Colon carcinoma cell glycolipids, integrins, and other glycoproteins mediate adhesion to HUVECs under flow

Monica M. Burdick, J. Michael McCaffery, Young S. Kim, Bruce S. Bochner, and Konstantinos Konstantopoulos


This study was undertaken to investigate the molecular constituents mediating LS174T colon adenocarcinoma cell adhesion to 4-h TNF-α-stimulated human umbilical vein endothelial cells (HUVECs) under flow. At 1 dyn/cm², ~57% of cells rolled and then became firmly adherent, whereas others continuously rolled on endothelium. Initial cell binding was primarily mediated by endothelial E-selectin. By using neuraminidase, glycolipid biosynthesis inhibitor d,l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl, trypsin, and flow cytometry, LS174T cells were shown to express sialyl Lewisα (sLeα)- and di-sLeα-decorated, glycolipid and glycoprotein ligands for E-selectin. The cells preferentially employed sialylated glycoproteins over glycolipids in adhesion as measured by conversion of rolling to firm adhesion, resistance to detachment by increased shear stress, and rolling velocity. However, a nonsialylated E-selectin counterreceptor also exists. Furthermore, LS174T α2, αα, and β1 integrins support a minor pathway in adhesion to HUVECs. Finally, tumor cell attachment specifically increases HUVEC endocytosis of E-selectin. Altogether, the data indicate the complexity of carcinoma cell-endothelium adhesion via sialylated glycoconjugates, integrins, and their respective counterreceptors.

E-selectin; sialyl Lewisα; glycolipid; shear stress

HEMATOGENOUS METASTASIS is a highly regulated and dynamic process in which cancerous cells separate from a primary tumor, migrate across blood vessel walls into the bloodstream, and disperse throughout the body to establish new colonies. In particular, it has been hypothesized that tumor cells may leave the bloodstream in a manner similar to that outlined for leukocytes during the inflammatory response to immunologic challenge. In this model, cells first loosely attach (tether) and roll on activated endothelial cells lining the blood vessel, and then stop, firmly adhere, flatten, and finally squeeze between endothelial cell junctions into the underlying inflamed tissue.

Endothelial E-selectin, a major receptor in the adhesion of leukocytes to the blood vessel wall, has been shown to support metastatic spread in vivo (7, 17) and to support breast and colon carcinoma cell tethering and rolling under dynamic flow conditions (13, 14, 47). Sialylation of a terminal Gal, and fucosylation of a GlcNAc, such as found on sialyl Lewisx (sLex) and sialyl Lewisα (sLeα) (12, 37), appear to be important structures capable of E-selectin binding (4, 49). Indeed, adhesion appears to involve sLeα- and/or sLeα-containing glycoconjugate ligands expressed on the tumor cell surface (14, 28, 40). Previous reports indicate that O-linked and/or N-linked glycoproteins may both be important in mediating carcinoma cell adhesion to E-selectin (17, 28, 40, 46), but these studies were performed under static (no flow) conditions. As has been argued in the literature, data obtained in vitro using static assays may not be relevant to the fluid dynamic environment of the vasculature. Furthermore, numerous reports profile the various roles of glycosphingolipids in metastasis (see review, Ref. 16), but data regarding a role in adhesion are limited. Only recently, through the use of a lipid glycosylation inhibitor, has a contributory role been identified for sLeα-decorated glycolipids in adhesion to E-selectin-transfected Chinese hamster ovary cells, albeit under static conditions and in the absence of O-linked glycoproteins (25). Such direct evidence of colon carcinoma sLeα-glycolipid involvement in adhesion is lacking (17, 28, 40). Notably, a growing line of evidence suggests that the natural E-selectin ligand on normal human granulocytes is a glycosphingolipid (6, 8, 26, 43, 44).

Whereas selectins primarily mediate the transient adhesive interactions of tethering and rolling, integrins and their ligands tend to mediate firm adhesion

Address for reprint requests and other correspondence: K. Konstantopoulos, Dept. of Chemical Engineering, Johns Hopkins Univ., 3400 North Charles St., Baltimore, MD 21218-2694 (E-mail: kkonsta1@jhu.edu).

http://www.ajp-cell.org

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
(9). More specifically, to stably attach to the endothelium, leukocytes use α4β2- and/or α6β1-integrins to interact with ICAM-1, and most (except neutrophils) employ α6β1 to bind VCAM-1 and the alternatively spliced connecting segment-1 of fibronectin (9). Prior work has provided evidence that the α4β1/VCAM-1 pathway may mediate binding and stable arrest of tumor cells to human umbilical vein endothelial cells (HUVECs), particularly melanoma cells, under conditions of flow (13). Moreover, through both static and dynamic adhesion assays, numerous integrins have been shown to mediate tumor cell attachment to components of the extracellular matrix (3, 5, 21, 39, 48), some of which may be expressed on the luminal surface of the endothelium (3, 5, 21, 39). However, Kitayama et al. (22) recently proposed a novel role in metastasis for immobilized E-selectin in mediating the firm arrest of flowing colon carcinoma cells via localized patches of sLeα, whereas randomly distributed sLeα residues are more important to tethering and rolling. Stable adhesion may otherwise involve non-integrin pathways by actin-mediated colocalization of selectin ligands or E-selectin. For example, the leukocyte actin network appears to concentrate P-selectin glycoprotein ligand-1 (PSGL-1) in adhesive “tails” to mediate firm adhesion to immobilized platelets expressing P-selectin (31a, 42). This finding, in combination with other static adhesion studies demonstrating that a myeloid cell line and human peripheral blood monocytes may induce clustering of endothelial E-selectin that could potentially affect dynamic adhesive interactions (50, 52), provided motivation to investigate whether integrins or selectin-selectin ligand-clustering was mediating firm adhesion of colon cancer cells to endothelium, similar to leukocytes.

This study was undertaken to investigate sLeα-negative metastatic LS174T colon adenocarcinoma cell adhesion to 4-h TNF-α-stimulated HUVECs in a system mimicking physiological flow conditions. The present work demonstrates that initial LS174T cell tethering and rolling on stimulated HUVECs is predominantly mediated by endothelial E-selectin. Tumor cells preferentially employ sLeα/dimeric-sLeα (di-sLeα)-decorated glycoproteins over glycolipids in their adhesive interactions with endothelial E-selectin, as assessed by conversion of rolling to firm adhesion, resistance to shear-induced detachment forces, and rolling velocity. Furthermore, a nonialylated ligand(s) supports E-selectin-dependent tethering. Unlike leukocytes, the LS174T actin cytoskeletal association/reorganization of selectin ligands is not involved in firm adhesion. Although it is unclear whether E-selectin clustering on the endothelial surface occurs after tumor cell binding, increased endocytosis of this receptor is induced upon LS174T cell attachment. Finally, LS174T α6β1 and α6β1 integrins may bind to counter-receptors expressed on the luminal HUVEC surface. Tumor cell adhesion to endothelium shares some similarities with leukocyte adhesion, but many differences exist as well.

**Materials and Methods**

**Monoclonal antibodies and adhesion antagonists.** All antibodies were murine anti-human IgG1 unless otherwise noted. The monoclonal antibody (MAb) AK4 (blocking anti-P-selectin) was from Pharmingen (San Diego, CA). Anti-E-selectin F(ab′)2 ENA2 was purchased from Monosan (Uden, The Netherlands), while nonblocking anti-EP-selectin MAb was obtained from R&D Systems (Minneapolis, MN). Anti-ICAM-1 BBIG-I1 was also purchased from R&D Systems; function-blocking anti-ICAM-1 F(ab′)2 MEM-111 was obtained from Caltag (Burlingame, CA). The blocking antibodies P3C4 (anti-VCAM-1), NKI-M9 (anti-α6), B3A (anti-β3), and ASC-3 (anti-β3) were from Chemicon (Temecula, CA). Blocking MAbs against α2 (G9), α4 (HP2/1), and α6 (GoH3, rat IgG2a) were acquired from Beckman Coulter (Miami, FL), and a blocking anti-β1 MAb 13 (rat IgG1) was a generous gift of Dr. S. A. Kakiya (National Institutes of Health, Research Triangle Park, NC). Anti-CD43 MAb DPT1 was purchased from Beckman Coulter. Anti-sLeα SNH3 IgM antibody was obtained as described previously (40). Anti-di-sLeα IgM MAb FH6 (which also recognizes related extended chain forms of sLeα) and anti-sLeα MAb NKKH1 were from Dr. A. Singhal (Biomembrane Institute, Seattle, WA). Isotype-matched IgG1 MAb was purchased from Sigma (St. Louis, MO), and IgM MAb was from Beckman Coulter. GRGDSP synthetic peptide was obtained from Life Technologies (Gaithersburg, MD). XV454 (a nonpeptide small-molecule α1β3 antagonist) and XT199 (a nonpeptide small-molecule α6β1 antagonist) were generously provided by Dr. S. A. Mousa (Albany College of Pharmacy, Albany, NY) (2), and c7E3 anti-α1β3 Fab fragment was from Centocor (Malvern, PA). Echistatin, an RGD-containing polypeptide, and glyceryl-ribohizin, an sLeα mimic, were purchased from Sigma.

**Cell culture.** The LS174T and HCT-8 human colon adenocarcinoma cells were obtained from the American Type Culture Collection and cultured in the recommended medium. Cells were detached from culture flasks by mild trypsinization (0.25% trypsin/EDTA for 2 min at 37°C; Life Technologies) and subsequently incubated at 37°C for 2.5 h to regenerate surface glycoproteins, as described previously (28, 31). Tumor cells were then washed, resuspended at 106 cells/ml in serum-free medium containing 0.1% bovine serum albumin (Sigma), and stored at 4°C for no longer than 5 h before use. HUVECs were harvested by collagenase digestion and cultured to confluence in gelatin-coated tissue culture flasks as described previously (24). Cells were then passaged into 1% gelatin-coated 35-mm tissue culture dishes. Before use in adhesion assays, first-passage HUVECs were stimulated for 4 h with 5 ng/ml TNF-α (R&D Systems) to obtain maximal levels of E-selectin expression.

**Enzyme and inhibitor treatments.** To remove terminal cell surface sialic acid residues, LS174T cells (107/ml) were incubated with 0.1 U/ml Vibrio cholerae neuraminidase (Roche Molecular Biochemicals, Indianapolis, IN) for 90 min at 37°C. To assess the contribution of glycoproteins, tumor cells (107/ml) were treated with tissue culture grade trypsin (Sigma) at 20 μg/ml for 90 min at 37°C. GPI-linked molecules were cleaved by treating cells with 1 U/ml phosphatidylinositol-specific phospholipase C (PI-PLC) (Glyko, Novato, CA and Sigma) for 1 h at 37°C (91). To remove surface-expressed glycosaminoglycans, tumor cells were simultaneously treated with 1.2–3.6 U/ml heparinase I (Sigma), 1.2–3.6 U/ml heparinase II (Sigma), and 1–2 U/ml chondroitinase ABC (Sigma) for 1 h at 37°C. For other studies, glycosylation inhibitors were added directly to cell cultures. The glycosphingolipid glycosylation inhibitor PPPP (d,l-threeo-1-phenyl-2-hexade-
canoylaminoo-3-pyrrrolidino-1-propanol HCl; Matreya, State College, PA) was added to LS174T cell cultures at 5 μM for 96 h. To prevent O-linked glycosylation or N-linked glycosylation of glycoproteins, cells were cultured for 48 h with 2 mM benzyl-N-acetyl-α-galactosaminide (Bzl-GalNAc; Sigma) or 200 ng/ml tunicamycin (Sigma), respectively (40). Another inhibitor of N-linked glycan maturation deoxymannojirimycin (DMJ; Sigma) was similarly applied for 48 h at 1 mM (32). Viability assessed by trypan blue exclusion was routinely ≥95% in response to the inhibitor treatments and dilute controls DMSO (for PPP) or Dulbecco’s phosphate-buffered saline (DPBS; for all others), similar to untreated cells.

To examine the possible involvement of the LS174T actin cytoskeleton in adhesion, cells were treated for 10 min with cytochalasins B or D (5–25 μg/ml; Sigma) or latrunculin A (1–4 μM; Calbiochem, San Diego, CA) before perfusion over purified E-selectin (see Flow adhesion assays). In some experiments, LS174T cells were fixed with 1% formalin for 15 min and then washed before use in attachment assays.

**Flow adhesion assays.** Tumor cell interactions with HUVECs were quantified under simulated physiological flow conditions using immunoelectron microscopy. Immunogold electron microscopy/digital image processing system (8, 24, 31, 45). Attachment assays were performed by perfusing cells (10^6/ml) at the appropriate flow rates to obtain wall shear stresses of 0.5 to 1.2 dyn/cm^2, thereby mimicking the fluid mechanical environment of the microcirculation and post-capillary venules (23). The total number of interacting cells in a single ×10 field of view (0.55 mm^2) during the 5-min perfusion period, the number of firmly adherent cells in five different fields of view after 5 min of flow, and the average rolling velocity were quantified by digital image processing (31). Interacting cells were defined as those that bound to HUVECs (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. Cells that arrested after rolling a short distance and cells that rolled continuously through the field of view were included in the counts. Firmly adherent cells were considered as those that remained stationary for at least 10 s at the end of the 5-min run. Rolling velocity was computed as the displacement by the centroid of the cell divided by the time interval of observation (31).

Controlled detachment assays were performed immediately after attachment assays by doubling the wall shear stress stepwise from 2 to 32 dyn/cm^2 every 30 s. The percentage of adherent cells was quantified as those firmly adherent cells remaining in the ×10 field of view at the end of each 30-s period relative to the number at the start of the detachment assay (8, 26, 45).

For some inhibition experiments, LS174T cells (10^6/ml) were pretreated for 15 min at room temperature (RT) with saturating concentrations of MAbs (10–20 μg/ml) before their perfusion over endothelial cell monolayers. Additionally, anti-β2-integrin and anti-ICAM-1 F(ab′)2 antibodies were maintained in the flow buffer. Peptides and nonpeptide antagonists were added to the flow buffer just before the LS174T cell perfusion. For other experiments, HUVEC monolayers were pretreated with MAbs (10–20 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. Immunofluorescence and flow cytometry with saturating concentrations of appropriate MAbs or irrelevant control MAb as described previously (24, 31). Values are reported as percent mean (±SE) fluorescence intensities relative to control (untreated) LS174T cell SNH3 or FH6 fluorescence using different batches of cells each time.

**Statistics.** Data are expressed as means ± SE. Statistical significance of differences between means was determined by one-way ANOVA. If means were shown to be significantly different, multiple comparisons by pairs were performed by the Tukey test. Probability values of P < 0.05 were considered statistically significant.

**RESULTS**

**TNF-α-stimulated HUVECs support LS174T cell adhesion under flow.** Treatment of HUVEC monolayers with TNF-α (5 ng/ml, 4 h) induced extensive attachment of metastatic LS174T colon adenocarcinoma cells under simulated physiological flow conditions. During the 5-min perfusion period, a consistent, progressive decrease in the extent of tumor cell tethering was observed between physiologically relevant shear stresses of 0.5 and 1.2 dyn/cm^2 (23), but no binding was observed at higher levels (Fig. 1). At 1 dyn/cm^2, an average of 148 ± 5 cells/mm^2 formed adhesive interactions with the HUVECs (Fig. 2). Of this number, 57 ± 3% of cells rolled and then became firmly adherent subsequent to tethering, whereas the remaining tethered cells continuously rolled on the HUVEC surface (Fig. 2). In contrast, LS174T cells perfused over unstimulated HUVECs did not attach to the monolayer (data not shown), consistent with previous studies performed under dynamic flow conditions (13, 14, 47).

**Roles of endothelial E-selectin and sialylated LS174T glycolipids and glycoproteins in adhesion.** As shown in Fig. 2, LS174T cell tethering to 4-h TNF-α-stimulated HUVECs is predominantly mediated by E-selectin, since antibody blockade with anti-E-selectin F(ab′)2 essentially abolished initial cell binding (Fig. 2). P-selectin blockade was without effect (data not shown). Tethering also required divalent cations as evidenced by abrogation of attachment by 5 mM EDTA to the flow
medium (data not shown). However, after ~2 min of exposure to EDTA, the HUVEC monolayer began to lose integrity as adjacent cells detached from one another.

Efforts were then made to characterize the LS174T cell counterreceptor(s) to endothelial E-selectin. Receptors bearing sialoieties such as sLex and sLeα support E-selectin-mediated adhesion (6, 15, 26, 43, 44). Through the use of indirect immunofluorescence and flow cytometry, LS174T cells were found to express only sLexα and di-sLexα identified with SNH3 and FH6 MAbs, respectively (Table 1), but not sLexα recognized by the NKH1 MAb. Treatment of LS174T cells with 0.1 U/ml neuraminidase, an enzyme that cleaves terminal sialic acid residues, led to greatly reduced adhesive strength such that only ~10% of tethered neuraminidase-treated cells were able to firmly adhere to HUVECs during attachment assays (Table 2). Consequently, a large increase in the number of rolling and thus total interacting cells resulted, but cell binding was not completely abolished (Fig. 2). To clarify whether neuraminidase treatment possibly led to an increase in tethering (i.e., due to removal of negatively charged sialic acid groups that could prevent intercellular binding) in addition to reduced adhesive strength, the total number of interacting cells was broken up into cells that tethered directly in the experimental field of view plus cells that rolled into the observational field having initially attached upstream. This analysis suggests that the tethering ability was not significantly impaired with enzyme exposure, because 134 ± 21 untreated control cells/mm² tethered in the field of view compared with 139 ± 23 neuraminidase-treated cells/mm² (n = 9). Furthermore, the increase in total interacting cells and weakened strength of adhesion could not be attributed to neuraminidase cleavage of the sialylated anti-adhesion molecule CD43 (leukosialin) (51), because flow cytometric analysis revealed that the LS174T cells did not constitutively express this antigen (data not shown). Moreover, higher concentrations of neuraminidase (0.5–1.0 U/ml), which resulted in complete cleavage of all sialic acid residues, also failed to further reduce LS174T attachment to HUVECs.

Table 1. Flow cytometric analysis of sLexα/di-sLexα expression on LS174T cells treated with enzymes and inhibitors

<table>
<thead>
<tr>
<th>Enzyme or Inhibitor</th>
<th>%Mean Fluorescence Intensity</th>
<th>SNH3 (sLexα)</th>
<th>FH6 (di-sLexα)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td></td>
<td>10.2 ± 2.7*</td>
<td>12.7 ± 1.9*</td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td>54.2 ± 3.7*</td>
<td>48.5 ± 1.9*</td>
</tr>
<tr>
<td>PPPP</td>
<td></td>
<td>70.8 ± 4.1*</td>
<td>48.8 ± 1.9*</td>
</tr>
<tr>
<td>Bzl-GalNAc</td>
<td></td>
<td>46.4 ± 2.6*</td>
<td>33.0 ± 6.3*</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td></td>
<td>72.5 ± 3.0*</td>
<td>64.6 ± 5.9*</td>
</tr>
</tbody>
</table>

Values are percentages of control mean (± SE) fluorescence intensities relative to control (untreated) LS174T cell SNH3 or FH6 fluorescence using different batches of cells each time (n = 6–13 cells). Mean fluorescence intensity for control (untreated) cells: IgM isotype control, 7.7 ± 0.6; SNH3, 512.4 ± 31.0; FH6, 53.9 ± 4.2. *P < 0.05 with respect to control. PPPP, d,l-threo1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl; Bzl-GalNAc, benzyl-N-acetyl-α-galactosaminide.

Table 2. Conversion of rolling to firm adhesion of LS174T cells treated with enzymes and inhibitors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%Total Interacting Cells</th>
<th>Firmly Adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.0 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>10.4 ± 2.3*</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>10.5 ± 1.4*</td>
<td></td>
</tr>
<tr>
<td>PPPP</td>
<td>21.7 ± 2.2*</td>
<td></td>
</tr>
<tr>
<td>Bzl-GalNAc</td>
<td>15.5 ± 3.3*</td>
<td></td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>17.0 ± 3.2*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE and are percentages of total interacting cells converting from rolling to firm adhesion at 1 dyn/cm² (n = 7–53 cells). LS174T cells were treated with enzymes or glycosylation inhibitors as described in MATERIALS AND METHODS. *P < 0.05 with respect to control.
HUVECs under flow (>600 total interacting cells/mm², ~40 firmly adherent cells/mm²).

Interestingly, similar results to neuraminidase treatment were obtained when the sLex mimic glycyrrhizin (20, 38) was maintained in the flow buffer at 10 mM (618 ± 136 total interacting cells/mm², 2 ± 1 firmly adherent cells/mm², n = 3) and when sLex⁻/low/ sLex⁻/negative nonmetastatic colon adenocarcinoma HCT-8 cells (18) were perfused over the HUVEC monolayer (555 ± 73 total interacting cells/mm², 4 ± 2 firmly adherent cells/mm², n = 6). Simultaneous perfusion of glycyrrhizin with neuraminidase-treated LS174T cells also failed to inhibit binding. Together, these findings indicate that a nonsialylated molecule(s) may be mediating tethering to HUVECs. Simultaneous treatment of LS174T cells with heparinase I, heparinase II, and chondroitinase ABC (to cleave glycosaminoglycans capable of E-selectin binding) (27, 29) did not affect binding compared with untreated control cells. These three enzymes also failed to reduce attachment when treated tumor cells were additionally exposed to neuraminidase or perfused with glycyrrhizin (data not shown).

To further classify the type of sialylated ligand(s) involved in adhesion, an inhibitor of glycosphingolipid glycosylation PPPP was used to demonstrate the potential involvement of LS174T sialylated glycolipid ligands in binding E-selectin. PPPP-treated cells expressed ~30 and ~50% less sLex and di-sLex, respectively, compared with untreated control cells (Table 1). These cells mainly rolled on HUVECs, given that only 22 ± 2% of total interacting PPPP-treated cells became stably adherent (Table 2). The effects of PPPP are specific to E-selectin-mediated adhesion, since this treatment failed to affect L-selectin-dependent binding of LS174T cells to neutrophils (19). Treatment of LS174T cells with the diluent DMSO affected neither sLex/di-sLex expression (~90% control values) nor adhesion to HUVECs significantly (~90% total interacting and firmly adherent cells relative to untreated control, n = 10), similar to the DPBS diluent controls for all other enzyme/inhibitor treatments.

LS174T cell attachment was also sensitive to trypsin treatment, which suggests the involvement of glycoprotein ligand(s) in the adhesion process in addition to glycolipids (Fig. 2). Surface-expressed sLex and di-sLex moieties were decreased by ~50% (Table 1), and ~90% of total interacting trypsin-treated cells rolled continuously rather than firmly arrested (Table 2), more than PPPP-treated cells but similar to neuraminidase-treated cells. A small but statistically significant additive effect on sLex and di-sLex expression was observed with dual trypsin/PPPP treatment (41.8 ± 2.8 and 28.8 ± 2.4% of control cell mean fluorescence intensity, respectively, n = 9). These cells rolled extensively and formed slightly less firm adhesion (23 ± 5 firmly adherent cells/mm², n = 9) than cells exposed to neuraminidase, trypsin, or PPPP alone.

Treatment with Bzl-GalNAc and tunicamycin, inhibitors of protein glycosylation, revealed that adhesive sialylated residues were carried on O-linked and N-linked glycans, respectively (Tables 1 and 2, Fig. 2). Results were almost identical to tunicamycin treatment when DMJ, an inhibitor that acts with less potentially toxic effects on cells (11), was used to disrupt N-linked glycan processing (data not shown). However, the glycoprotein counterreceptors did not appear to be GPI-linked, because PI-PLC exposure failed to significantly influence tumor cell attachment to endothelial cells (118 ± 17 and 110 ± 5% of control total interacting and firmly adherent cells, respectively, n = 4), even though flow cytometry confirmed that the GPI-linked molecule CD24 was cleaved (data not shown).

Detachment assays were performed to further characterize the effects of the various LS174T cell treatments on adhesive strength to HUVECs. All enzyme and inhibitor treatments, with the exception of the DMSO or DPBS diluent controls, resulted in significantly decreased adhesive strength relative to untreated cells. As shown in Fig. 3A, trypsin-treated cells detached more readily than neuraminidase-treated cells, which in turn detached faster than PPPP-treated cells. It is noteworthy that this trend did not exactly correspond to total levels of sLex/di-sLex expression (Table 1). More specifically, a shear stress of 8 dyn/cm² was needed to achieve ~50% detachment of untreated cells, whereas approximately half of trypsin-treated cells detached with 2 dyn/cm². Only ~30% of neuraminidase-treated LS174T cells were removed with 2 dyn/cm², and this value was statistically significant relative to trypsin-treated cells at the same shear stress (P < 0.05). PPPP-cultured cells required 4 dyn/ cm² to detach 52 ± 5%. As observed in attachment assays, a small but consistent additive effect was observed with trypsin/PPPP treatment (~10% over trypsin treatment alone at 2–4 dyn/cm²). Furthermore, cells cultured with Bzl-GalNAc or tunicamycin/DMJ consistently showed similar reduced resistance to shear stress (Fig. 3B), even though Bzl-GalNAc-treated cells displayed less sLex/di-sLex than tunicamycin/ DMJ-treated cells (Table 1).

LS174T cell rolling velocities on HUVECs were dependent on shear stress, as expected (Fig. 3C) (8, 13, 14, 24, 31, 45, 47). More specifically, mean rolling velocities at 1 dyn/cm² were determined to be 8.6 ± 0.8 μm/s (n = 30 cells), 5.0 ± 0.6 μm/s (n = 28), and 4.8 ± 0.8 μm/s (n = 29) for neuraminidase-, trypsin-, and PPPP-treated cells, respectively, and these velocities increased with increasing shear stress. However, unlike detachment, rolling velocities were dependent on sLex/di-sLex expression, particularly at higher shear stresses (Table 1, Fig. 3C); that is, cells exposed to neuraminidase rolled faster on HUVECs than those treated with trypsin, which rolled faster than PPPP-treated cells.

Dependence on integrins of LS174T cell adhesion to HUVECs. We next focused on identifying additional molecules that also participate in LS174T-HUVEC adhesive interactions by testing a panel of function-blocking MAbs and peptides. Antibodies against endothelial VCAM-1 and LS174T α₁ and α₅ integrin receptors (31) did not reduce tethering or firm adhesion (data not shown).
Fig. 3. LS174T cell detachment from HUVECs. Comparison of neuraminidase-, trypsin-, and PPPP-treated (A) and Bzl-GalNAc- and tunicamycin-treated (B) LS174T cell resistance to shear stress in detachment assays. Cells were treated with various enzymes and inhibitors affecting sialylated surface molecule expression as described in MATERIALS AND METHODS. Immediately following the attachment assay at 1 dyn/cm², detachment assays were performed by doubling shear stress every 30 s from 2 to 32 dyn/cm². Percent adhesion is indicated relative to the number of cells in the field of view at the start of the detachment assay. Values are means ± SE (n = 6–18). * and †P < 0.05 with respect to control at the same shear stress for neuraminidase-, trypsin-, and PPPP-treated cells, respectively, in A. * and †P < 0.05 with respect to control at the same shear stress for Bzl-GalNAc- and tunicamycin-treated cells, respectively, in B. C: effect of shear stress on LS174T cell rolling velocities. Experimental conditions were as in A. Average rolling velocities were calculated as described in MATERIALS AND METHODS. Values are means ± SE (n = 12–23 cells). * and †P < 0.05 with respect to control at the same shear stress for neuraminidase-, trypsin-, and PPPP-treated cells, respectively.

Fig. 4. Effects of antibodies on LS174T cell binding to HUVECs. Open bars represent total interacting cells as a percentage of control counted throughout the entire 5-min perfusion period, whereas filled bars represent the stable adherent cells as a percentage of control counted at the end of perfusion (stationary for >10 s). LS174T cells were treated with saturating concentrations of antibodies for 15 min at RT before perfusion (10%/ml) at 1 dyn/cm² over HUVECs stimulated with TNF-α (5 ng/ml) for 4 h. MAb 13 (blocking β1 MAb), ASC-3 (blocking β4 MAb), Gi9 (blocking α2 MAb), GoH3 (blocking α9 MAb). Values are means ± SE (n = 5–9, except n = 2 for ASC-3). *P < 0.05 with respect to control.

shown). MABs against α1β3 and β3 and nonpeptide antagonists of α1β3 (XV454) and α6β3 (XT199) failed to affect binding, because β3 integrins are not present on LS174T cells (31). Furthermore, treatment of HUVECs with ICAM-1 P(ab)2 antibody did not influence LS174T adhesion, in accord with the lack of β2 integrins on unstimulated LS174T cells (31), even though this MAB effectively inhibited neutrophil adhesion to the endothelial cell monolayer (data not shown). However, statistically significant decreases in total interacting cells and firm adhesion were achieved through blockade of LS174T cell α5, α6, and β1 integrin subunits (Fig. 4). Combined α5 and α6 MAB application did not show increased inhibition over single MAB treatments (data not shown), and β4 MAB failed to influence adhesion (Fig. 4). When HUVECs alone were incubated with the anti-β1 MAB 13, no difference in adhesion was observed compared with untreated cells (data not shown). Together, α5β1 and α6β1, but not α6β4 integrins on tumor cells may play minor redundant roles in mediating adhesion to endothelial cells, perhaps by binding surface-expressed laminin (3, 5, 21, 39). In agreement with binding of α5β3 and α6β3 integrins to laminin (35), GRGDS peptide and RGD-containing polypeptide echistatin also failed to inhibit tumor cell-HUVEC adhesion (data not shown).

E-selectin internalization is induced by LS174T cell attachment. We further wished to examine whether the LS174T actin cytoskeleton regulates colon carcinoma cell adhesion to HUVECs, in a manner similar to that of leukocytes to immobilized platelets expressing P-selectin (31a, 42). Actin disruptive agents latrunculin A and cytochalasins B and D failed to influence LS174T cell tethering (data not shown) and shear-induced detachment from purified E-selectin-coated surfaces (range ±7% of control cell detachment, up to 16 dyn/cm²) or rolling velocity (data not shown). Furthermore, fixation of protein structure and inhibition of LS174T
metabolic function by exposure with 1% formalin also failed to significantly affect cell binding and conversion to firm adhesion on HUVECs (86 ± 4 and 83 ± 9% of control, respectively, n = 3). Together, these data indicate that neither actin-mediated receptor reorganization nor another cellular metabolism-dependent process within tumor cells was mediating firm adhesion.

The possibility that E-selectin clustering may be induced upon tumor cell attachment to HUVECs, as has been demonstrated for peripheral blood monocytes and the HL60 leukocyte cell line (50, 52), was next investigated by using immunogold electron microscopy. The 4-h TNF-α-stimulated HUVEC monolayers failed to label with P-selectin MAbs, verifying that the anti-E-/P-selectin MAbs identified only E-selectin under these conditions, consistent with previous studies (36, 50). Gold particles labeling E-selectin were found on the HUVEC surface as expected, but clustering was not apparent on any endothelial cells, let alone those with adherent LS174T cells. However, early endosomes containing label were evident at 2 min after the start of cell perfusion in HUVECs with bound tumor cells (Fig. 5, B and C). Furthermore, later stage membrane proximal multivesicular endosomes containing label were occasionally found at 2 min (Fig. 5D) but were prominent at 6 min (Fig. 5, E–G), again in endothelial cells with adherent LS174T cells. HUVECs without attached tumor cells never contained these structures (Fig. 5A). Similar results were obtained with perfusion/attachment of peripheral blood neutrophils (Fig. 5H) and THP-1 monocytic cells (data not shown). The internalization was E-selectin specific, because labeled ICAM-1 was found only on the surface of HUVECs but not in endosomes (data not shown). The presence of E-selectin in early/multivesicular endosomes in direct proximity to adherent tumor cells has numerous potential mechanistic implications toward adhesion and signaling, discussed in detail below.

DISCUSSION

In this work, we demonstrate that sLeα-negative, sLeα/di-sLeα-positive LS174T colon adenocarcinoma cells utilize multiple glycoconjugates to adhere to 4-h TNF-α stimulated HUVECs under flow conditions. More specifically, adhesion mechanisms, involving sialylated glycolipids and glycoproteins, nonsialylated molecule(s), integrins, and their respective ligands are revealed. Firm adhesion does not appear to be dependent on active E-selectin counterreceptor reorganization on the tumor cells, but E-selectin internalization is increased in HUVECs with attached tumor cells, a phenomenon with possible ramifications on adhesion.

Sialylated molecules, clearly, are greatly important to the binding of LS174T cells to HUVECs. A glycolipid role in E-selectin-dependent adhesion has largely been speculated as a result of reduction, but not complete abrogation, of binding through the use of proteases and inhibitors of protein glycosylation (17, 28, 40). In this study, we show that sLeα/di-sLeα-decorated glycolipids participate in dynamic adhesion to endothelium expressing E-selectin, through the use of the lipid glycosylation inhibitor PPPP. Glycolipids have a lesser role in adhesion as opposed to glycoproteins, as evidenced by generally stronger adhesion when comparing conversion of rolling to firm adhesion, rolling velocities, and resistance to detachment by increased shear stress of PPPP-treated vs. trypsin-treated cells. These data are consistent with the notion that glycoproteins tend to extend farther beyond the cell surface, whereas glycolipids tend to be smaller and closer to the cell surface, alone a suggestion of a minor role for glycolipids compared with glycoproteins. Similarly, Laskowska et al. (25) recently reported a contributory role in E-selectin-mediated adhesion for sLeα-bearing glycolipids in the absence of O-linked glycoproteins (25). However, this particular study was performed under static (no flow) conditions, and sLeα expression was not examined. It should be noted that a prior investigation did not demonstrate adhesive capabilities for colon carcinoma sLeα-glycolipids to HUVECs under static conditions through the use of d-threo-l-phenyl-2-decanoylamino-3-morpholino-1-propanol (17), a glycolipid biosynthesis inhibitor approximately four times less active than PPPP (1), but differences in cell lines may also be a factor. Regardless, our results provide direct evidence for sLeα-glycolipid receptors in carcinoma cell rolling on endothelium through E-selectin.

Though increased rolling velocities corresponded to decreased sLeα/di-sLeα expression, detachment did not similarly correlate. Trypsin-treated LS174T cells detached more easily from HUVECs than neuraminidase-treated cells up to 4 dyn/cm², despite the observation that the former possessed ample sialylated molecules whereas the latter expressed almost none (Table 1). This finding may indicate the involvement of another (possibly nonsialylated) glycoprotein ligand(s) participating in firm adhesion, on which detachment assays were based. Furthermore, LS174T cells cultured with BzI-GalNAc expressed less sLeα/di-sLeα than tunicamycin-treated cells, but detachment was almost equivalent. Assuming no other differences than target glycoprotein expression, these data suggest that although there are fewer N-linked sialylated glycoproteins, the individual molecules bind with greater strength to E-selectin than their more numerous O-linked counterparts. It is important to recognize that although overall weakened adhesive strength of enzyme/inhibitor-treated cells reflected decreased total sLeα/di-sLeα expression compared with control (untreated) LS174T cells, additional factors may be of influence: membrane presentation of glycolipids vs. glycoproteins, overlapping/redundant function, other important binding epitopes along the counter-receptor backbone, and/or inherent receptor-ligand characteristics (binding kinetics, reactive compliance, tensile strength, etc.) (9).

Surprisingly, tethering of LS174T cells could not be fully attributed to sLeα/di-sLeα residues, because numerous rolling cells were observed after treatment with neuraminidase and perfusion of sLeα mimic gly-
cyrrhizin, alone or simultaneously. These data suggest that a nonsialylated ligand(s) is present on LS174T cells, but no role was identified for tumor cell surface-expressed heparin and other glycosaminoglycans, molecules previously shown to block E-selectin binding (27, 29). Another possible E-selectin counterreceptor is sulfatide (34), but sulfatides and other sulfated glycolipids are more widely implicated in L- and P-selectin binding (33, 34). At this point, therefore, the identity of any remaining ligand(s) is unknown.

The LS174T cell α2, α6, and β1 integrins (i.e., laminin binding α2β1, α6β1) that support a minor adhesion pathway are not likely the aforementioned unknown ligand(s), because the β1 integrin subunit is trypsin sensitive (data not shown). Blocking tumor cell integrin function reduced both the amounts of total interacting and firmly adherent cells; the (possibly redundant) roles of these receptors are not solely in mediating stable adhesion. Nevertheless, our work is in agreement with a recent report stating that LS174T cells can adhere to surface-expressed laminin on stimulated HUVECs in static adhesion assays in a β1-dependent manner (3), as well as other studies demonstrating functions of various integrins in dynamic adhesion to purified laminin, although which integrins participate depends on the type of laminin present and individual experimental conditions (21, 48).

Soluble E-selectin has been previously shown to support firm adhesion of colon carcinoma cells, which was mediated by high-density areas of sLeα-presenting ligands, whereas tethering and rolling was more reliant on sLeα (22). In contrast, the sLeα-negative, sLeα/di-sLeα-positive LS174T cells rolled continuously on purified E-selectin-coated substrates, and actin and metabolic function inhibitors applied to the tumor cells failed to influence binding strength. These findings indicate that active receptor reorganization to achieve localized high-density sites capable of mediating stable adhesive interactions was not necessary in adhesion, unlike leukocyte PSGL-1 association with the actin network in firm adhesion to immobilized platelets (31a, 42). On the other hand, the sialylated epitopes may be constitutively localized on the LS174T cell surface but not associated with the actin cytoskeleton.

Through the use of immunogold electron microscopy, early/multivesicular endosomes specifically containing gold-labeled E-selectin were observed in endothelial cells with attached tumor cells, but not in those without. E-selectin clustering, seen previously with human peripheral blood monocytes and HL60 cells under static conditions (50, 52), was not observed, likely as a result of clustering/internalization that occurs within 2 min of tumor cell attachment. Therefore, the presence of endosomal structures may in themselves be an indication of E-selectin distribution/localization, and though short-lived, these clusters are potentially capable of transiently mediating stronger interactions, such as assisting in the conversion of rolling to firm adhesion or else firm arrest itself. For example, internalization-competent P-selectin-transfected cells (i.e., cells capable of normal or enhanced endocytosis) support slower rolling and generally stronger adhesion of leukocytes compared with internalization-incompetent cells (41). These adhesive differences are attributed to membrane molecular distributions rather than to disparities in the internalization process itself: localized P-selectin in internalization-competent cells, but diffuse distribution otherwise (41). At the very least, a signaling mechanism exists such that E-selectin-specific endocytosis is increased in HUVECs with adherent LS174T cells. E-selectin itself acts as a signaling molecule once cross-linked by adhering cells or MAbs (52), and its cytoplasmic domain contains a serine residue that allows for increased E-selectin internalization over normal bulk membrane flow (10). The increase in E-selectin endocytosis may provide a pathway to clear this molecule from the surface to allow another firm adhesion mechanism to be upregulated or to ease a further downstream event such as transmigration.

Cumulatively, the results of this study demonstrate that LS174T colon adenocarcinoma cell adhesion involves sialylated glycolipids, both O- and N-linked sialylated glycoproteins, possibly a nonsialylated ligand, and integrins to bind stimulated HUVECs in a system mimicking physiological flow conditions. Notably, the total glycoprotein involvement is greater than that of the glycolipids. Furthermore, upregulated endocytosis of E-selectin was observed in endothelial cells with attached tumor cells, an indirect indication that firm adhesion may in part be mediated by E-selectin clustering induced by LS174T cell adhesion. These findings help advance our understanding of the molecular mechanisms underlying blood-borne colon cancer metastasis, thus providing insight toward the development of novel therapeutics to combat the spread of cancer.

We thank Dr. G. L. Sexton (Johns Hopkins University (JHU) Integrated Imaging Center) for microscopy assistance and Dr. R. L. Schnaar (Dept. of Pharmacology and Molecular Sciences, JHU School of Medicine) for insightful discussions.

This work was supported by a Whitaker Foundation Grant (to K. Konstantopoulos), National Science Foundation (NSF) Grant BES 9978160 (to K. Konstantopoulos), National Institute of Allergy and Infectious Diseases Grant AI-45115 (to B. S. Bochner and K. Konstantopoulos), an NSF Graduate Research Fellowship (to M. M. Burdick), NSF Grant DBI 0099705 (to J. M. McCaffrey), and the Department of Veterans Affairs Medical Research Service (to Y. S. Kim).
REFERENCES


