Selectin-mediated binding of tumor cells to platelets, leukocytes, and vascular endothelium may regulate their hematogenous spread in the microvasculature. We recently reported that CD44 variant isoforms (CD44v) on LS174T colon carcinoma cells possess selectin binding activity. Here we extended those findings by showing that T84 and Colo205 colon carcinoma cells bind selectins via sialidase-sensitive O-linked glycans presented on CD44v, independent of heparan and chondroitin sulfate. To assess the functional role of CD44v in selectin-mediated binding, we quantified the adhesion to selectins of T84 cell subpopulations sorted based on their CD44 expression levels and stable LS174T cell lines generated using CD44 short hairpin RNA. High versus low CD44-expressing T84 cells tethered more efficiently to P- and L-selectin, but not E-selectin, and rolled more slowly on P- and E-selectin. Knocking down CD44 expression on LS174T cells inhibited binding to P-selectin and increased rolling velocities over P- and L-selectin relative to control-transfected cells, without affecting tethering and rolling on E-selectin, however. Blot rolling analysis revealed the presence of alternative sialylated glycoproteins with molecular masses of ~170 and ~130 kDa, which can mediate selectin binding in CD44-knockdown cells. Heparin diminishes the avidity of colon carcinoma cells for P- and L-selectin, which may compromise integrin-mediated firm adhesion to host cells and mitigate metastasis. Our finding that CD44v is a functional P-selectin ligand on colon carcinoma provides a novel perspective on the enhanced metastatic potential associated with tumor CD44v overexpression and the role of selectins in metastasis.
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To date, the selectin ligands on colon carcinoma cells have yet to be identified and characterized other than by general classifications (i.e. siafofucosylated mucin-like glycoproteins). The binding affinity of selectins for isolated monovalent sLeα and its isomer sLeα is very low. Consequently, neither expression of the sLeα nor the sLeα groups per se correlates with the properties of endogenous selectin ligands on cellular targets. As has been argued appropriately in the literature (16), distinctions must be drawn between structures than can bind to selectins under certain conditions in vitro and structures that actually do interact with selectins in vivo. To this end, a “functional” selectin ligand should fulfill certain criteria (16) as follows: it should be expressed in the right place at the right time; the ligand should bind with some selectivity and relatively high affinity; and removal or absence of the ligand should prevent cell adhesive interactions.

We recently reported that O-linked siafofucosylated glycans on CD44 variant isoforms (CD44v) on LS174T colon carcinoma cells possess selectin binding activity (17, 18). However, these studies did not reveal a functional role for LS174T CD44v in selectin-mediated adhesion. Moreover, it is not clear whether the selectin binding activity of CD44v is shared by other metastatic colon carcinoma cells. Here we employ two distinct metastatic colon carcinoma cell lines, LS174T and T84, expressing similar surface levels of CD44, and two complementary strategies to modulate CD44 expression to assess whether CD44v is a functional selectin ligand on colon carcinoma cells under physiological shear conditions. By perfusing subsets of T84 cells, sorted based on their CD44 expression levels, over purified selectin substrates, we show that high versus low CD44-expressing cells (mean fluorescence intensity (MFI), 1731 versus 364) bind more efficiently to P- and L- but not E-selectin and roll more slowly on P- and E-selectin. These data highlight the role of avidity in CD44v binding to P- or L-selectin and implicate CD44v as an auxiliary E-selectin ligand on colon carcinoma, which is engaged in the stabilization of tumor cell-endothelial cell adhesive interactions against fluid shear. Furthermore, stable LS174T cell lines generated using CD44 short hairpin (sh)-RNA display markedly reduced (>95%) CD44 surface expression and tether to P- but not L- or E-selectin with an ~55% efficiency relative to untreated or control-transfected cells. Interestingly, this intervention did not alter the rolling velocity of CD44-knockdown LS174T cells relative to control cells on E-selectin. The lack of significant inhibition of cell tethering to L-selectin and the absence of modulation of the rolling velocity on E-selectin are attributed to the presence of siafofucosylated glycans on alternative selectin ligands with apparent molecular masses of ~130 and ~170 kDa in CD44-knockdown cells. Taken altogether, these data provide functional evidence that CD44v is a major functional P- but not L- or E-selectin ligand on colon carcinoma cells. Our findings offer a unifying perspective on the apparent enhanced metastatic potential associated with tumor cell CD44v overexpression and the critical role of selectins in metastasis.

EXPERIMENTAL PROCEDURES

Adhesion Molecules, Antibodies, and Reagents—The chimeric forms of L-selectin-IgG Fc (L-selectin), P-selectin-IgG Fc (P-selectin), and E-selectin-IgG Fc (E-selectin), consisting of the lectin, epidermal growth factor, and consensus repeat domains for human L-, P-, or E-selectin linked to each arm of human IgG1, were generous gifts of Wyeth External Research (Cambridge, MA) (19). Anti-CD44 (2C5) monoclonal antibody (mAb) was from R&D Systems (Minneapolis, MN). Alkaline phosphatase (AP)- and horseradish peroxidase (HRP)-conjugated anti-mouse IgG and AP-conjugated anti-rat IgM were from Southern Biotech Associates (Birmingham, AL). All other unlabeled and phycoerythrin (PE)- or fluorescein isothiocyanate-conjugated antibodies were from BD Biosciences. All other reagents were from Sigma unless otherwise stated.

Cell Culture—The human colorectal carcinoma cell lines T84, Colo205, and LS174T were obtained from the American Type Culture Collection (Manassas, VA) and cultured in the recommended media. Prior to cell lysis, colon carcinoma cells were detached from culture flasks using Enzyme Free Cell Disassociation Media (15 min at 37 °C; Chemicon, Phillipsburg, NJ). For flow cytometric/sorting and flow-based adhesion assays, T84 and LS174T cells were harvested by mild trypsinization (0.25% trypsin/EDTA for 5 min at 37 °C) and subsequently incubated (10⁷ cells/ml) at 37 °C for 2 h to allow regeneration of surface glycoproteins (7, 8, 20). Colo205 cells were detached by nonenzymatic means, as described above, and used immediately. CHO cells, stably transfected with cDNA encoding full-length E- (CHO-E) or P-selectin (CHO-P), were kindly donated by Affymax (Palo Alto, CA) and processed as described previously (18). Cell lines were routinely checked and confirmed to be negative for mycoplasma infection (8).

Colon Carcinoma Cell Lysis and Immunoprecipitation of CD44—Whole cell lysate was prepared by membrane disruption using 2% Nonidet P-40 followed by differential centrifugation (18, 21, 22). CD44 was immunoprecipitated from colon carcinoma cell lysate with an anti-CD44 mAb, 2C5, using recombinant protein G-agarose beads (Invitrogen) (18).

SDS-PAGE and Western Blotting—Whole cell lysate or immunopurified CD44 was diluted with reducing sample buffer and separated using 4–20% SDS-polyacrylamide gels (Bio-Rad) (17, 18). Resolved proteins were transferred to Sequi-blot or Immoblot polyvinylidene difluoride or Sequi-blot nitrocellulose membrane (Bio-Rad) and blocked with StartingBlock (Pierce) for 15 min. Immunoblots were stained with HECA-452 or anti-CD44 (2C5) mAbs and rinsed with Tris-buffered saline, 0.1% Tween 20. In all cases, duplicate immunoblots were stained in parallel with irrelevant isotype control primary antibodies to assess nonspecific binding to protein bands. Subsequently, blots were incubated with appropriate AP- or HRP-conjugated secondary antibodies. Western Blue AP substrate (Promega, Madison, WI) and SuperSignal West Pico chemiluminescent substrate (Pierce) were used to develop the AP- and HRP-conjugated antibody-stained immunoblots, respectively.

Blot Rolling Assay—Blots of immunopurified CD44 from T84 or Colo205 whole cell lysate or whole cell lysate from untreated, mammalian scramble, or CD44-knockdown LS174T cells were stained with anti-CD44 (2C5) or HECA-452 mAbs and rendered translucent by immersion in 90% D-PBS, 10% glycerol (23). The blots were placed under a parallel plate flow chamber, and human peripheral blood lymphocytes (18) or CHO trans-
fectants, resuspended at $5 \times 10^6$ cells/ml in 90% D-PBS, 10% glycerol, were perfused at the shear stress of 0.5 dyne/cm$^2$. 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 lane was averaged over $10^4$ fields of view (0.55 mm$^2$ each). Nonspecific adhesion was assessed by perfusion by adding 10 mM EDTA in the flow medium.

**Preparation of CD44-coated Microspheres**—Immunoprecipitated CD44 from control and metabolically inhibited T84 whole cell lysate was diluted to desired concentrations with binding buffer (0.2 M carbonate/bicarbonate buffer, pH 9.2), and incubated with 10-μm polystyrene microspheres (2.5 $\times$ 10$^7$ microspheres/ml; Polysciences Inc., Warrington, PA) overnight at 4 °C with constant rotation (18). Microspheres were washed twice with D-PBS and subsequently blocked with D-PBS, 1% BSA for 30 min at room temperature. Microspheres were resuspended (2 $\times$ 10$^6$ microspheres/ml) in D-PBS, 0.1% BSA for use in flow cytometric and flow chamber assays. Site densities of CD44-coated microspheres were determined by flow cytometry (18).

**Enzymatic Treatments**—To remove terminal sialic acid residues, T84 CD44-coated microspheres were incubated with 0.1 unit/ml *Vibrio cholerae* sialidase (Roche Applied Science) for 90 min at 37 °C (18). In select experiments, CD44-coated microsphere suspensions were incubated for 6 h at 37 °C with 3.6 units/ml *Flavobacterium heparinum* heparitinase to specifically digest heparan sulfate glycosaminoglycans (GAGs) (24, 25). To cleave chondroitin sulfate GAGs from CD44 isoforms, CD44-coated microsphere suspensions were incubated 2 h at 37 °C with 1 unit/ml *Proteus vulgaris* chondroitinase ABC (4, 26). Site densities of CD44 adsorbed onto microspheres following enzymatic treatments were determined by flow cytometry and confirmed to be equivalent to untreated controls before use in adhesion assays.

**Inhibitor Treatments**—Prior to metabolic inhibitor studies, T84 cell suspensions (10$^7$ cells/ml) were pretreated with 0.1 unit/ml *V. cholerae* sialidase for 60 min at 37 °C to remove terminal sialic acid residues and to ensure de novo synthesis of newly generated HECA-452 reactive carbohydrate structures (17). Complete removal of sialic acid was confirmed via flow cytometry using the mAb HECA-452 that recognizes sialic acid-bearing epitopes. Subsequently, T84 cells were cultured for 48 h at 37 °C in medium containing either 2 mm benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (benzyl-GalNAc) to inhibit O-linked glycosylation (18) or 10 μg/ml *Castanospermum australe* casanompermine to inhibit N-linked processing of glycoproteins (27); D-PBS diluent was used for control untreated cells.

**Flow Cytometry**—CD29, CD44, and HECA-452 expression levels on LS174T and T84 cells as well as CD44 and HECA-452 site densities on microspheres were quantified by single-color immunofluorescence and flow cytometry (FACSCalibur; BD Biosciences) using PE-conjugated anti-CD29 (MAR4), anti-CD44 (515), or HECA-452 antibodies. Background levels were determined by incubating cell or microsphere suspensions with properly matched PE-conjugated isotype control antibodies (18).

**Cell Sorting by Flow Cytometry**—T84 colon carcinoma cells (10$^7$ cells/ml) were incubated with an anti-CD44 mAb (fluorescein isothiocyanate-conjugated G44-26 or PE-conjugated 515) or a fluorescently labeled isotype control antibody for 1 h at room temperature in the dark. Using a flow cytometer/sorter (FACSARia; BD Biosciences), the highest and lowest (above isotype control staining) 10% of CD44-expressing T84 cells were collected into separate growth medium-filled centrifuge tubes. Subsequently, cell suspensions were centrifuged, resuspended to a concentration of 5 $\times$ 10$^7$/ml in D-PBS, 0.1% BSA, and immediately used in flow-based adhesion assays.

**Preparation of CD44 siRNA Oligonucleotides**—Short interfering (si)RNA oligonucleotides (19 nucleotides) targeting exons 1–5 and 15–20 of CD44 were generated using the W1 siRNA design program (Whitehead Institute, Massachusetts Institute of Technology). Identified target sequences were subjected to BLAST search of the human genome and subsequently filtered to remove sequences that were not specific to CD44. The siRNA sequences were used to construct 60-mer short hairpin (sh)RNA oligonucleotides, which were then synthesized (Operon, Inc., Huntsville, AL), and ligated into the pSUPER.neo.gfp product was transformed into competent DH5α Escherichia coli cells, amplified in the presence of ampicillin, and the plasmid was purified using the EndoFree maxi kit (Qiagen, Valencia, CA). Sequence insertion was verified by restriction digestion and confirmed by direct sequencing. A tested mammalian scramble sequence (Oligoengine, Inc) was used as a negative control in all siRNA experiments.

**Generation of Stable CD44 Knockdown Colon Carcinoma Cell Lines**—8 $\times$ 10$^6$ LS174T cells were plated in 100-mm dishes and grown overnight reaching an ~50% confluency. The cells were then transfected with 32 μg of pSUPER.neo.gfp.CD44S using Lipofectamine 2000 for 24 h. Upon reaching confluency, transfected LS174T cells were passed and 5 $\times$ 10$^6$ cells seeded per Petri dish in growth medium in triplicate. After 24 h, the medium was replaced by a fresh aliquot containing 500 μg/ml neomycin. Cells were then grown continually without passing for 15 days, replenishing the neomycin-containing medium every 2–3 days. Single cell colonies were isolated and cultured using standard techniques.

**Flow-based Adhesion Assays**—To simulate the physiological shear environment of the vasculature, colon carcinoma cells or T84 CD44-coated microspheres suspended in D-PBS, 0.1% BSA were perfused over immobilized E- or L-selectin-coated plates at prescribed wall shear stresses using a parallel plate flow chamber (250 μm channel depth, 5.0 mm channel width) (4, 18). The extent of adhesion was quantified by perfusing cells/microspheres at either 2 $\times$ 10$^7$/ml and averaging the total number of cells/beads interacting during 15-s intervals over six $\times$ 10 fields of view (0.55 mm$^2$ each) or 1 $\times$ 10$^7$/ml and enumerating the total number of tethering events in a single
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×10 field of view during a 5-min period. Average rolling velocities were computed as the distance traveled by the centroid of the translating cell/microsphere divided by the time interval at the given wall shear stress (4, 7, 18). In select experiments, LS174T cells were preincubated for 10 min at room temperature with either 5 units/ml heparin or diluent (D-PBS, 0.1% BSA) and used in perfusion assays.

Statistical Analysis—Data are expressed as the mean ± S.E. for at least three independent experiments. Statistical significance of differences between means was determined by analysis of variance. If means were shown to be significantly different (p < 0.05), multiple comparisons were performed by the Tukey test.

RESULTS

The Selectin Binding Determinants of CD44v on Colon Carcinoma Cells Are Displayed on Sialofucosylated O-Linked Glycans and Are Independent of GAGs—We recently reported that the colon carcinoma cell line LS174T expresses CD44 variant isoforms that display selectin binding activity, implicating these molecules as a link between the up-regulation of CD44v expression and increased metastatic potential (17, 18). We sought to quantify the expression of these isoforms on other colon carcinoma cell lines, such as T84 and Colo205, to begin to reveal the breadth of its distribution as a selectin ligand as well as to further characterize its selectin binding characteristics. Western blot analysis of CD44 immunopurified from whole cell lysates of 5 × 10⁶ T84 or Colo205 cells, using the anti-CD44 mAb 2C5, revealed the presence of a relatively weak level of CD44s at ~100 kDa and a more prominent CD44 signal at ~150 kDa, which corresponds to CD44v (Fig. 1A, T84, lane 1; Colo205, not shown). Fig. 1A, lane 2, stained with HECA-452, identified the presence of sialofucosylated epitopes, such as sLex, solely on the variant isoforms of CD44 (Fig. 1A, T84, lane 2; Colo205, not shown). A blot rolling assay was next employed to assess the ability of immunopurified CD44 to mediate selectin-dependent adhesion under physiologically relevant levels of shear stress. To this end, L-selectin-expressing human peripheral blood lymphocytes or P- or E-selectin-transfected CHO cells, perfused over SDS-PAGE-resolved immunopurified CD44 protein bands, tethered predominantly over the ~150-kDa region that corresponds to sialofucosylated CD44v, whereas minimal binding was detected at the ~100-kDa band of CD44s (T84, Fig. 1B; Colo205; data not shown). Taken together, these data suggest that CD44v, but not CD44s, on T84 and Colo205 colon carcinoma cells is capable of interacting efficiently with selectins under physiological flow conditions.

We next used a cell-free flow-based adhesion assay to evaluate the adhesion capabilities of microspheres coated with CD44 immunopurified from T84 cells over immobilized E-, P-, or L-selectin. This technique allows quantitative comparisons of CD44-mediated adhesion to purified selectin substrates at identical concentrations under physiological flow conditions. Microspheres, coated with T84 CD44, which displays strong HECA-452 reactivity (supplemental Table 1S), tethered and rolled over all three selectins, albeit with varying efficiencies which correlate with those of intact T84 cells, with E-selectin mediating the slowest rolling and L-selectin the fastest rolling (supplemental Fig. 1S). To characterize the biochemical nature of CD44-selectin binding, we treated T84 CD44-coated microspheres with highly selective enzymes that cleave specific carbohydrate and GAG moieties from the glycoprotein. In accord with our previous findings using LS174T CD44 (18), sialidase treatment of T84 CD44-coated microspheres, which eliminated HECA-452 reactivity without affecting CD44 expression (supplemental Table 1S), nearly abrogated microsphere adhesion to P- and L-selectin, whereas partially reduced binding to E-selectin relative to appropriate controls (supplemental Fig. 2S). It is noteworthy that although untreated CD44-bearing microspheres displayed slow rolling behavior over E-selectin, sialidase treatment converted these interactions to swift tethers.
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Colon Carcinoma Cells; the Emergent Role of Alternative Selectin Ligands from Selective Knockdown of CD44 Function—To assess the functional role of CD44v in selectin-mediated adhesion under flow, we employed two complementary strategies to modulate CD44 expression on colon carcinoma cells. First, T84 cells were sorted on a FACSAria into the following two populations: high (MFI, 1731) and low (MFI, 364) CD44-expressing cells (Fig. 2A). Sorted T84 cells were resuspended in D-PBS, 0.1% BSA at a concentration of \(5 \times 10^5\) cells/ml and perfused over selectins for 2 min at a wall shear stress of 1 dyne/cm\(^2\). As shown in Fig. 2B, low CD44-expressing T84 cells tethered and rolled on P- and L-selectin substrates at a rate significantly lower than that of high CD44-expressing cells, whereas no difference was detected in the extent of adhesion to E-selectin. Moreover, the rolling velocities of sorted T84 cells over P-selectin correlated inversely with CD44 expression levels, whereas no correlation was noted for the L-selectin-dependent binding (Table 1). Notably, although the E-selectin adhesion levels were not altered by the cell surface density of CD44 (Fig. 3B), low relative to high CD44-expressing T84 cells rolled significantly faster on E-selectin (Table 1), illustrating the reduced capacity of the former population to engage E-selectin in shear flow. These data reveal that a significant correlation between CD44 expression levels and L- and P-selectin-mediated tethering in shear flow, implicating CD44v as a functional L- and P-selectin ligand on intact T84 cells. Moreover, it appears likely that CD44 functions as an auxiliary E-selectin ligand, stabilizing the colon carcinoma adhesive interactions with E-selectin in shear flow. However, we cannot exclude the possibility that the affinity of CD44 for E-selectin is dramatically higher than that for L- and P-selectin, and thus, a more pronounced reduction in the cell surface CD44 density might be required to detect diminished T84 cell binding to E-selectin.

To address this issue, we generated stable CD44-knockdown LS174T cell lines by transfecting cells with a CD44 shRNA plasmid, isolating single cell clones and propagating these clones in neomycin-containing media. As shown in Fig. 3, A–C, this procedure resulted in the generation of LS174T cells with markedly...
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The mammalian scramble control plasmid using the anti-CD44 mAb, 2C5, revealed the presence of an intense band at ~150 kDa that corresponds to CD44v, and a lighter band at ~100 kDa that depicts CD44s (Fig. 3D, lanes 1 and 2). In distinct contrast, little CD44 immunostaining was detected in whole cell lysates prepared from two distinct CD44-knockdown LS174T cell lines (Fig. 3D, lanes 3 and 4). Notably, probing with the HECA-452 mAb revealed the absence of an immunoreactive band at the ~150-kDa region in CD44-knockdown LS174T cell lysates (Fig. 3D, lanes 7 and 8), which is in accord with the lack of CD44v immunostaining, and the concurrent presence of two distinct bands at ~130 and ~170 kDa.

In flow-based adhesion assays, CD44-knockdown LS174T colon carcinoma cells relative to mammalian scramble controls displayed a markedly reduced capacity (~50%) to tether to purified P-selectin substrates at a wall shear stress of 1 dyne/cm² (Fig. 3E). A slight reduction, which did not reach a statistically significant level, was noted in the binding of CD44-knockdown LS174T cells to L-selectin under flow (Fig. 3E), although they rolled markedly faster than mammalian scramble or untreated controls (Table 2). However, no difference in the extent of adhesion to purified E-selectin was noted at 1 dyne/cm² (Fig. 3E) or 1.5 dynes/cm² (data not shown). In distinct contrast to our flow cytometric/sorting findings using T84 cells, CD44 knockdown on LS174T colon carcinoma cells did not alter the rolling velocity on E-selectin relative to mammalian scramble controls, although it drastically increased the velocity on L- and P-selectin substrates (Table 2). This discrepancy in E-selectin-mediated rolling can be reconciled by the potential presence of alternative selectin ligands on CD44-knockdown cells, which can participate in stabilizing cell rolling on E-selectin against fluid shear. To test this hypothesis, blot rolling assays using whole cell lysates from CD44-knockdown, mammalian scramble control, or untreated LS174T cells were performed. As shown in Table 3, maximal binding of CHO-E cells to SDS-PAGE-resolved lysates from untreated control LS174T cells occurred at ~150 kDa, which corresponds to the molecular

reduced surface CD44 expression (>95% decrease in MFI) relative to LS174T cells transfected with a mammalian scramble control plasmid or untreated LS174T cells, as evidenced by flow cytometry using the anti-CD44 mAb 2C5. Evidence for the specificity of this intervention was provided by the flow cytometric analysis of other LS174T cell surface adhesion molecules such as CD29 and HECA-452 (Fig. 3, A–C) whose expression levels remained intact. In accord with our prior results using intact LS174T cells (17, 18), immunoblot analysis of whole cell lysates from untreated LS174T cells or cells transfected with the molecular...
weight of CD44v, whereas substantially lower binding occurred at all other regions. In marked contrast, the binding of CHO-E cells to the ~150-kDa region from CD44-knockdown LS174T cells was nearly abrogated, whereas pronounced binding was detected at ~170 and ~130 kDa relative to untreated LS174T cells or mammalian scramble controls (Table 3). Moreover, as shown in Fig. 3F, both peripheral blood lymphocytes and CHO-P cells interacted with HECA-452-stained bands from CD44 siRNA whole cell lysate at ~170 and ~130 kDa. Taken together, these data suggest the existence of alternative selectin ligands on LS174T cells with a molecular mass of ~170 and ~130 kDa, which mediate selectin-dependent adhesion in CD44-knockdown cells, and shield the potential role of CD44 as a functional L-selectin or an auxiliary E-selectin ligand. Nevertheless, our findings provide clear evidence that CD44v is a major functional P-selectin ligand.

**Heparin Interferes with Colon Carcinoma Cell Rolling on L- and P-selectin**—Several lines of evidence suggest that selectins enhance carcinoma metastasis and that heparin can prevent this enhancement (10, 11, 28). To this end, we evaluated the effects of heparin (5 units/ml) on colon carcinoma cell binding to purified selectins under physiological flow conditions. This pharmacological intervention reduced the avidity of LS174T cells for L- and P-selectin but did not affect E-selectin in shear flow, as manifested by the inhibition of cell tethering to P-selectin (Fig. 4), and strikingly increased rolling velocities over P- and L-selectin substrates (Table 4). Interestingly, heparin treatment nearly abrogated the extent of CD44-knockdown cell binding to P-selectin and significantly inhibited L-selectin but not E-selectin-dependent tethering. Our findings are in line with prior observations (16, 29) suggesting that heparin interferes with L- and P-selectin binding to sLe^a^, which decreases in P- and L-selectin avidity may be sufficient to compromise integrin-mediated firm adhesion to host cells and to mitigate metastasis.

**DISCUSSION**

CD44 participates in a diverse array of pathophysiological processes ranging from hematopoiesis to wound healing and cell signaling (30). Perhaps the most intriguing is its potential role in metastasis. Accumulating evidence suggests that up-regulation of CD44v by carcinoma cells confers metastatic potential in vivo (31–33) and results in a poor prognosis (34). Although others have hypothesized that CD44-mediated tumor cell adhesion to hyaluronic acid is a dominant factor regulating metastasis of colon carcinoma cells (35), the possibility of CD44-dependent adhesion to selectins has been largely overlooked. Interestingly, selectins have been implicated in the hematogenous spread of colon carcinoma cells (10–13). We recently reported that O-glycosylated CD44 variant isoforms on LS174T colon carcinoma cells possess selectin binding activity (17, 18). However, it is not clear whether the selectin binding activity of CD44v is restricted to LS174T cells or shared by other metastatic colon carcinoma cell lines. Most importantly, these previous studies did not provide evidence for a functional role of CD44v in supporting tethering/rolling of colon carcinoma cells to selectins under flow. Accordingly, we report that CD44 expressed on T84 and Colo205 colon carcinoma cells possess similar selectin-binding earmarks.
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to those of LS174T CD44. Furthermore, by implementing two complementary strategies to modulate CD44 expression on T84 and LS174T cells, we demonstrate that CD44v is a functional P-selectin ligand.

By sorting T84 cells based on their CD44 expression levels, we illustrate that high versus low CD44-expressing cells bind more efficiently to P- and L- but not E-selectin in shear flow. On the other hand, stable LS174T cell lines generated using CD44 siRNA express near background levels of CD44 and tether to P- but not L- and E-selectin with a markedly reduced capacity relative to mammalian scramble controls or untreated cells. Cumulatively, these findings provide concrete evidence for a functional role of CD44v in P-selectin-dependent binding of colon carcinoma cells under physiological flow conditions. Interestingly, low relative to high CD44-expressing T84 cells roll significantly faster on E-selectin, suggesting an auxiliary role for CD44 in stabilizing colon carcinoma cell adhesion interactions with E-selectin against shear. However, this inverse correlation between CD44 expression levels and E-selectin-dependent rolling was not noted in mammalian scramble control versus stable CD44-knockdown LS174T cell lines. This discrepancy may be because of alternative selectin ligands on CD44-knockdown cells that can mediate E-selectin binding, and to a lesser extent L-selectin binding, under flow. Immunostaining of CD44-knockdown LS174T cell lysates with HECA-452 reveals the absence of an immunoreactive band at the ~150-kDa region, which is in accord with the lack of CD44v immunostaining, and the presence of two distinct bands at ~130 and ~170 kDa, which exhibit selectin ligand activity as evidenced by blot rolling analysis. Interestingly, E-selectin-dependent adhesion to ~170- and ~130-kDa regions is markedly enhanced in CD44-knockdown cells relative to untreated and mammalian scramble control cells. Although no significant difference in HECA-452 immunoreactivity at ~170 and ~130 kDa was detected in CD44-knockdown relative to untreated or mammalian scramble control cell lysates, their capacity to support enhanced E-selectin binding may be because of one of more compensatory mechanisms. CD44 knockdown may result in the emergence of a new selectin-binding glycoform(s) not detected by the HECA-452 mAb. Alternatively, the spatial distribution of existing HECA-452 reactive epitopes on the polypeptide core may change to enhance E-selectin binding. Finally, changes in the glycoprotein polypeptide in the region of HECA-452 glycans (i.e. tyrosine sulfation) may render the glycoproteins more effective as selectin ligands. Cumulatively, these yet unidentified glycoproteins with apparent molecular masses of ~170 and ~130 kDa can interact efficiently with selectin in shear flow, and shield the potential role of CD44 as a functional L-selectin and an auxiliary E-selectin ligand.

Our findings are in contrast with a recent report, which suggested by the use of siRNA lentiviral technology, that sialofucosylated CD44v is the predominant glycoprotein ligand mediating LS174T cell binding to E-selectin (36). Although this genetic intervention did not alter the extent of LS174T cell adhesion to E-selectin relative to vector alone-transduced cells at 1 dyne/cm², it markedly increased their average rolling velocity (36). We speculate that the discrepancy between these recently published data (36) and our results may be attributed to diminished levels of HECA-452 reactivity on CD44 siRNA-treated LS174T cells relative to vector alone-transduced cells. This possibility is further substantiated by the fact that CD44 siRNA-transduced LS174T cell lysates failed to support CHO-E binding in a blot rolling assay (36), which is in distinct contrast with our findings. Clearly, this reduced HECA-452 reactivity following CD44 gene silencing (36) might also explain the more pronounced reduction of siRNA-treated LS174T cell binding to L-selectin under flow noted in that study.

Accumulating evidence suggests that selectins facilitate the hematogenous dissemination of tumor cells, although the underlying mechanisms for this process remain rather elusive. In vivo studies have documented that when colon carcinoma cells enter the bloodstream, they form complexes with platelets (10, 11) via a P-selectin-dependent pathway (6, 7, 20). It is believed that platelets, by adhering to tumor cells, provide a protective shield that masks them from the cytotoxic activity of natural killer cells (37). Alternatively, platelets may promote colon carcinoma cell adhesion to the endothelial vessel wall, and thus assist carcinomas to escape from the circulation and seed metastatic foci. This enhanced carcinoma adhesion is achieved via a platelet-bridging mechanism, whereby platelets adhere to an endothelium-bound carcinoma cell capture free-flowing carcinomas in a P-selectin/α₁β₃-dependent manner (3). The extravasation of metastatic cells is also regulated by molecular events involving the initial E-selectin-dependent “direct” binding of tumor cells to endothelium, and their subsequent migration across blood vessel walls. On the other hand, the contribution of L-selectin and leukocytes to cancer metastasis is less developed. It is believed that tumor cells can form multicellular complexes with platelets and leukocytes (via an L-selectin-dependent mechanism (8, 9)), which can then arrest in the microvasculature of distant organs, and eventually extravasate and establish metastatic colonies. Thus, selectins can act cooperatively to promote tumor-host cell interactions and cancer metastasis. To date, the synergistic effects of L- and P-selectin in the facilitation of metastasis have been demonstrated in an in vivo study (11).

Previous work has shown that heparin significantly reduces the extent of established metastasis of LS174T colon carcinoma cells in immunodeficient mice by interfering with P-selectin-mediated events (10). Our data indicate that this pharmacological intervention diminishes the avidity of colon carcinoma cells to P- and L-selectin, which may compromise the integrin-mediated firm adhesion to host cells and mitigate metastasis.

Altogether, our data offer a unifying perspective on the apparent enhanced metastatic potential associated with CD44v overexpression on many types of tumor cells and the critical role of selectins in metastatic spread. Our findings support further research to investigate CD44v as a potential therapeutic target to combat metastasis and contribute to the complexity of the possible functions from this ubiquitous adhesion molecule.

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