

Distinct Selectin Ligands on Colon Carcinoma Mucins Can Mediate Pathological Interactions among Platelets, Leukocytes, and Endothelium

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Selectins are adhesion molecules that mediate calcium-dependent cell-cell interactions among leukocytes, platelets, and endothelial cells. The naturally occurring vascular ligands for the selectins are mostly mucin-type glycoproteins. Increased expression and altered glycosylation of mucins are known to be prominent features of carcinoma progression. We have previously shown that all three selectins bind to colon carcinoma cell lines in a calcium-dependent fashion and that carcinoma growth and metastasis formation are attenuated in P-selectin-deficient mice. Here we show that the three recombinant soluble selectins recognize ligands within primary colon carcinoma tissue samples. Affinity chromatography showed that the ligands for all three selectins are O-sialoglycoprotease-sensitive mucins that are recognized in a calcium- and sialic acid-dependent manner. Furthermore, there are separate binding sites on the mucins for each selectin, allowing cross-binding of a single mucin molecule by more than one selectin. We also show that the selectin ligands on purified carcinoma mucins can mediate at least four different pathological interactions among platelets, leukocytes, and endothelial cells. These findings could explain some of the adhesive events of blood-borne tumor cells reported to occur with leukocytes, platelets, and endothelial cells, which are believed to play a part in modulating some early events in tumor metastases. (Am J Pathol 1999, 155:461–472)

Many interactions that occur among leukocytes, platelets, and endothelial cells in the vasculature have been shown to be mediated by the selectin family of cell adhesion molecules.^{1–12} The naturally occurring ligands for the three selectins are mostly mucin-type glycoproteins carrying sialylated, fucosylated glycans.^{1–3,7,9,11–15} Recent studies suggest that selectin interactions with carcinoma cells may play pathological roles in tumor biol-

ogy. Interestingly, progression and poor prognosis of carcinomas are associated with enhanced expression of sialylated, fucosylated epithelial mucins. Mucins are large rod-like glycoproteins with extensive O-linked glycosylation.^{16,17} The interactions of selectins with O-glycan chains involves primarily their N-terminal lectin and epidermal growth factor-like domains.^{2,4,5,11,18} Sialylated and/or sulfated Lewis^{x/a} and related structures are the most common glycan recognition elements for the selectins.^{2,7,9,11,12} However, monovalent oligosaccharide ligands such as Sialyl Lewis^x (Sia α 2–3Gal β 1–4(Fuc α 1–3)GlcNAc) and Sialyl Lewis^a (Sia α 2–3Gal β 1–3(Fuc α 1–4)GlcNAc) bind with low affinity to the selectins, and O-glycans released from the natural high-affinity ligands do not show easily detectable rebinding.¹⁹ One suggested explanation for the preferred recognition of mucin ligands by selectins is simple multivalency of oligosaccharide presentation.²⁰ Another proposal is a more complex presentation of combinations of the sugar chains, closely spaced on a mucin polypeptide backbone.^{2,21,22} A variation on the latter is the combined recognition of glycans and immediately adjacent tyrosine sulfate residues on the leukocyte ligand P-selectin glycoprotein ligand-1 (PSGL-1).¹¹

Increased expression and altered glycosylation of mucins are known to be prominent features of carcinoma progression.^{23–26} For example, sialylated T and Tn antigens and sialylated Lewis blood group antigens were originally described as tumor-associated antigens on mucins.^{27,28} Sialyl-Lewis^{x/a} expression has also been strongly correlated with poor prognosis in a variety of human carcinomas.^{23,24,26,29–36} Thus, it has been suggested that E-selectin-bearing endothelial cells may be interacting with carcinoma cells via these sialylated, fucosylated epitopes. Indeed, *in vitro* experiments suggest that these antigens are potential mediators of extravasation of metastatic cells through endothelial cells via binding to E-selectin.^{33,35,37–39} Metastasis of tumor cells *in*

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vivo could also be diminished by reduction of cancer cell O-glycosylation,^{23,40} redirected by the transgenic expression of E-selectin⁴¹ or inhibited by infusion of a soluble E-selectin.⁴² Recently, we found that carcinoma growth and metastasis formation is attenuated in P-selectin-deficient mice.⁴³ We have also previously shown that all three selectins can bind to colon carcinoma cell lines in a calcium-dependent fashion.⁴⁴ This recognition could be largely abolished by a mucin-specific endopeptidase called O-sialoglycoprotease. Thus, our working hypothesis is that cell surface and/or secreted tumor mucins bearing selectin binding sites may interact in the bloodstream with leukocytes, platelets, and endothelial cells that are expressing selectins, and that these interactions can play roles in tumor biology. In this report, we have therefore examined primary human colon carcinoma samples for the presence of selectin ligands. We also explored the cross-binding and competition assays among the selectins for the carcinoma mucins. Finally, we showed that the carcinoma mucins can mediate a variety of pathological interactions among blood cells and endothelium. In so doing, we have provided a potential link between the selectins and the previously reported association of hematogenously borne cancer cells with leukocytes, platelets, and endothelium during the early events of tumor metastasis.^{45–50}

Materials and Methods

Chemicals and Reagents

Unless specified, all chemicals and reagents were from Sigma (St. Louis, MO). Frozen sections of primary human colon carcinomas were from the University of California San Diego Cancer Center Histology Core. O-sialoglycoprotease (OSGPase) was from Cedarlane (Hornby, ON). LS180 colon carcinoma cells (ATCC CL 187) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Recombinant selectin immunoglobulin-Fc fusion proteins (selectin-Igs) were prepared and purified as reported.^{19,51} For some experiments, we used selectin-Ig proteins incorporating the FLAG epitope tag (Eastman Kodak; details of these constructs will be reported elsewhere). The α 2–3 linkage specific sialidase L was a kind gift from Dr. Y.-T. Lee of Tulane University (New Orleans, LA). Blocking antibodies against the selectins were kindly provided by M. Bevilacqua (H18/7, anti-E-selectin), R. McEver (C138A, anti-P-selectin), and T. Tedder (LAM 1.14, anti-L-selectin).

Immunohistochemistry

Paraffin or frozen sections (fixed in 10% formalin/0.1 mol/L cacodylic acid for 30 minutes at room temperature) of human colon carcinomas or of LS180 cells were probed for selectin binding. For tissue sections, endogenous peroxidase was destroyed with 0.03% H₂O₂ for 10 minutes at room temperature, followed by a rinse in binding buffer (3% bovine serum albumin (BSA) in 20 mmol/L HEPES, 100 mmol/L NaCl, 2 mmol/L CaCl₂, 2 mmol/L

MgCl₂, pH 7.4). Sialic acid side chains were next selectively oxidized with 2 mmol/L periodate in phosphate buffered saline (PBS) for 30 minutes in the dark at 4°C, followed by biotinylated or FLAG-epitope-tagged recombinant selectins in binding buffer for 1 hour at room temperature. FLAG-epitope-tagged selectins were detected with mouse anti-FLAG-IgG, followed by biotinylated horse anti-mouse IgG. Biotin groups were then detected with streptavidin-peroxidase conjugates (Binding Site, Birmingham, UK) and enzymatic color development performed at room temperature using VIP substrate (Vector Labs, Burlingame, CA) or 3'3'-9-aminoethyl carbazole (Sigma). Slides were finally counterstained with Mayer's hematoxylin and rinsed in binding buffer without BSA. Control slides were incubated with either 5–10 mmol/L EDTA in place of calcium or without the primary probe.

Selectin Affinity Chromatography

Each selectin-Ig (~6 nmol, 0.5 mg protein) was incubated with 1 ml of Protein-A Sepharose resin (PAS) (Pharmacia) in pH 8.0 buffer for several hours with end-over-end rotation, and the resin then prepared as a column in a 2-ml Pasteur pipette.¹⁹ Secreted or detergent-extracted glycoconjugates from LS180 cells metabolically radiolabeled with [6-³H]glucosamine were enriched for labeled mucins by Jacalin-Sepharose chromatography and gel filtration on Sephacryl S200.¹⁹ These were dissolved in 0.5 ml binding buffer, loaded onto each of the selectin columns, and 0.5-ml fractions collected under gravity flow (~0.05 ml/min). After ~10 column volume washes, bound materials were eluted with 20 mmol/L MOPS, 100 mmol/L NaCl, 5 mmol/L EDTA, 2 mmol/L MgCl₂. Bound and run-through fractions were pooled, the latter adjusted for divalent cations, and aliquots reloaded onto each selectin column to test capacity and cross-binding. Aliquots of bound material were also treated with sialidase L in sodium acetate, pH 5.5, or with OSGPase in binding buffer, boiled, adjusted into binding buffer as needed, and reapplied to the selectin columns.

Liquid Scintillation Counting

Appropriate background subtraction was determined with blank vials. Samples were counted at a constant quench level in aqueous-compatible scintillation fluid for a period long enough to give a 95% confidence level. Thus, although there were low levels of radioactivity available for some analyses, the signal-to-noise ratios were acceptable.

Preparation of Mucins from Colon Carcinomas Grown in Immunodeficient Mice

Confluent LS180 cells were released in 5 mmol/L EDTA containing medium, washed in PBS and 2 × 10⁶ cells subcutaneously injected into the flanks of immunodeficient Rag-2 null mice (Taconic, Germantown, NY).⁵² After several weeks, the tumors (~2 cm in diameter) were

excised, any necrotic regions discarded, and homogenized in $0.5 \times$ PBS with $40 \mu\text{g/ml}$ aprotinin, $1 \mu\text{g/ml}$ leupeptin, $0.7 \mu\text{g/ml}$ pepstatin, and $20 \mu\text{g/ml}$ phenylmethylsulfonyl fluoride. The insoluble debris was removed at $100,000 \times g$ for 1 hour at 4°C and the viscous supernatant fractionated on a Superose CL-4B column run at ~ 1 ml/minute flow in PBS with the protease inhibitor cocktail detailed above. High molecular weight fractions (void volume) were pooled and further enriched for mucins using a CsCl_2 gradient. Fractions with a density of 1.45 or greater were pooled, dialyzed, lyophilized, and further enriched for O-linked glycoproteins using Jacalin affinity chromatography. Purification of the mucins was monitored using bovine submaxillary mucin as a standard, with A_{280} readings for protein, and the 2-thiobarbituric acid (TBA) or 1,2-diamino-4,5-methylene dioxybenzene (DMB) assays for sialic acids. Typically, ~ 5 g of tissue yielded ~ 1 mg of carcinoma mucin. For some studies, mucin samples were also extracted with chloroform:methanol (1:1, 10 volumes) or treated with heparin lyase II to exclude contamination by glycolipids or heparan sulfate, respectively.

Enzyme-Linked Immunosorbent Competition Assay

Selectin-Igs were biotinylated using 0.5 mg sulfo-NHS-biotin (Pierce, Rockford, IL) dissolved in DMSO, and mixed with $100 \mu\text{g}$ of selectin-Igs in PBS at 4°C for 1 hour. Reactions were quenched with 100 mmol/L glycine and the samples extensively dialyzed against binding buffer. Biotin content per molecule was estimated by Western blot analysis in comparison to standards. Preservation of selectin activity after biotinylation was confirmed using an enzyme-linked immunosorbent assay (ELISA) method involving binding to polyacrylamide-sLe^x absorbed on microtiter plates.⁵³ Purified mucins from tumors were absorbed onto 96-well Xeno-bind plates in sodium carbonate buffer, pH 9.5, at $5 \mu\text{g/ml}$ concentration. (Xeno-bind plates aid covalent attachment of the highly glycosylated mucins.) Absorption of mucins was confirmed using tracer amounts of [³H]mucin from LS180 cells and by detection with wheat germ agglutinin (WGA). Plates were blocked with 1% BSA in binding buffer, and incubated with various mixtures of biotinylated and non-biotinylated selectins at 4°C for 3 hours. After washing, Neutravidin-alkaline phosphatase conjugate was used to detect the biotinylated selectin binding. Cleavage of 4-methylumbelliferyl phosphate by the bound phosphatase was detected after 4 hours (product release was linear to this point) using a 96-well fluorescent reader (CytoFluor II, Perseptive Biosystems, Bedford, MA).

Cross-Binding of Carcinoma Mucins by Multiple Selectins

Corning ELISA plates were coated with 200 ng of E-, P-, or L-selectin-Ig by overnight incubation at 4°C in $100 \mu\text{l}$ of 50 mmol/L sodium carbonate/bicarbonate buffer, pH 9.5.

Plates were blocked with 0.1% BSA in Hanks' balanced salt solution (HBSS, Sigma) for 30 minutes at room temperature. Purified carcinoma mucin in HBSS was added ($1 \mu\text{g}$ per well) and incubated for 1 hour at room temperature. During the mucin incubation, 200 ng of selectin Ig chimeras with the FLAG epitope tag were precomplexed with monoclonal anti-FLAG M2 antibody (Sigma) at 1:300 dilution. Controls including 30 mmol/L EDTA in this step were used. Plates were washed twice with HBSS and the precomplexes added to plates for 1 hour at room temperature. Plates were washed with HBSS and incubated with alkaline phosphatase-conjugated goat-anti-mouse antibody (Biorad) at concentration 1:1000 for 1 hour at room temperature. Plates were again washed four times with HBSS, developed with p-nitrophenyl phosphate (Sigma), quenched by addition of $40 \mu\text{l}$ of 3 mol/L NaOH and the absorption at 405 nm read on a microplate reader (Molecular Devices).

Platelet Aggregation

Blood from normal individuals who had not recently consumed caffeine or medications was collected into acid citrate dextrose, and platelet-rich plasma prepared by centrifugation. Platelets were rewashed into 10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L CaCl_2 , 0.5% BSA, and 20 nmol/L PGE_1 added for stabilization. Aggregation was monitored in a standard platelet aggregometer (Chromo-log), following addition of human thrombin (0.1 U- 1.0 U/ml). Forty micrograms of tumor mucins prepared as described above were added before or after thrombin addition at concentrations that showed submaximal aggregation. No detectable aggregation was observed with high amounts ($100 \mu\text{g}$) of purified LS180 mucins alone.

Platelet:Endothelium Interaction Assay

Human umbilical vein endothelial cells (HUVECs) from the American Type Culture Collection (Manassas, VA) were passaged with trypsin/EDTA (Gibco) and grown to near confluence in a 6-well tissue culture plate with 20% FCS/ $50 \mu\text{g/ml}$ endothelial growth supplement/ $100 \mu\text{g/ml}$ bovine lung heparin/Medium 199 (Gibco, Becton Dickinson). Human platelets were prepared as described above. Both HUVECs and platelets were washed with 10 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L CaCl_2 , and 0.5% BSA (interaction buffer), and 5×10^6 platelets/well were added with or without $40 \mu\text{g/ml}$ of carcinoma mucins and mixed for 5 minutes. Human thrombin (0.1 U/well) was added and the plates were shaken vigorously for 10 minutes. All wells were then washed several times with the same buffer and observed under phase contrast.

Aggregation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) from normal human volunteers were prepared by Ficoll-Hypaque density gradient centrifugation, washed with interaction

buffer, and incubated in the same buffer, in the presence or absence of 40 $\mu\text{g/ml}$ of carcinoma mucins. In addition, PBMCs were preincubated with L-selectin blocking antibody for 5 minutes or in presence of 1 mmol/L EDTA before adding 50 $\mu\text{g/ml}$ of carcinoma mucins. After gentle shaking for 10 minutes at room temperature, the cells were mounted on slides using cytospin and observed under phase contrast.

Mononuclear Cell:Endothelium Interaction Assay

HUVECs were preincubated for 4 hours in complete medium containing 0.5 $\mu\text{g/ml}$ TNF- α and washed once in interaction buffer. Peripheral blood mononuclear cells (1×10^6) were added in presence/absence of 40 $\mu\text{g/ml}$ of carcinoma mucins. In addition, E-selectin blocking antibody was added to HUVECs before adding of carcinoma mucins, and PBMCs were incubated with L-selectin blocking antibody before adding them to HUVECs in the presence of carcinoma mucins. After 10 minutes of gentle shaking, all wells were washed 3 times with the same buffer and observed under phase contrast.

Results

Human Colon Carcinomas Can Express Calcium-Dependent Ligands for All Three Selectins

Frozen sections of 18 human colon carcinoma specimens were immunostained with FLAG-epitope-tagged human selectin-Ig chimeras, and the results were compared with those obtained from similarly immunostained adjacent normal colonic tissue. All three selectin chimeric probes recognized epitopes localized along the luminal edges of the epithelial cells within many of the colon carcinomas tissue samples studied (see Figure 1 and Table 1). Normal colonic epithelial cells also showed some reactive epitopes in most specimens. However, these regions were localized mostly to goblet cells and secreted mucins and to a few other epithelial cells, with all reactivity strictly facing the lumen. The E-selectin and L-selectin probes recognized epitopes within 16 of 18 carcinomas studied, whereas P-selectin immunostained 10 of 18 (see Figure 1A for an example of a tumor with ligands for all three selectins and Table 1 for a semiquantitative summary). Immunostaining was inhibited in control sections using EDTA chelation, indicating specific recognition of ligands via the C-type lectin domain. Immunostaining with the antibody CSLEX-1 showed that Sialyl-Le^x structures were also expressed in these tumors (data not shown). Serially cut sections probed with the three selectins showed a heterogeneous pattern of immunostaining, with certain areas within the tumor sections staining more intensely than others. These sections also showed that each of the three selectins immunostained different foci within the same tumor sample. However, occasionally similar areas within the same tumor

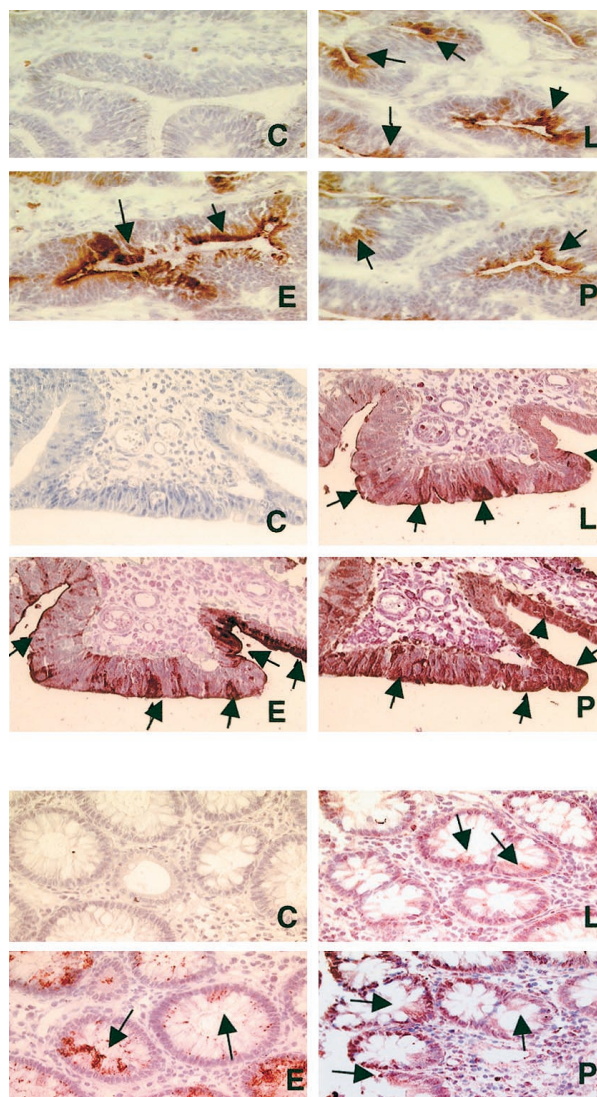


Figure 1. Heterogeneous expression of selectin ligands in human colon carcinoma samples. Frozen (upper panel) or paraffin (middle and lower panels) sections of primary human colon carcinomas (upper and middle panels) or normal colon (lower panel) were stained with the recombinant soluble FLAG-epitope tagged selectin probes in the presence of calcium as described in Materials and Methods. Each panel shows a similar region of one of the samples studied: L, L-selectin-FLAG-Rg; E, E-selectin-FLAG-Rg; P, P-selectin-FLAG-Rg. In each panel, the control (C) shows an example of loss of binding on incubation of E-selectin-Rg in EDTA-containing buffer.

samples were immunostained to varying degrees of intensity by all three selectin chimeric probes, as demonstrated in Figure 1A. In addition to prominent ligand expression along the luminal aspect of cells, mucin lakes also stained strongly with all three probes, and this was particularly evident with the E-selectin probe. Mononuclear cells as well as some mast cells scattered throughout the muscularis layers and sometimes within the invading carcinoma also reacted variably with all three selectins. These data indicate that potential ligands for all three selectins can be found in many primary colon carcinoma tissues and that the carcinoma cells express both cell-bound and secreted forms of these ligands. A range of immunostaining intensity of individual tumors by the three selectins was seen and not all tumors were equally

Table 1. Quantitation of Recombinant Selectin Binding to Primary Human Colon Carcinoma Sections

Selectin probe	Staining intensity (number of tumors)			
	0	+	++	+++
E-selectin-Rg	2	12	3	1
L-selectin-Rg	2	11	4	1
P-selectin-Rg	8	6	2	2

Frozen sections of 18 primary human colon carcinomas were stained with the soluble FLAG-epitope-tagged selectin probes in the presence of calcium as described in Materials and Methods. Staining was quantitated on a semiquantitative scale of 0 to +++. (See Figure 1 for an example of a tumor that is ++ positive for L- and E-selectin staining and + positive for P-selectin).

positive for all three selectins (See Table 1 for a summary). Paraffin sections gave similar results (Figure 1B). This differential pattern of staining among the selectins raises the possibility that there may be distinct mucin-like ligands for each selectin.

Colon Carcinoma Cell Lines Express Both Cell Surface and Secreted Mucins that Can Bind to All Three Selectins

To explore potential differences in selectin interactions with carcinoma mucins, we studied LS180 colon carcinoma cells, one of the cell lines we had previously demonstrated is recognized by all three selectins.⁴⁴ Direct binding of biotinylated selectin-Rg chimeras indicated that only a small fraction of these cells actually expressed ligands for each of the three selectins. The extent of immunostaining by each selectin was also highly heterogeneous, which was always eliminated by EDTA treatment. This recognition was also abrogated by sialidase or O-sialoglycoprotease, confirming that the ligands are sialylated cell surface mucin-type glycoproteins (data not shown).

To determine if the interactions with the three selectins involve overlapping recognition sites, we metabolically radiolabeled the LS180 cells with [³H]glucosamine and studied the labeled glycoconjugates by selectin affinity chromatography. Generally similar results were initially obtained with both membrane-associated and secreted glycoconjugates (data not shown). The secreted molecules were chosen for further study to avoid artifacts caused by detergents, cellular proteases, or glycosidases. Labeled molecules from the spent media were enriched in O-linked glycoproteins by binding to Jacalin-Sepharose columns, and the high-molecular mucins collected by gel filtration chromatography. When aliquots of these labeled preparations were treated with heparin lyase II and chondroitinase ABC, no degradation products were seen in a gel filtration profile, indicating no major contamination with labeled secreted proteoglycans. These samples, at physiological pH and salt concentration, were then passed over columns of immobilized selectin-Ig chimeras in the presence of calcium.⁵³ A significant portion of the labeled material (ranging from 30 to 60% in various experiments) was bound and eluted with EDTA from each selectin column (See Table 2).

Table 2. Rebinding and Cross-Binding of Intact and Protease-Digested Colon Carcinoma Mucins to Immobilized Recombinant Selectins

Original binding Treatment	Rebinding to selectin		
	E-selectin	L-selectin	P-selectin
E-selectin			
None	96%	51%	67%
OSGPase	86%	19%	50%
Pronase	29%	<3%	<3%
L-selectin			
None	85%	96%	96%
OSGPase	32%	72%	76%
Pronase	<3%	7%	<3%
P-selectin			
None	95%	69%	96%
OSGPase	73%	40%	74%
Pronase	<3%	<3%	9%

Metabolically labeled mucins from LS180 cells were subjected to selectin affinity chromatography. EDTA-eluted fractions from each run (primary selectin binding) were pooled, recalcified, and aliquots passed over columns of all three selectins, either directly or after proteolysis with OSGPase or Pronase, boiling, and resuspension in binding buffer. The percentage of EDTA-eluted binding material in each secondary run is shown (for the amount of radioactivity loaded, the reliable detection limit for rebinding was ~3%). These data represent typical results from studies that were repeated 2 or 3 times.

Bound and unbound fractions were repassaged through the same columns to confirm that binding capacity had not been exceeded. In each case, the unbound fractions continued to run through the columns (data not shown), whereas the bound fractions were able to rebind completely after recalcification (Table 2). The importance of calcium was reconfirmed by the lack of rebinding in the presence of EGTA (data not shown). Treatment with an α 2-3 linkage-specific sialidase L abrogated rebinding to the selectins by 80 to 90% in each case, showing the importance of α 2-3-linked sialic acids for recognition (data not shown). Taken together, these data show that colon carcinoma cells express cell surface and secreted O-linked glycoproteins that carry sialic acid- and calcium-dependent binding sites for all three selectins.

Protease Treatments Release Some Fragments that Can No Longer Bind

To confirm that these secreted selectin ligands are mucins, bound samples were treated with O-sialoglycoprotease and reloaded onto the columns to which they had originally bound. This endopeptidase will cleave mucins at a few sites adjacent to clustered O-linked chains. In each case studied, a portion of the resulting glycopeptides no longer re-bound (see Table 2). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with autoradiography also showed the expected pattern for mucins, with a high molecular weight smear ranging from the top of the gel to about 100 kd, and the treatment with O-sialoglycoprotease causing a reduction in molecular weight (data not shown). Sepharose CL-4B gel filtration further confirmed that these high molecular weight, soluble mucins were cleaved by this specific protease, showing a partial decrease in apparent size on treatment

(data not shown). A similar degree of size heterogeneity was seen among mucins that bound and did not bind to the selectins (data not shown). Because O-sialoglycoprotease only cleaves mucins at occasional sites within the less heavily glycosylated regions and the radioactivity is in the sugar chains, large labeled fragments carrying even one binding site for a selectin would be expected to rebind to the selectin columns. Treatment with a more broad-spectrum protease such as Pronase was therefore tried and, indeed, caused a further loss in binding. Especially for P- and L-selectin, extensive Pronase digestion substantially reduced the population of rebinding glycopeptide fragments (Table 2).

Lack of Complete Overlap in Recognition of Mucin and Mucin Fragments by the Selectins

The data above indicate not only that the underlying polypeptide apomucin component of the mucin ligands is critical for recognition, but also that, on cleavage of the apomucin, only some fragments can carry the high-affinity binding sites. This could occur either because of loss of avidity arising from the loss of close proximity of the common glycans (eg, SLe^x or SLe^a) recognized by all three selectins, or by disruption of more complex arrangements of glycans that generate high-affinity binding sites specific for each selectin. In the case of simple multivalency, one would expect essentially complete overlap of recognition of fragments by the three selectins. Mucin molecules that bound to each of the selectins were therefore studied for their ability to bind to the other two selectins by affinity chromatography. As shown in Table 2, the overlap in binding to the three selectins was not complete. Corroborating data were obtained by showing that molecules that did not bind to any one selectin could partially bind to the other two (data not shown). These data indicate that multiple selectin binding sites may reside within a given mucin molecule and that there is lack of complete overlap in binding. Intact mucin molecules that are completely specific for only one of the selectins could not be purified by sequential affinity chromatography (data not shown). However, the mucin molecules are very large and could have distinct binding sites along the length of their chains. We therefore looked for mucin fragments that were specific for each of the three selectins. The total mucin preparations were fragmented with O-sialoglycoprotease or Pronase and then studied for cross-binding among each of the three selectins. With O-sialoglycoprotease, some partial overlap in binding was preserved among the resulting glycopeptides (Table 2). With Pronase digestion, there was substantial loss of rebinding activity to the selectins. However, in each instance there was a fraction remaining after extensive Pronase digestion that bound only to the original selectin and not to the other two (Table 2). Because these represented a low fraction of the total material, it proved difficult to isolate adequate amounts for more detailed study.

Demonstration of Non-Overlapping, Unique Binding Sites for Each of the Selectins on the Mucins

Taken together, the data presented above indicate that the mucin molecules may carry at least some binding sites along their length that are distinctly recognized by each of the selectins. To confirm and quantitate this matter, competition ELISAs were performed on plates coated with intact mucins. Studies were done with mucins purified from the culture medium of LS180 cells (see above) and from tumors grown in immunodeficient mice. In the latter case, a cesium chloride density gradient was included in the purification protocol, and heparin lyase digestion and lipid extraction were also done to confirm the absence of potential contaminating ligands such as heparan sulfate and sulfatide, respectively. The mucin preparations were coated onto 96-well plates and the binding of biotinylated selectin-Ig chimeras used as a measure of specific recognition sites. The signal obtained was relatively low, indicating that the number of high-affinity binding sites is limited. However, the specificity of the selectin-Ig chimera binding was confirmed by an increasing linear signal over the background, obtained using pooled human IgG as a control (data not shown). Competition studies with unlabeled selectins were then used to determine the degree of overlap in selectin recognition. As shown in Figure 2A, each biotinylated selectin bound to unique sites on the same mucins that could only be effectively competed by the corresponding nonbiotinylated selectin, but not by the other two molecules. These results are all the more remarkable considering the potential for steric hindrance of adjacent binding sites by the relatively large selectin-Ig chimeras. To directly confirm the presence of multiple binding sites on individual carcinoma mucin molecules, we captured mucin molecules to ELISA plate-bound recombinant selectins, and then showed the presence of additional binding sites for each of the three selectins on the bound mucin molecules (Figure 2B). Taken together, the data indicate that there are multiple, distinct, and separate binding sites for each selectin along the length of the carcinoma mucin molecules.

Purified Carcinoma Mucins Can Potentiate Platelet Aggregation

There are many possible mechanisms by which selectin-mucin interactions could facilitate the hematogenous spread of tumor cells. In this regard, we have recently found that P-selectin deficiency in mice attenuates the growth, seeding, and organ colonization of the LS-180 colon carcinoma cells.⁴³ We also explored the basis for this finding by showing that the tumor cells interact *in vitro* and *in vivo* with activated mouse platelets in a P-selectin-dependent fashion.⁴³ However, such studies of interactions between intact tumor cells and normal cells are confounded by other variables that are difficult to control (eg, unrelated adhesion molecules or receptor ligands on either cell type). The types of glycosylation expressed on

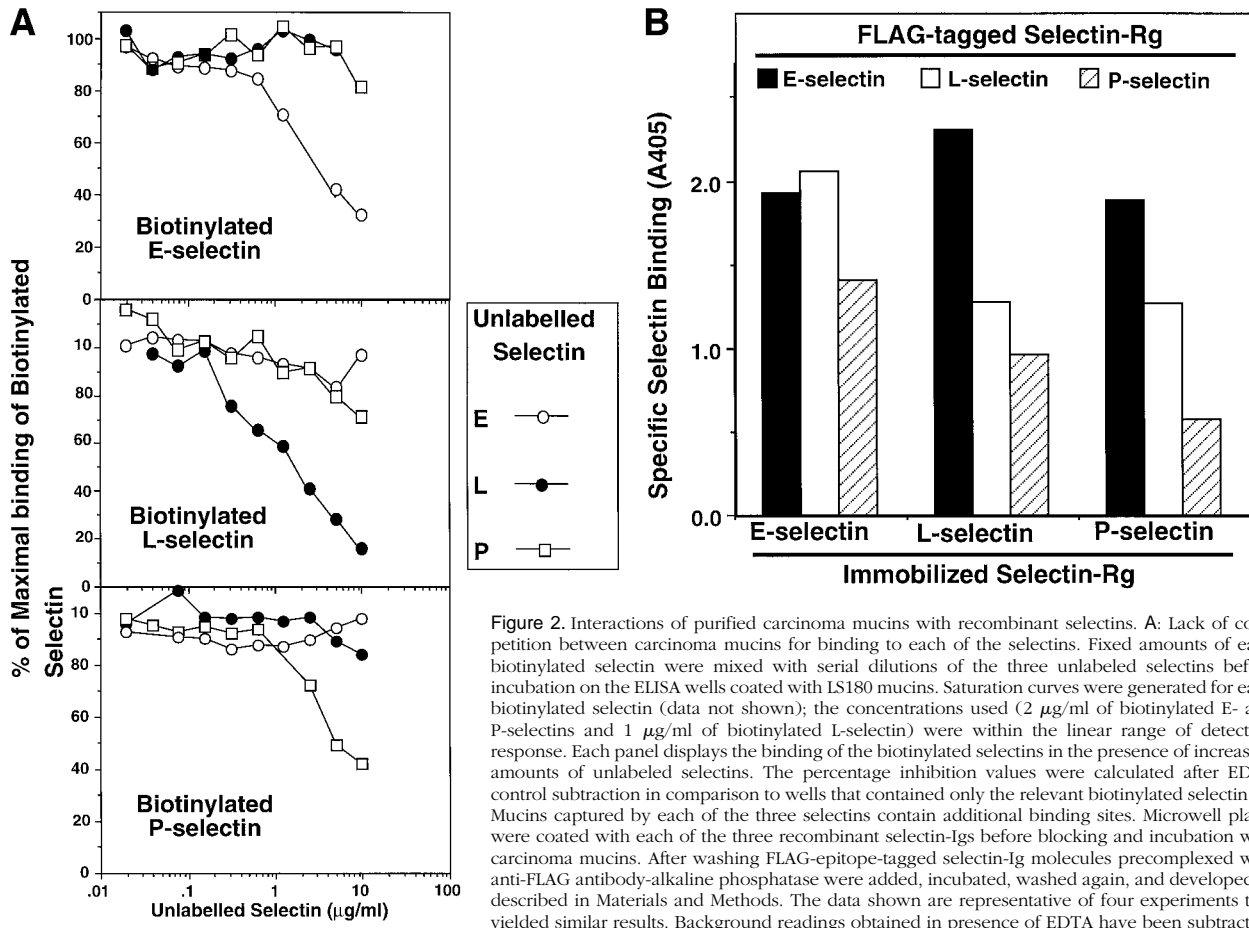


Figure 2. Interactions of purified carcinoma mucins with recombinant selectins. **A:** Lack of competition between carcinoma mucins for binding to each of the selectins. Fixed amounts of each biotinylated selectin were mixed with serial dilutions of the three unlabeled selectins before incubation on the ELISA wells coated with LS180 mucins. Saturation curves were generated for each biotinylated selectin (data not shown); the concentrations used (2 µg/ml of biotinylated E- and P-selectins and 1 µg/ml of biotinylated L-selectin) were within the linear range of detection response. Each panel displays the binding of the biotinylated selectins in the presence of increasing amounts of unlabeled selectins. The percentage inhibition values were calculated after EDTA control subtraction in comparison to wells that contained only the relevant biotinylated selectin. **B:** Mucins captured by each of the three selectins contain additional binding sites. Microwell plates were coated with each of the three recombinant selectin-Igs before blocking and incubation with carcinoma mucins. After washing FLAG-epitope-tagged selectin-Ig molecules precomplexed with anti-FLAG antibody-alkaline phosphatase were added, incubated, washed again, and developed as described in Materials and Methods. The data shown are representative of four experiments that yielded similar results. Background readings obtained in presence of EDTA have been subtracted.

cell surface-bound mucins are typically reiterated on secreted mucins derived from the same cell, because both types of molecules are exposed to the same Golgi enzymes during biosynthesis. To pursue potential tumor mucin interactions, we therefore examined the ability of the purified soluble mucins from primary colon carcinoma tumors grown in mice to mediate selectin-dependent interactions among blood cells. As shown in Figure 3, when freshly isolated platelets were incubated with the carcinoma mucins in a standard platelet aggregometer, no detectable aggregation was seen. However, the mucins markedly potentiated platelet aggregation caused by low levels of thrombin, which is known to induce translocation of P-selectin to the cell surface. This potentiation was abrogated by a P-selectin-blocking monoclonal antibody known to sterically inhibit the binding activity of its lectin domain and not by a nonblocking antibody (Figure 3, A and B), confirming that it is the P-selectin:mucin interaction that facilitates platelet aggregation. To further establish this finding, the platelets were initially treated with subthreshold levels of thrombin and subsequently shown to aggregate upon addition of the carcinoma mucins (Figure 3C). It should be noted that in our recent *in vivo* study,⁴³ intravenously injected tumor cells became coated with endogenous platelets in a P-selectin-dependent fashion, without the need to coinject a platelet-activating agent such as thrombin. We speculate that this is because the tumor cells can produce agonists such as

tissue factor that can generate thrombin *in vivo*. Alternatively, at any one time a small fraction of circulating platelets might be already expressing P-selectin *in vivo*, and these would be picked up by the tumor cells.

Carcinoma Mucins Can Mediate Platelet Interactions with Endothelium when Both Are Expressing P-Selectin

In the above instance, the carcinoma mucins are potentiating a naturally occurring aggregation phenomenon and thus could have a role in forming microthrombi of platelets with each other or with tumor cells. We hypothesized that the carcinoma mucins could also mediate the direct binding of activated platelets to activated but undamaged endothelium. To study this possibility, platelets were incubated with cultured endothelial cells, with or without the addition of thrombin or mucins. Although thrombin causes aggregation of platelets, these aggregates cannot bind to endothelial cells that are simultaneously activated by the thrombin. However, as shown in Figure 4, on addition of the carcinoma mucins, the aggregated platelets bound well to the activated endothelium. This interaction requires calcium and is reduced by a blocking antibody against P-selectin. Thus, the soluble mucins can act as a bridge between P-selectin mole-

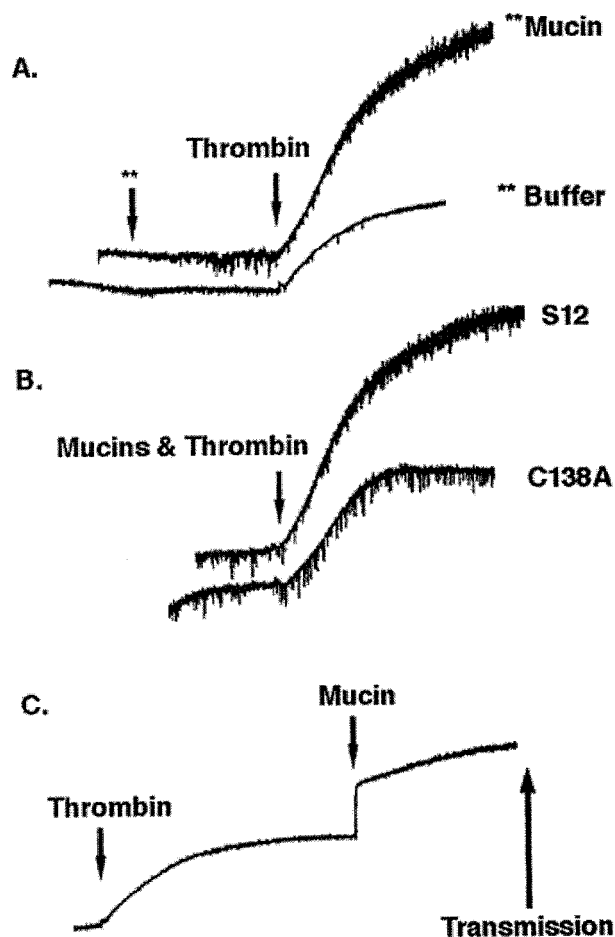


Figure 3. Carcinoma mucins potentiate platelet aggregation. Freshly prepared human platelets were studied for aggregation in a platelet aggregometer after various dosages of thrombin and/or mucin. The results shown are representative of several experiments. **A:** $\sim 20 \times 10^6$ human platelets were initially incubated in the presence of $0.11 \mu\text{g}/\mu\text{L}$ of carcinoma mucins without any effect. When 0.5 U of human thrombin was added thereafter, maximal aggregation was noted. Without mucins (buffer only), the same amount of thrombin was suboptimal, giving only partial aggregation. **B:** Mucins and thrombin were added together at the same concentrations as in **A**. Mucin potentiation of aggregation was inhibited by a blocking anti-P-selectin antibody (C138A), but not with a nonblocking P-selectin antibody (S12). **C:** The reverse order of addition gives similar results; mucin added after suboptimal dose of thrombin induced further aggregation.

cules expressed on platelets and on endothelium. If these mucins were on the surface of tumor cells, they would have involved these cells as partners in a multicellular complex with the platelets and endothelium.

Carcinoma Mucins Can Mediate Agglutination of Unactivated Peripheral Blood Mononuclear Cells Via L-Selectin

Many peripheral blood leukocytes constitutively express L-selectin. Under normal conditions, the only major ligand available for L-selectin on other leukocytes is PSGL-1. However, for unknown reasons, this receptor: ligand pair does not engage unless the leukocytes are activated. We reasoned that if the carcinoma mucins carried multiple binding sites for L-selectin, they might be able to directly cross-link unactivated leukocytes bearing

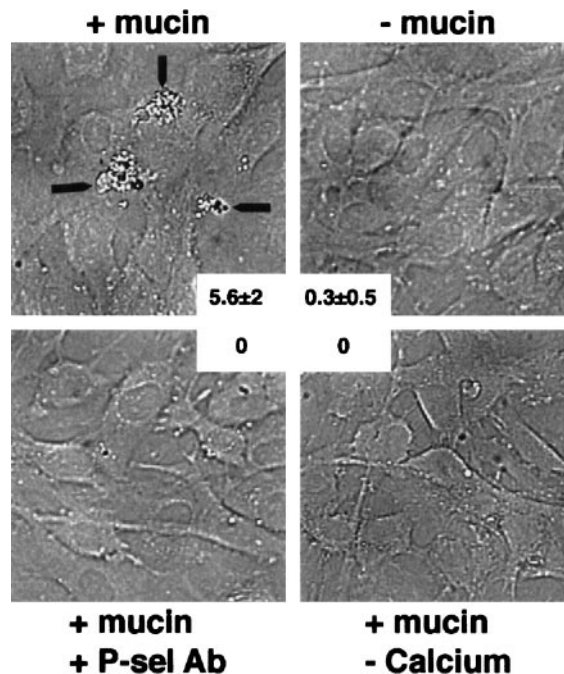


Figure 4. Carcinoma mucins cause interactions of activated platelets with activated endothelium. Human platelets (5×10^6) were added to HUVEC cells in a 6-well plate with or without LS180 carcinoma mucins ($40 \mu\text{g}/\text{ml}$) or thrombin ($0.1 \text{ U}/\text{well}$) as described in Material and Methods. No aggregation or attachment of platelets occurred in the absence of a thrombin activation, regardless of whether mucin was added (data not shown). In all panels shown, both HUVEC cells and platelets were activated with thrombin. The upper panels show examples (arrows) of aggregates of platelets attached to the HUVEC cells in the presence of mucins and a marked decrease in the absence of mucin. Aggregates were essentially undetectable even with mucin added in the presence of a P-selectin-blocking antibody or in the absence of calcium ions. The numbers indicate the number of aggregates seen per high-power field (mean \pm SD of 10 fields).

L-selectin. Indeed, as shown in Figure 5, the purified carcinoma mucins caused agglutination of a mixture of human PBMCs. This mucin-dependent agglutination is markedly reduced by chelating calcium or by a blocking antibody against L-selectin (Figure 5).

Carcinoma Mucins Can Mediate Interactions of Unactivated Peripheral Blood Mononuclear Cells with Activated Endothelium Expressing E-Selectin

We also explored whether the carcinoma mucins can cross-link between leukocytes bearing L-selectin and cytokine-activated endothelium, expressing E-selectin. Nonactivated PBMCs were incubated with cultured endothelial cells (pre-activated for 4 hours with $\text{TNF-}\alpha$ to stimulate new synthesis of E-selectin), with or without the addition of carcinoma mucins. As shown in Figure 6, there is some baseline binding of PBMCs (presumably expressing endogenous E-selectin ligands) to the activated endothelium. On addition of carcinoma mucin, there was a marked enhancement of binding of the PBMCs to the activated endothelium. All PBMC binding was abrogated by antibodies to E-selectin, while the mucin-induced enhancement of binding was specifically

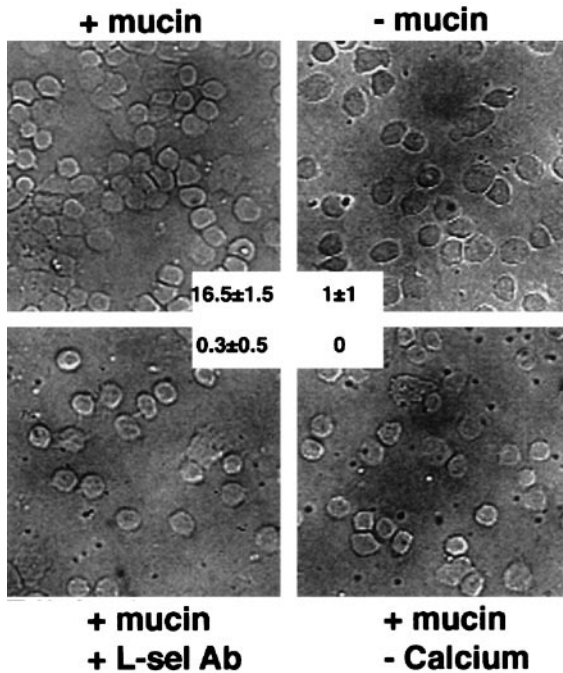


Figure 5. Carcinoma mucins agglutinate peripheral blood leukocytes in a calcium- and L-selectin-dependent manner. Human PBMCs were washed and incubated with or without carcinoma mucins (40 $\mu\text{g}/\text{ml}$) as described in Materials and Methods. Examples are shown of the agglutination observed in the presence of mucins, which was markedly reduced by calcium chelation with 1 mmol/L EDTA, or by adding a blocking anti-L-selectin antibody. The numbers indicate the number of leukocyte aggregates of >5 cells seen per high-power field (mean \pm SD of 10 fields).

blocked by antibodies to L-selectin (Figure 6). All of these interactions were dependent on calcium and on TNF- α preactivation of endothelium (data not shown). Taken together, all of the above data with intact cells also support the finding with soluble mucins (Figure 2B) that individual molecules may have multiple distinct binding sites for each of the selectins.

Discussion

An extensive body of literature reports a consistent correlation between the expression of mucins bearing altered oligosaccharide structures, and tumor progression and poor clinical prognosis of many human carcinomas, including colon cancer. In particular, tumor progression has been associated with expression of antigens such as SLe^x and SLe^a.^{23,24,26,29-36} The observation that the family of adhesion molecules, the selectins, recognize such structures suggested that these endogenous lectins might interact with carcinoma cells via those epitopes. Most previous reports have focused on potential E-selectin ligands expressed on carcinoma cell lines,^{37-39,41,42,54-57} and a few on P-selectin ligand expression.^{43,58-62} We previously showed that some colon carcinoma cell lines can be recognized by all three selectins.⁴⁴ Here we show by immunohistology that both secreted and cell-bound calcium-dependent selectin ligands can be expressed in a heterogeneous fashion on colon carcinoma cell lines and on primary colon cancer

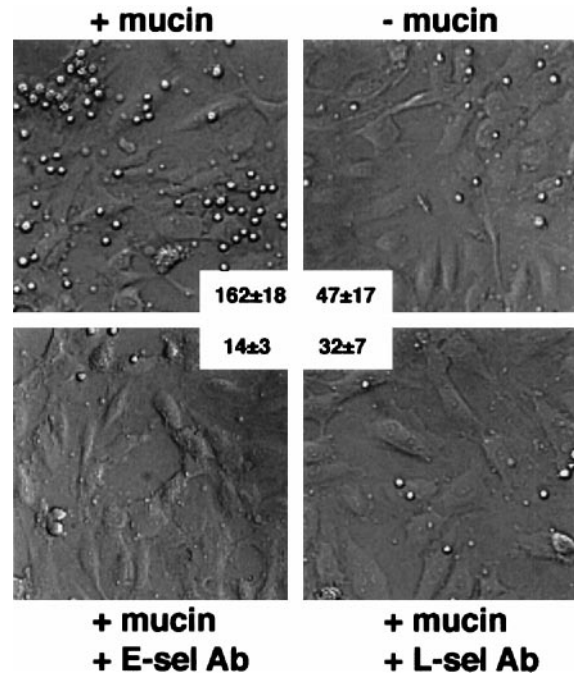


Figure 6. Carcinoma mucins mediate interactions of human PBMCs with activated endothelium via L- and E-selectin. 1×10^6 washed PBMCs were added to HUVECs grown in a 6-well plate that had been previously incubated for 4 hours in complete medium containing 500 $\mu\text{g}/\text{ml}$ of TNF- α and washed once in the interaction buffer (see Material and Methods). The incubations were carried out with or without carcinoma mucins (40 $\mu\text{g}/\text{ml}$) as indicated with gentle shaking at room temperature for 10 minutes, and the wells washed three times in the same buffer. The binding of the PBMCs to the HUVECs was enhanced by the presence of mucin, markedly reduced by an E-selectin-blocking antibody, and partially reduced by an L-selectin-blocking antibody (to about the level seen without mucin). The numbers indicate the number of attached leukocytes seen per high-power field (mean \pm SD of 10 fields). PBMC binding was almost completely absent if the HUVECs' preincubation did not include TNF- α or, in the absence of calcium ions, regardless of whether carcinoma mucins were present (data not shown).

tissues as well. In keeping with this, we show that all three selectins can also bind to the mucins secreted by the colon carcinoma cells. Our results indicate that simple multivalency of Lewis^{x/a} may not account for binding by the three selectins. Rather, each selectin appears to bind to multiple sites along a particular carcinoma mucin molecule, distinct from those recognized by the other two selectins. These unique binding sites are presumably created by distinct combinations of different O-glycans presented by the apomucin backbone. The possibility that tyrosine sulfate residues are also involved needs to be explored. The precise structural delineation of these binding sites will be a challenging task.

It is interesting to note that mucins from the normal bronchus¹⁹ and normal salivary gland⁶³ have been shown to be recognized by L-selectin *in vitro*. Likewise, we have noted selectin-binding sites in normal colon epithelial specimens, localized primarily to goblet cells and secreted mucins, and to a few other epithelial cells, with all reactivity strictly facing the lumen. However, because epithelial mucins are normally expressed and secreted vectorially toward the lumen, they would not normally encounter the selectins, with the possible exception of L-selectin-positive leukocytes that happen to exit into the lumen of these organs. The loss of normal topology

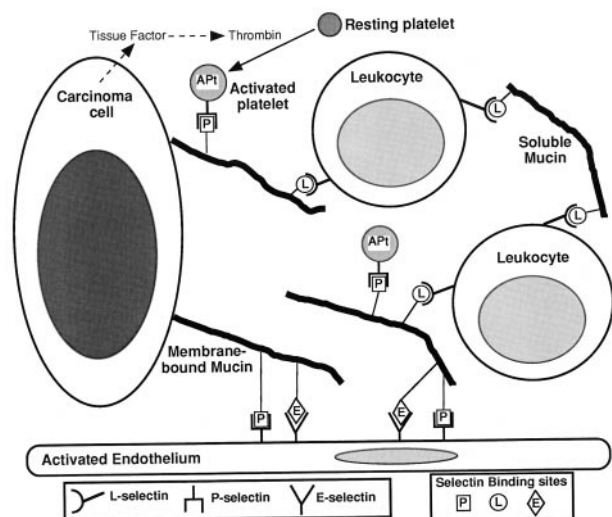


Figure 7. Potential interactions between vascular selectins and the multiple binding sites on colon carcinoma mucins. The composite schematic shows potential interactions of cell surface or secreted carcinoma mucins with selectins expressed on leukocytes, platelets, or endothelium; it was assumed that activation of endothelium and/or platelets could occur in the setting of a systemic malignancy. Although all of these interactions may not occur at the same time, multiple interactions could potentially occur in various combinations. Several of these have been demonstrated in this study using purified carcinoma mucins. Interactions involving the natural endogenous cell surface E- and P-selectin ligands, present on many leukocytes, are not shown.

and disruption of polarity of epithelial cells in malignancy can result in aberrant secretion of carcinoma mucins bearing selectin binding sites into the bloodstream. Tumor cells that enter the bloodstream and invade tissues would also present such selectin binding sites on their cell surfaces, allowing them to interact with platelets, leukocytes, and endothelial cells.

It should be noted that we used mild periodate oxidation of sialic acid side chains to increase the sensitivity of recognition by the recombinant selectins in the tissue immunostaining studies.⁵¹ We also used EDTA-containing buffer to confirm that the interactions are calcium-dependent and adjusted salt concentrations to more closely approximate the physiological state.⁵³ Although some staining for selectin ligands was also seen in goblet cells of normal colonic epithelium, the polarity and fate of these cells (eventually shed into the lumen of the gut) assures that these ligands would never be exposed to the vascular compartment in the normal situation. In contrast, the strong expression of ligands for all three selectins in many malignant tumors provides an opportunity for interactions by tumor cells invading the bloodstream with leukocytes, platelets, and endothelial cells.

Earlier studies hypothesized a simple model wherein such cells would be recognized by E- or P-selectin on endothelial cells, thus permitting extravasation from the bloodstream into metastatic sites. The fact that L- and P-selectin (present on leukocytes and platelets) can also recognize the mucins on these tumor cells generates many more possibilities for potential interactions between selectin-ligand-positive carcinoma cells and host cell components that are known to play a role in tumor biology (see Figure 7). Furthermore, the fact that these elongated mucin molecules bear multiple distinct binding sites for

each of the selectins makes possible some complex cellular interactions that would not normally occur *in vivo*, eg, the direct binding of activated platelets to activated endothelium and facilitation of the formation of tumor emboli coated simultaneously with both leukocytes and platelets. Indeed, we demonstrate here that purified carcinoma mucins can mediate many of the interactions shown in Figure 7 in a selectin-dependent manner (note that our studies with soluble mucins are directly relevant to the potential interactions of cell surface mucins, which have similar selectin binding sites). These observations may also provide a link between the selectins and the well-known physical association of hematogenously borne tumor cells with leukocytes, platelets, and endothelium during the process of metastasis.⁴⁵⁻⁵⁰

Of course, because the mucins carrying multiple selectin binding sites can be either secreted or bound to the cell surface (see Figure 7), one must also consider some diametrically opposing possibilities. Thus, for example, while cell surface mucins could facilitate interactions of tumor cells with L-selectin-positive leukocytes and P-selectin-positive platelets or endothelium, secreted forms of the same mucins could be acting as soluble inhibitors of such interactions. Also, such soluble mucins could prevent the trafficking of tissue homing T lymphocytes that use P- and E-selectin to extravasate into tissues.^{9,64}

This could be a factor in explaining the poor response of the cellular immune system to tumor antigens. Although all of the interactions shown in Figure 7 may not occur *in vivo* at the same time, multiple interactions could potentially occur in various combinations, especially if it is assumed that activation of endothelium and/or platelets can occur in the setting of a systemic malignancy, causing expression of E- and/or P-selectin. More work will be needed to identify which of the many possible interactions actually occurs at high frequency *in vivo* and generates the selection pressure favoring expression of sialylated, fucosylated mucins in naturally occurring carcinomas. In this regard, our recent finding that P-selectin deficiency attenuates tumor growth and metastasis⁴³ makes it likely that at least the P-selectin:mucin interactions are biologically relevant. Finally, our findings may also explain the clinical association of mucin-producing carcinomas with platelet-containing microthrombi, microangiopathic hemolytic anemia, and lowered circulating platelet counts (Trousseau's syndrome). In this regard, it is known that patients with carcinomas can have substantial levels of circulating soluble mucins in the bloodstream,^{37,65} sometimes in the low microgram range, similar to the amounts we used to generate pathological interactions *in vitro*.

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