hematopoietic stem cells. Indeed, HCELL is the most potent E-/L-selectin ligand yet identified (23, 24, 39). Moreover, the robust selectin binding is retained despite the different linkages of the relevant sialofucosylated selectin binding determinants (such as sLeα and sLeβ) displayed on the core CD44 protein; the E-/L-selectin binding determinants of the hematopoietic HCELL are expressed primarily on N-glycans of sCD44 on hematopoietic stem cells (24, 39) while principally on O-glycans of vCD44 on colon cancer (LS174T) cells (17, 18). Furthermore, HCELL can bind selectins as well as HA. This characteristic is unusual, insofar as sialic acid is necessary for the selectin ligand activity of HCELL (17, 18, 23, 24, 39), yet this carbohydrate modification carries a net negative charge that can dampen CD44 binding to HA by interfering with the positively charged HA binding site (38). Notably, anti-CD44 mAb known to block CD44 binding to HA had no effect on HCELL binding to E-selectin either in parallel plate flow chamber assays using HUVEC or in blot rolling assays (data not shown), indicating that the selectin binding site on HCELL is distinct from that of HA. In any case, the ability of HCELL to engage selectins and HA provides a unifying perspective on the heretofore independent observations of the involvement of CD44, selectins, and HA in promoting metastasis.

Collectively, the current study has demonstrated that the HCELL glycoform of CD44 on colon cancer cells is the predominant mediator of shear resistant binding to E- and L-selectin. Our results offer a shear-stress-dependent model whereby this molecule mediates colon cancer cell capture on endothelial E-selectin, as well as promoting L-selectin-dependent binding of leukocytes to tumor cells, thus forming heterotypic emboli capable of amplifying metastatic potential. The increased understanding of HCELL as a mediator of tumor cell adhesion to vascular cells and leukocytes in flow provides compelling logic to explore HCELL-directed therapeutic interventions as a means to combat hematogenous colon cancer metastasis.

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