American Journal of Pathology, Vol. 149, No. 5, November 1996

A Human Colon Carcinoma Cell Line Exhibits Adhesive Interactions with P-Selectin under Fluid Flow *via* a PSGL-1-Independent Mechanism

Douglas J. Goetz,* Han Ding,*
William J. Atkinson,* Gloria Vachino,†
Raymond T. Camphausen,†
Dale A. Cumming,† and
Francis W. Luscinskas*

From the Vascular Research Division, Departments of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, and the Small Molecule Drug Discovery Group,† Genetics Institute, 87 Cambridge Park Drive, Cambridge, Massachusetts*

It has been postulated that endothelial cell adhesion molecules involved in leukocyte recruitment play a role in metastasis. Using an* in vitro *flow model, we studied the adhesion of the human colon carcinoma cell line KM12-L4 to P-selectin, an inducible endothelial-expressed adhesion molecule involved in leukocyte recruitment. Recombinant forms of P-selectin and Chinese hamster ovary cells stably expressing P-selectin supported attachment and rolling of KM12-L4 cells at 1 to 2 dynes/cm^2. The adhesive interactions to P-selectin were abolished by pretreatment of the KM12-L4 cells with neuraminidase but were unaltered by pretreatment of the KM12-L4 cells with O-sialoglycoprotein endopeptidase, an enzyme that cleaves mucin-type glycoproteins such as P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is the only counter-receptor for P-selectin known to mediate myeloid cell adhesion to P-selectin under flow. Flow cytometric and Northern blot analyses revealed that KM12-L4 cells did not express PSGL-1 and monoclonal antibody PL1, a function-blocking monoclonal antibody to PSGL-1, had no inhibitory effect on KM12-L4 adhesion to P-selectin under flow. Compared with HL-60 cells, which express PSGL-1, the KM12-L4 cells exhibited a slightly lower rate of attachment to P-selectin and rolled at a significantly higher velocity. In summary, KM12-L4 human colon carcinoma cells interact with P-selectin, under flow, through a PSGL-1-independent adhesion pathway. (Am J Pathol 1996, 149:1661–1673)

A critical step in the recruitment of leukocytes to a site of inflammation is the adhesion of the leukocytes to the vascular endothelium in the fluid dynamic environment of the microcirculation. This adhesion process involves a cascade of adhesive events including initial attachment, rolling, spreading, and ultimately transendothelial migration.[1–4] *In vivo* and *in vitro* studies have shown that the inducible endothelial cell adhesion molecules P-selectin (CD62P) and E-selectin (CD62E) are involved in leukocyte initial attachment and rolling on activated vascular endothelium.[5–8] The third known member of the selectin family, L-selectin (CD62L), is expressed only on leukocytes and also is involved in leukocyte recruitment.[2,9–11] A notable feature of the selectins is their amino-terminal, lectin-like domain that binds carbohydrate moieties in a Ca^{2+}-dependent manner.[12] Thus, several carbohydrate ligands for P- and E-selectin have been proposed including SLe^x and SLe^a.[13–15] Recently, P-selectin glycoprotein ligand-1 (PSGL-1), a mucin-like glycoprotein, has been identified as a high-affinity counter-receptor that presents carbohydrate ligands such as SLe^x to P-selectin.[16–18] A monoclonal antibody (MAb) to PSGL-1 (PL1) blocked the adhesion of neutrophils and HL-60 cells to P-selectin under fluid flow, implying that PSGL-1 mediates attachment of these cells to P-selectin.[17,19] To date, PSGL-1 is the only P-selectin counter-receptor known to play such a role.[17,19]

The above paradigm of leukocyte recruitment to a site of inflammation is analogous in several respects

Supported by National Institutes of Health grants T32HL07627 (D. J. Goetz) and HL36028 and HL47646 (F. W. Luscinskas).

Accepted for publication July 15, 1996.

Address reprint requests to Dr. Francis W. Luscinskas, Brigham and Women's Hospital, Department of Pathology, 221 Longwood Avenue, Boston, MA 02115.

to the latter steps of the metastatic cascade. In particular, at a secondary site of potential colonization, metastatic tumor cells presumably interact with the vascular endothelium in the fluid dynamic environment of the microcirculation. Based on this analogy, and the fact that several carcinoma cell lines express SLe^a and SLe^x,[20–22] it has been proposed that E- and P-selectin play a role in metastasis.[20–27] Indeed, *in vitro* adhesion assays conducted under static[21] and flow[22,26,27] conditions have demonstrated that E-selectin plays a role in the adhesive interactions between carcinoma cell lines and interleukin-1β or tumor necrosis factor-α stimulated cultured vascular endothelial monolayers. Studies performed under flow have revealed that carcinoma cells roll on interleukin-1β stimulated endothelium and substrates coated with soluble forms of E-selectin[22,26] in a sialylated-moiety-dependent manner.[22] This rolling behavior is similar to that seen for T lymphocytes rolling on tumor necrosis factor-α activated endothelium[8] (ie, initial attachment followed by slow (<10 μm/second) rolling).

It has been reported that, under static conditions, several carcinoma cell lines adhere to activated human platelets that express P-selectin[25] and surfaces coated with purified P-selectin.[24] In addition, P-selectin-IgG chimera molecules have been shown to bind to carcinoma tissue sections and cell lines derived from the various carcinomas.[23] However, there are very few data on the ability of carcinoma cells to interact with P-selectin-expressing substrates under flow. The salient feature of the selectins is their ability to mediate attachment and rolling of cells under flow conditions.[5,6,11,22] This appears to imply that the selectin-ligand bonds have high on and off rates, support a tensile force, and eventually dissociate in response to the applied force.[28,29] Static assays cannot reveal whether a given selectin interaction has these characteristics. In addition, as argued by Varki et al,[30] results generated *in vitro* under static conditions may not be relevant to the fluid dynamic conditions present *in vivo*. Hence, we have studied the interaction of a model human colon carcinoma cell line, KM12-L4, with P-selectin under flow.

The present study demonstrates that P-selectin supports KM12-L4 cell adhesive interactions under flow. In contrast to leukocytes, however, KM12-L4 cells interact with P-selectin in a PSGL-1-independent manner, implying that KM12-L4 cells express a non-PSGL-1 counter-receptor(s) for P-selectin that can mediate cell attachment and rolling under flow.

Materials and Methods

Materials

Alpha media, Dulbecco's phosphate-buffered saline with 1.5 mmol/L Ca^{2+} and Mg^{2+} ($DPBS^+$) or without cations (DPBS), Hanks balanced salt solution (HBSS) containing phenol red without Ca^{2+} and Mg^{2+}, and RPMI-1640 containing 25 mmol/L HEPES and L-glutamine were obtained from BioWhittaker (Walkersville, MD). Fetal bovine serum (FBS) and dialyzed FBS were obtained from Hyclone (Logan, UT). Cell dissociation medium, a non-enzymatic harvesting solution for KM12-L4 cells, was purchased from Specialty Media (Lavallette, NJ). *O*-sialoglycoprotein endopeptidase (OSGE) was obtained from Accurate Chemicals (Westbury, NY). All other chemicals were of the highest grade available from J.T. Baker (Phillipsburg, NJ).

Antibodies

Function-blocking murine MAb to E-selectin MAb 7A9 (IgG_1) was obtained from American Type Culture Collection (Rockville, MD; clone HB 10135) and used as purified IgG. Function-blocking murine MAb to E-selectin MAb H18/7 (IgG_{2a}) was used as purified $F(ab')_2$.[31] Leukocyte function-blocking murine anti-P-selectin MAb HPDG2/3 (IgG_1)[18] was used as purified IgG and $F(ab')_2$ (10 μg/ml). The following antibodies recognize human PSGL-1 and have been described previously[18,32]: nonblocking murine MAb PSL-275 (IgG_1; purified IgG, 20 μg/ml) and polyclonal rabbit sera, Rb3026 (1:20 dilution). Preimmune rabbit serum (1:20 dilution) was used as a negative control for Rb3026. PL1 (IgG_1), a function-blocking MAb to PSGL-1, was obtained from Drs. Roger P. McEver and Kevin L. Moore (University of Oklahoma Health Sciences Center, Oklahoma City, OK) and was used as purified IgG.[17] Murine anti-L-selectin MAbs LAM1-3, LAM1-5, LAM1-4, and LAM1-14 (all IgG_1) were obtained from Dr. Thomas Tedder (Duke University Medical Center, Durham, NC).[33] LAM1-14, LAM1-5, and LAM1-4 were used as purified IgG at 10 μg/ml whereas LAM1-3 was used as ascites (diluted 1:200). MAb to sialyl $Lewis^x$ (IgM; 20 μg/ml), CSLEX-1, was obtained from Becton Dickinson (San Jose, CA). MAb to sialyl $Lewis^a$ (IgG_1; 20 μg/ml), KM23,[34] was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). MAb to the β_1 integrin (IgG_1; 10 μg/ml), 18D3, was obtained from Endogen (Boston, MA) and used as purified IgG. MAb K16/16 (IgG_1) was used as undiluted hybridoma supernatant and served as a nonbinding

control. Fluorescein isothiocyanate (FITC)-labeled secondary antibodies (1:50 dilution), goat $F(ab')_2$ anti-mouse IgG, goat $F(ab')_2$ anti-rabbit IgG, and goat $F(ab')_2$ anti-mouse IgM were obtained from Caltag Laboratories (San Francisco, CA).

E- and P-Selectin Molecules

The E- and P-selectin-IgG_1 chimeras consisted of the amino-terminal lectin, epidermal-growth-factor-like, and six and four, respectively, CR-like repeat domains fused to the Fc region of human IgG_1 as previously described.[18,35] The full-length recombinant P-selectin molecule contained the extracellular portion of P-selectin as previously described.[18,35] Purified human IgG_1 (Sigma Chemical Co., St. Louis, MO) was used as a negative control for the selectin-IgG_1 chimeras.

Cell Culture

KM12-L4a (KM12-L4) human colon carcinoma cells[36] were obtained from Dr. I. J. Fidler (University of Texas, MD Anderson Cancer Center, Houston, TX). Tumor cells were maintained in RPMI-1640 containing 10% FBS, antibiotics, and sodium pyruvate and used in experiments after reaching approximately 60% confluence. Chinese hamster ovary (CHO) cells expressing a stably transfected human E-selectin cDNA (CHO-E) and CHO cells expressing a stably transfected human P-selectin cDNA (CHO-P) were grown in Alpha media containing 10% dialyzed FBS as previously detailed.[18] The HL-60 cell line was obtained from American Type Culture Collection and cultured in RPMI containing 10% FBS and antibiotics. Cell size was determined by image analysis of videotape recordings made of HL-60 or KM12-L4 cells and a calibrated stage micrometer using a 40× objective.

Harvest of KM12-L4 Cells

KM12-L4 cells were washed once in DPBS without divalent cations and harvested with a non-enzymatic cell dissociation solution (described above in Materials). The tumor cells were recovered in RPMI-1640 containing 10% FBS, washed twice in $DPBS^+$, and finally resuspended to 1×10^7 cells/ml in HBSS, pH 7.4, containing 25 mmol/L HEPES. Harvested KM12-L4 cells were held in this buffer at 4°C for no longer than 4 hours before use. Unless otherwise noted, an aliquot of the unicellular suspension of KM12-L4 cells was diluted to 1×10^6/ml in 37°C perfusion buffer (DPBS containing 1.5 mmol/L Ca^{2+} and Mg^{2+} and 0.5% bovine serum albumin (BSA)) immediately before use in the flow chamber assay.

Parallel Plate Flow Chamber Analysis of KM12-L4-Selectin Interactions

The flow chamber apparatus used in these studies has been described in detail.[8,11] Briefly, the chamber consists of two stainless steel plates separated by a silastic gasket. Coverslips containing either a cell monolayer or purified molecules were placed in the flow chamber, and fluid was drawn through the chamber at defined flow rates using a syringe pump (model 44, Harvard Apparatus, Natick, MA). Shear stresses at the adhesive surface were calculated from the equation $\tau = 3\ \mu Q/2ba$,[2] where a is one-half the distance between the top and the bottom plates of the flow chamber, 0.125 mm, b is the width of the flow field, 5 mm, Q is the flow rate of the fluid drawn through the chamber, and μ is the viscosity of the fluid assumed to be the viscosity of water at 37°C, 0.7 cp. Temperature was maintained at 37°C by a heating plate. The flow apparatus was mounted on an inverted microscope (Nikon Diaphot), and the entire perfusion period was recorded on videotape by a video camera and VCR. KM12-L4 cell adhesion was quantified as follows: for the E-selectin studies, the number of adherent cells in three to four random fields of view was determined after 3 minutes of cell perfusion. These values were averaged to give n = 1. For the P-selectin data, the number of cells that interacted with the P-selectin-presenting substrate in a given field of view in a 30-second time period was determined in several random fields, and these values were averaged to give n = 1. To determine the rate of attachment, the objective of the microscope was positioned 3 cm from the inlet of the chamber before the start of the flow of the fluid. The fluid flow was initiated and the field of view was observed for 3 to 4 minutes. The number of attachment events as a function of time was determined. The HL-60 rolling velocities were determined by measuring the distance the cells translated in 10 seconds. The KM12-L4 rolling velocities were determined by measuring the length of time required for the cells to translate a distance of greater than 400 μm. All observations were made with a 10× objective.

Preparation of Soluble-Selectin-Coated Surfaces and EDTA Protocol

Circular glass coverslips (25-mm diameter; Fisher Scientific, Medford, MA) were sterilized in 100% eth-

anol and a 1-cm diameter region was washed three times with DPBS. After the wash, a 40-μl drop containing 10 μg/ml of the selectin-IgG chimera, human IgG_1 (diluted in DPBS), or full-length recombinant P-selectin (diluted in 0.1 mol/L $NaHCO_3$, pH 9.2) was added and incubated at room temperature for 2 hours. This region was washed three times with DPBS, and the entire surface was blocked with $DPBS^+$ containing 1% BSA for 1 hour at room temperature. Immediately before the assay, the coverslip was treated with 1% Tween 20 in $DPBS^+$ for 1 minute to block nonspecific binding of the KM12-L4 cells to the protein-coated surface.[5] To demonstrate that the adhesive interactions to the soluble forms of P-selectin were cation dependent, KM12-L4 or HL-60 cells were resuspended in DPBS, 0.5% BSA, and 5 mmol/L EDTA at pH 7.4 for use in the flow chamber assay.

Antibody Blocking Experiments

The CHO-E or CHO-P cell monolayers were treated with anti-E-selectin (MAb 7A9) or anti-P-selectin (MAb HPDG2/3) in Alpha medium/10% FBS for 15 minutes at 37°C before the adhesion assays described above. For the E-selectin-IgG, the P-selectin-IgG chimera, or the full-length recombinant P-selectin experiments, saturating levels of MAb (50 μg/ml 7A9, 10 μg/ml HPDG2/3 to block KM12-L4 adhesion, and 20 μg/ml HPDG2/3 to block HL-60 adhesion) were added to the $DPBS^+$, 1% BSA blocking solution 15 minutes before the adhesion assay. MAb 7A9 (10 μg/ml) or MAb HPDG2/3 (20 μg/ml) was added to the perfusion buffer to block the KM12-L4 cell/E-selectin or the HL-60 cell/P-selectin interactions, respectively. For the PL1 blocking experiments, an aliquot of HL-60 or KM12-L4 cells was resuspended in 37°C perfusion buffer at 1×10^6 cells/ml and 10 μg/ml PL1 was added to the cell suspension. The suspension was incubated for 10 minutes at room temperature and subsequently used in the flow chamber assay. Control cells were prepared in exactly the same manner only without the MAb.

Flow Cytometric Analysis of KM12-L4 Cell Expression of β_1, PSGL-1, L-Selectin, SLe^x, and SLe^a

A unicellular suspension of KM12-L4 cells was washed in RPMI-1640 containing 2% FBS. Replicate samples (50 μl) containing 10^6 cells and saturating levels of primary MAb in RPMI-1640/2% FBS were incubated for 30 minutes on ice. After incubation, the cells were washed and the primary MAb detected with 50 μl of the appropriate FITC-labeled secondary antibodies. The KM12-L4 cells were incubated for 30 minutes at 4°C, washed twice in RPMI-1640/2% FBS and once in $DPBS^+$, and fixed with 2% formaldehyde in $DPBS^+$. FITC fluorescence was determined on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) by measuring the fluorescence of either 5,000 or 10,000 cells. The data are presented as single-parameter histograms.

Flow Cytometric Analysis of Selectin-IgG Chimera Binding to KM12-L4 Cells

P-selectin-IgG chimera or human IgG_1 (40 μg/ml) were precoupled with anti-P-selectin MAb HPDG2/3 $F(ab')_2$, anti-E-selectin MAb H18/7 $F(ab')_2$ (80 μg/ml), or medium alone for 1 hour at 4°C. An aliquot of this complex was treated with 4 μg/ml FITC-labeled protein A (Zymed, South San Francisco, CA) and incubated at 4°C for 2 hours. This was termed the Fc/FITC complex. Replicate samples of a unicellular suspension of KM12-L4 cells (10^6 cells) were preincubated in RPMI/2% FBS for 30 minutes at 4°C and then incubated further with either the P-selectin chimera or human IgG Fc/FITC complex. After 30 minutes at 4°C, cells were washed twice in RPMI containing 2% FBS and once in $DPBS^+$ and fixed with 2% formaldehyde in $DPBS^+$. Fluorescence was quantified using a FACScan.

Sialidase Treatment of KM12-L4 Cells

Harvested KM12-L4 cells (10^7) were washed twice in RPMI-1640, resuspended in 1 ml of RPMI-1640, and incubated for 1 hour at 37°C with 0.1 U/ml neuraminidase (Boehringer Manheim, Indianapolis, IN) derived from *Vibrio cholerae*. Another sample was prepared in an identical manner but incubated without neuraminidase. The cells were resuspended in RPMI-1640/10% FBS and washed twice with $DPBS^+$. The cells were resuspended in HBSS containing 25 mmol/L HEPES and 0.5% BSA and held on ice until use in the flow assay. A third sample of KM12-L4 cells was treated exactly as described with the neuraminidase but in the presence of 20 mmol/L 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid, a competitive inhibitor of neuraminidase (Sigma).

O-Sialoglycoprotein Endopeptidase Treatment

Harvested KM12-L4 or HL-60 cells were washed twice in $DPBS^+$ and were resuspended to 3×10^7 cells/ml in $DPBS^+$ pH 7.4 containing 0.2% FBS and 25 mmol/L HEPES. OSGE (80 μg/ml) was added and the cell suspension was incubated for 1 hour at 37°C with occasional mixing to keep the cells in suspension. KM12-L4 or HL-60 cells were washed once with $DPBS^+$, resuspended to 1×10^7 cells/ml in HBSS containing 25 mmol/L HEPES, and held at 4°C until use in the flow assay.

Northern Blot Analyses

Total cellular RNA from KM12-L4 and HL-60 cells was prepared using the RNAzol method (Tel-Test, Friendswood, TX). Poly(A)$^+$ RNA was prepared from ~2 mg of total RNA using oligo-(dT)20-30 cellulose columns as detailed by the manufacturer (FastTrack kit, Invitrogen, San Diego, CA). RNA samples (3 μg of poly (A)$^+$ or 25 μg of total cellular RNA) were resolved on a 1% agarose-formaldehyde gel, transferred to nylon filters (Hybond N+, Amersham Corp., Arlington Heights, IL), and immobilized by ultraviolet irradiation with an ultraviolet Stratalinker 2400 (two cycles; Stratagene, La Jolla, CA). Equal loading of RNA to each lane of the gel was determined both by ethidium bromide fluorescence and by hybridization with a probe for glyceraldehyde phosphate dehydrogenase.[37] Blots were prehybridized at 42°C for 4 hour and hybridized overnight at 65°C with [^{32}P]dCTP-labeled probes (Megaprime kit, Amersham).[37] The PSGL-1 probe was a full-length cDNA[18] and the full-length cDNA probe for glyceraldehyde phosphate dehydrogenase was obtained from American Type Culture Collection. The filters were exposed to x-ray films at −70°C with intensifying screens.

Statistics

All differences were evaluated by a two-tailed Student's *t*-test. *P* values represent the results of these *t*-tests and values <0.05 were considered significant. All error bars represent SEM.

Results

Soluble Fluid Phase P-Selectin Binds Specifically to KM12-L4 Cells

Previous studies have shown that P-selectin binds to the cell surface of a number of cell lines derived from colon carcinomas.[23] Hence, as a first step, we tested the ability of a P-selectin-IgG chimera to bind to the human colon carcinoma cell line, KM12-L4. KM12-L4 cells were treated with either P-selectin-Fc/FITC complex or human IgG_1-Fc/FITC complex. KM12-L4 cells treated with the P-selectin-Fc/FITC complex consistently showed an increase in the FITC fluorescence relative to the fluorescence of KM12-L4 cells treated with FITC-labeled protein A alone (Figure 1). Binding of the P-selectin chimera to the KM12-L4 cells was inhibited by a $F(ab')_2$ fragment of function-blocking MAb to P-selectin, HPDG2/3, whereas a function-blocking $F(ab')_2$ MAb to E-selectin, H18/7, had no effect (Figure 1). The fluorescence of KM12-L4 cells treated with the IgG_1-Fc/FITC complex was essentially identical to cells treated with FITC protein A alone (Figure 1). These data indicate a specific interaction between fluid-phase P-selectin and moieties expressed on the surface of KM12-L4 cells.

KM12-L4 Cells Exhibit Specific Adhesive Interactions with the P-Selectin-IgG Chimera and CHO-P Monolayers under Flow

As the time scale of interaction between P-selectin and the KM12-L4 counter-receptor in the above equilibrium experiment is on the order of minutes and the interaction of a flowing KM12-L4 cell with a particular segment of the endothelium may be on the order of seconds or less, results generated under equilibrium conditions may not reliably predict what occurs under *in vivo* fluid flow conditions.[30,38] In addition, an investigation into the adhesive behavior under fluid flow gives insight into the nature of the bonds that mediate the adhesion processes.[28] Hence, we tested the ability of the P-selectin-IgG chimera to support KM12-L4 cell adhesion in an *in vitro* assay[8,11] designed to mimic laminar blood flow conditions. We observed that the P-selectin-IgG chimera adsorbed to glass did support attachment and rolling of KM12-L4 cells under flow at 1.5 dynes/cm^2 (Figure 2a). By live-time videomicroscopy it was observed that KM12-L4 cells attached to the P-selectin-IgG chimera-coated glass and proceeded to roll at velocities significantly lower than the theoretical velocity of a KM12-L4 cell not interacting with the glass surface.[39] In addition, KM12-L4 cells were observed to roll into the field of view indicating that they had attached upstream of the field of view under observation. The majority of the KM12-L4 cells in contact with the P-selectin-IgG chimera-coated portion of the glass slide remained in contact for their entire transit through the field of view. In contrast, rolling KM12-L4

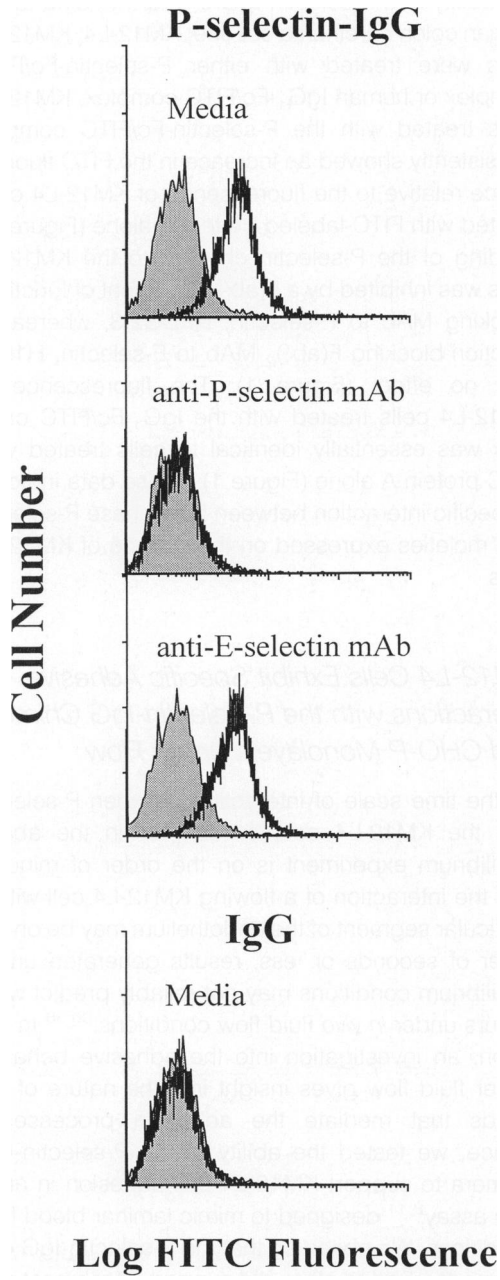

Figure 1. *Fluid phase P-selectin-IgG chimera binds specifically to KM12-L4 cells. A P-selectin-IgG chimera was incubated with medium alone, F(ab')$_2$ MAb fragments to P-selectin (HPDG2/3), or E-selectin (H18/7) and coupled to FITC-labeled protein A before addition to an aliquot of KM12-L4 cells. The P-selectin-IgG chimera-protein A complex bound to the KM12-L4 cells* (top panel). *The binding was blocked by the antibody to P-selectin* (second panel) *but unaffected by the antibody to E-selectin* (third panel). *Human IgG1 precoupled to protein A did not bind to the KM12-L4 cells* (bottom panel). *Shaded histograms are KM12-L4 cells treated with FITC-labeled protein A alone; open histograms are KM12-L4 cells treated with the P-selectin-IgG chimera (or IgG) precoupled with FITC-labeled protein A and no antibody (media) or the antibody listed in the figure.*

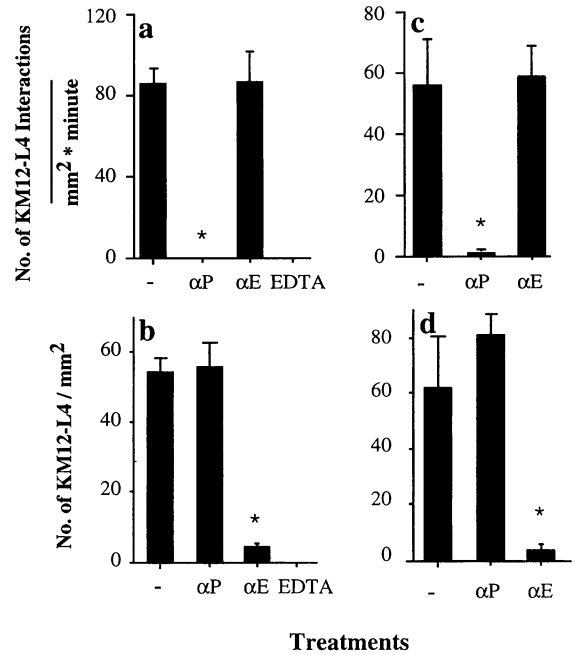

Figure 2. *KM12-L4 cells exhibited specific adhesive interactions with P- and E-selectin under flow.* a *and* c: *KM12-L4 cells attached and rolled on a P-selectin-IgG chimera and CHO-P cell monolayers. The adhesive interactions were blocked by MAb HPDG2/3 to P-selectin but were unaffected by MAb 7A9 to E-selectin. In the absence of divalent cations, no interactions were observed between the KM12-L4 cells and the P-selectin-IgG chimera.* b *and* d: *KM12-L4 cells attached and rolled on an E-selectin-IgG chimera and CHO-E cell monolayers. The adhesive interactions were blocked by MAb 7A9 but unaffected by MAb HPDG2/3. In the absence of divalent cations, no interactions were observed between the KM12-L4 cells and the E-selectin-IgG chimera. Shear stress = 1.5 dynes/cm^2 over the purified proteins and 1.0 dyne/cm^2 over the CHO cells; *P < 0.05; n = 4.*

cells immediately detached into the flow stream upon reaching a region of the glass slide not coated with the P-selectin-IgG chimera, indicating that the observed adhesive interactions were solely dependent upon the P-selectin-IgG chimera.

The adhesive interactions were blocked by MAb HPDG2/3, whereas anti-E-selectin MAb 7A9 had no effect (Figure 2a). As predicted, these antibodies had the opposite effect on the adhesion of KM12-L4 cells to an E-selectin-IgG chimera (Figure 2b). KM12-L4 cells resuspended in DPBS containing 5 mmol/L EDTA, ie, no divalent cations, did not interact with either the P- or E-selectin chimera (Figure 2, a and b). To control for the IgG portion of the P-selectin chimera molecule, we performed studies with KM12-L4 cells and human IgG_1. No adhesive interactions were observed (data not shown).

To determine whether P-selectin expressed by an intact cell monolayer can support KM12-L4 adhesive interactions under flow, we investigated the interaction of KM12-L4 cells to CHO-P cells in our *in vitro* model. CHO-P monolayers supported attachment and rolling of KM12-L4 cells at 1.0 dynes/cm^2 (Figure 2c). As shown in the photomicrographs in Figure 3, KM12-L4 cells initially attached to the CHO-P monolayers, rolled and skipped anywhere from 2 to

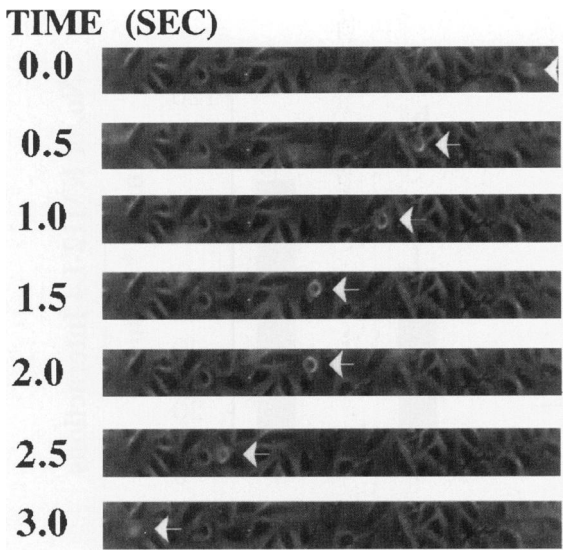

Figure 3. *KM12-L4 cells interacting with CHO-P cells under fluid flow. A typical interaction between a KM12-L4 cell and CHO-P cells is shown. At time 0 seconds, the KM12-L4 cell was moving at the free-stream velocity and hence appears as a blur. By 0.5 seconds, the KM12-L4 cell has attached to the CHO-P cells and proceeded to translate at a nonconstant velocity for approximately 2 seconds. By 3.0 seconds, the KM12-L4 cell has detached from the CHO-P cells and again was moving at the free-stream velocity. This type of behavior (attachment, rapid translation for between 2 and 15 cell diameters, and detachment) was typical for KM12-L4 cells that exhibited an adhesive interaction with the CHO-P cells. Length of the field of view shown = 430 μm. Shear stress = 1.0 dynes/cm².*

30 cell diameters and then detached from the CHO-P surface, releasing into the fluid stream. This behavior was in contrast to that observed over the P-selectin-IgG chimera-coated surfaces where KM12-L4 cell skipping and detachment were rarely observed. The adhesive interactions were blocked by anti-P-selectin MAb HPDG2/3 but were unaffected by anti-E-selectin MAb 7A9 (Figure 2c). These antibodies had the opposite effect on the interaction of KM12-L4 cells with CHO-E monolayers (Figure 2d). Taken together, these data demonstrate that P-selectin can support adhesive interactions of KM12-L4 cells under defined fluid flow conditions.

Sialylated Moieties Expressed on the Surface of KM12-L4 Cells Are Involved in the Adhesive Interactions between KM12-L4 Cells and P-Selectin under Fluid Flow

A recurring observation in selectin-mediated leukocyte adhesion under flow is that the adhesive interactions are sensitive to neuraminidase treatment of the leukocytes.[40,41] In addition, we have previously found that treatment of KM12-L4 cells with neuraminidase significantly reduces the level of adhesive interactions of KM12-L4 cells to E-selectin under fluid flow.[22] Hence, we investigated the effect of neuraminidase treatment on the adhesive interactions between KM12-L4 cells and P-selectin. Flow cytometric analysis revealed that KM12-L4 cells express SLe^x and SLe^a, reported ligands for P-selectin,[14] as well as the β_1 integrin (Figure 4, top panel). The epitopes recognized by the MAbs to SLe^x and SLe^a were dramatically reduced by a 1-hour treatment with neuraminidase whereas β_1 expression was unchanged (Figure 4, middle panel). Treatment with neuraminidase in the presence of the competitive inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid did not significantly alter the level of SLe^x and SLe^a detected on the surface of KM12-L4 cells (Figure 4, bottom panel). This observation, along with the fact that the level of detection of the β_1 integrin was unaltered after neuraminidase treatment, indicates that the removal of the SLe^x and SLe^a was probably due to the neuraminidase and not due to a nonspecific proteolysis. Treatment of the KM12-L4 cells with neuraminidase before use in the flow chamber assay significantly reduced the level of adhesive interactions between the KM12-L4 cells and the P-selectin-IgG chimera (Figure 4b). This effect was prevented by 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Figure 4b). Hence, it appears that sialylated moieties are involved in the adhesion between KM12-L4 cells and P-selectin under flow.

Treatment of KM12-L4 Cells with O-Sialoglycoprotein Endopeptidase Had No Detectable Effect on the Adhesive Interactions between KM12-L4 Cells and P-Selectin under Fluid Flow

Counter-receptors for P-selectin, PSGL-1,[42–44] and L-selectin, CD34,[45] are mucin-like glycoproteins that are susceptible to proteolytic cleavage by OSGE.[42,46] Indeed, pretreatment of HL-60 cells with OSGE significantly reduces the level of adhesion of HL-60 cells to P-selectin under flow.[19] In addition, several carcinoma cell lines adhere to P-selectin under static conditions via mucins expressed on the surface of the carcinoma cells.[24] Therefore, we tested the effect of OSGE on the adhesive interactions between KM12-L4 cells and soluble P-selectin. Since HL-60 cells were used as a positive control for the activity of OSGE, we chose to use a recombinant form of P-selectin containing the entire extracellular portion of P-selectin[18] as the substrate rather than the P-selectin-IgG chimera to avoid potential Fc-mediated adhesion of HL-60 cells. Preliminary studies demonstrated that the adhesive interactions of

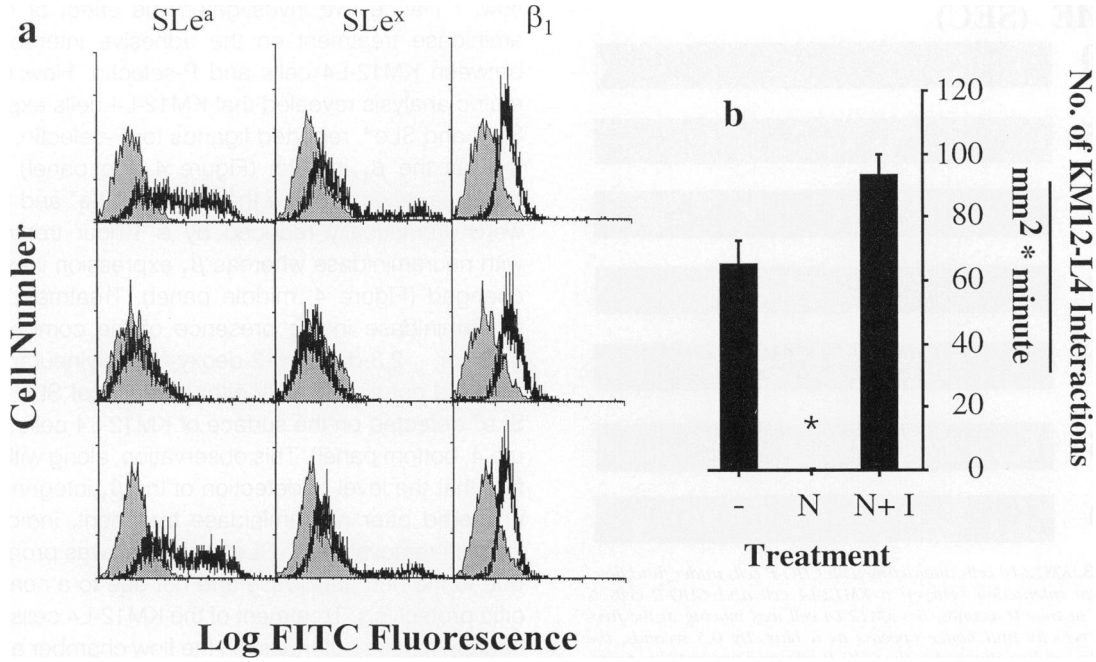

Figure 4. *Sialylated moieties are involved in the adhesive interactions between KM12-L4 cells and P-selectin.* a: *KM12-L4 cells express SLe^a, SLe^x, and the β_1 integrin* (top panels). *The sialylated moieties were removed by treatment with neuraminidase whereas the expression of the β1 integrin was unaffected* (middle panels). *The neuraminidase effect was inhibited by a competitive inhibitor to neuraminidase, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid* (bottom panels). *Open histograms are cells stained with an antibody to the antigen listed at the top of the panels and shaded histograms are cells stained with an isotype-matched control antibody.* b: *Pretreatment of the KM12-L4 cells with neuraminidase abolished the adhesive interactions between KM12-L4 cells and P-selectin. Again, the neuraminidase activity was blocked with the competitive inhibitor to neuraminidase, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. −, negative control; N, cells treated with neuraminidase; N+I, cells treated with neuraminidase and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. Shear stress = 1.5 dynes/cm2; *P < 0.05; n = 4.*

both HL-60 cells and KM12-L4 cells with recombinant P-selectin were completely inhibited by anti-P-selectin MAb HPDG2/3 and were divalent cation dependent (data not shown). KM12-L4 cells and HL-60 cells were treated with OSGE (80 μg/ml for 1 hour at 37°C) before introduction into the flow chamber. Treatment of the KM12-L4 cells with OSGE had no detectable effect on the adhesive interactions between the KM12-L4 cells and P-selectin (Figure 5). In contrast, treatment of the HL-60 cells with OSGE dramatically reduced the adhesion of the HL-60 cells to P-selectin (Figure 5) consistent with recent reports by Patel and colleagues.[19]

PSGL-1 Was Not Detected on KM12-L4 Cells

The above result suggests that the adhesive interactions between KM12-L4 cells and P-selectin is not mediated by mucin-like structures, implying that PSGL-1 is not involved. To test this hypothesis, we assayed for the presence of PSGL-1 on KM12-L4 cells by flow cytometry and for appropriate mRNA transcripts by Northern blot analysis. Flow cytometry did not detect PSGL-1 on the surface of the KM12-L4 cells using either a MAb to PSGL-1, PSL-275 (Figure 6a), or a rabbit polyclonal immune serum to PSGL-1, Rb3026 (data not shown). In addition, L-selectin, which has been shown in some assays to interact with E- and P-selectin,[47] was not detected on the surface of the KM12-L4 cells (Figure 6a). PSGL-1 was detected on the surface of HL-60 cells whereas very little, if any, L-selectin was detected on the HL-60 cells (Figure 6a). All of these antibodies consistently detected their respective antigens on the surface of human monocytes (Figure 6a). Northern blot analysis of total cellular RNA or mRNA did not detect the appropriate 2.5-kb mRNA transcript for PSGL-1 in KM12-L4 cells (Figure 6b, lanes 4 and 2 respectively). In contrast, the appropriate size mRNA transcript for PSGL-1 was detected in HL-60 cells (Figure 6b, lanes 1 and 3) and monocytes (data not shown). Hence, it appears that KM12-L4 cells do not express detectable levels of PSGL-1.

A Function-Blocking MAb to PSGL-1 Did Not Inhibit the Adhesive Interactions between KM12-L4 Cells and P-Selectin under Flow

Anti-PSGL-1 MAb PL1 has been shown to inhibit neutrophil and HL-60 cell adhesion to P-selectin un-

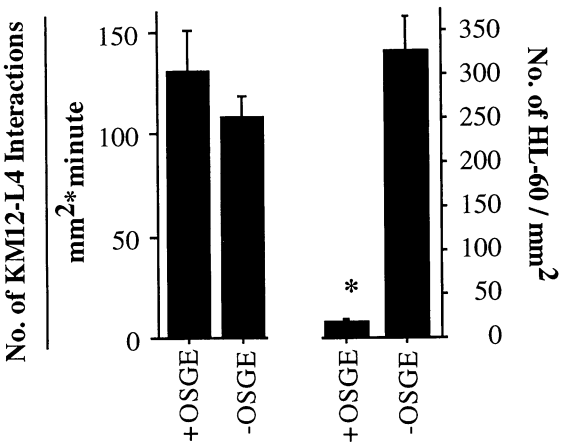

Figure 5. *OSGE treatment of KM12-L4 cells does not affect the level of adhesive interactions observed between KM12-L4 cells and P-selectin under flow. KM12-L4 cells or HL-60 cells were treated with OSGE for 1 hour at 37°C before use in the flow chamber assay. Treatment of KM12-L4 cells with OSGE did not alter KM12-L4 cell interactions with P-selectin under flow. In contrast, treatment of the HL-60 cells with OSGE significantly reduced the adhesion of HL-60 cells to P-selectin under flow. Shear stress = 1.5 dynes/cm²; *P < 0.05; n = 3.*

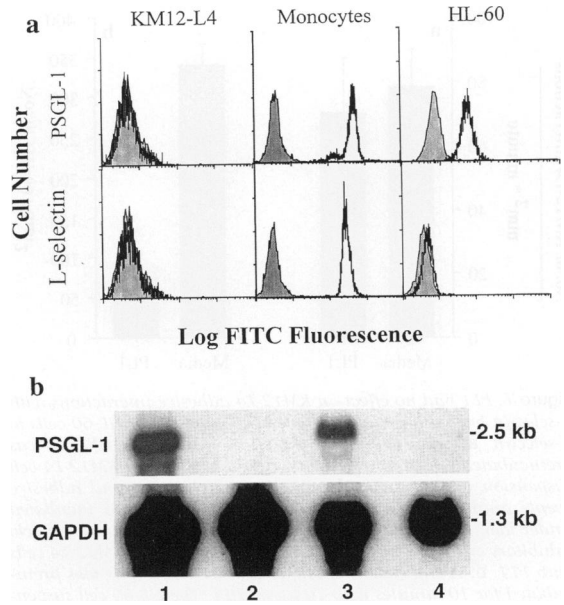

Figure 6. *KM12-L4 cells do not appear to express PSGL-1.* a: *KM12-L4 cells were incubated with a MAb to PSGL-1 (PSL-275) or a MAb to L-selectin (LAM1-5) (open histograms), an isotype-matched control MAb (monocytes and HL-60 cells), or the secondary antibody alone (KM12-L4 cells) (shaded histograms). Neither PSGL-1 nor L-selectin were detected on KM12-L4 cells, whereas both were detected on purified human monocytes. PSGL-1 was also detected on HL-60 cells whereas a very small amount, if any, of L-selectin was detected (n > 4). A polyclonal antibody to PSGL-1 (Rb3026) gave similar results as did several other MAbs to L-selectin, LAM 1–4, LAM1-14, and LAM1-3 (data not shown).* b: *RNA transcripts for PSGL-1 were not detected in mRNA* (lane 2) *or total cellular RNA preparations* (lane 4) *from KM12-L4 cells. However, transcripts were detected in mRNA* (lane 1) *and total cellular RNA* (lane 3) *prepared from HL-60 cells. Similar amounts of glyceraldehyde phosphate dehydrogenase transcripts were observed in each lane.*

der flow conditions.[17,19] Hence, we tested the ability of PL1 to block the interactions of KM12-L4 cells with recombinant P-selectin under flow conditions. PL1 had no inhibitory effect on KM12-L4 cell adhesive interactions with recombinant P-selectin under flow (Figure 7a). In agreement with previous reports,[17,19] however, PL1 significantly (>75%) reduced the adhesion of HL-60 cells to recombinant P-selectin under flow (Figure 7b).

Comparison of KM12-L4 Cell and HL-60 Cell Binding to P-Selectin under Flow

HL-60 cells interact with P-selectin under fluid flow *via* PSGL-1.[17,19] Hence, we compared the KM12-L4 cell adhesive interactions to the HL-60 cell adhesive interactions with P-selectin under identical conditions. For this analysis, we again used full-length recombinant P-selectin, as opposed to the P-selectin-IgG chimera, to avoid potential Fc-receptor-mediated HL-60 cell adhesion. We measured the rate of attachment at three different shear stresses and the rolling velocity at 1.5 dynes/cm^2. The change in the number of cells that interact with the P-selectin-coated surfaces, *Ni*, over time can be expressed mathematically as *dNi/dt* and is equal to the instantaneous rate of attachment, k_a. If k_a varies little with time, integration yields $Ni = k_a t$. Hence, a plot of *Ni versus t* should yield a straight line, the slope of which is equal to the rate of attachment. This analysis was performed for both KM12-L4 and HL-60 cell attachment to P-selectin. To avoid cell depletion of a subpopulation of cells, we made the observations at the P-selectin-coated field of view that was closest to the inlet of the flow chamber.[48] It was typically observed that the number of attachment events increased linearly with time. Hence, linear regression was used to determine k_a. The nominal rate of attachment of KM12-L4 cells was not significantly different from the rate of attachment of HL-60 cells at 1.5 dynes/cm^2 and 2.0 dynes/cm^2 (Figure 8a). In contrast, at 2.5 dynes/cm^2, KM12-L4 cells did appear to have a lower rate of attachment than HL-60 cells ($P < 0.05$). Finally, KM12-L4 cells exhibited a rolling velocity that was significantly higher than that exhibited by the HL-60 cells (Figure 8b).

Discussion

We have used an *in vitro* flow model to examine the adhesive interactions between the KM12-L4 colon carcinoma cell line and P-selectin. The results demonstrate that KM12-L4 cells attach to and roll on recombinant forms of P-selectin adsorbed to glass as well as P-selectin stably expressed by CHO cell

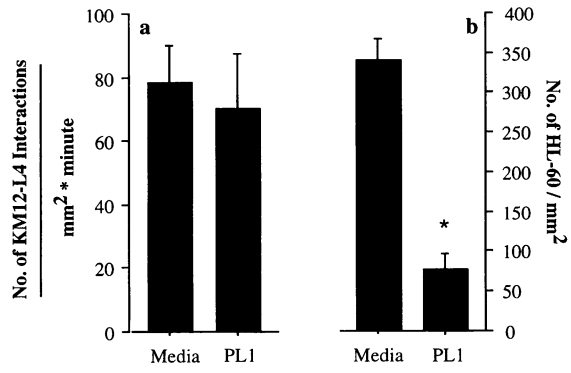

Figure 7. *PL1 had no effect on KM12-L4 adhesive interactions with P-selectin but significantly inhibited the adhesion of HL-60 cells to P-selectin.* a: *A suspension of KM12-L4 cells (1 × 10⁶/ml) was preincubated for 10 minutes with 10 μg/ml PL1. The KM12-L4 cell suspension was introduced into the flow chamber and adhesive events quantified. As a control, KM12-L4 cells were incubated under identical conditions only in the absence of the antibody. No inhibitory effect was observed due to treatment of the KM12-L4 cells with PL1.* b: *A suspension of HL-60 cells (1 × 10⁶/ml) was preincubated for 10 minutes with 10 μg/ml PL1. The HL-60 cell suspension was introduced into the flow chamber and adhesive events quantified. As a control, HL-60 cells were incubated under identical conditions only in the absence of the antibody. Treatment with PL1 significantly inhibited the interactions between the HL-60 cells and P-selectin. Shear stress = 1.5 dynes/cm²; *P < 0.05; n = 2.*

monolayers. A MAb to P-selectin inhibited the adhesive interactions whereas a MAb to E-selectin had no effect, demonstrating the specificity of the interactions. This pair of antibodies had the opposite effect on KM12-L4 cell adhesive interactions with E-selectin. In addition, the adhesive interactions with the recombinant forms of P-selectin required divalent cations and involved sialylated moieties, two hallmarks of selectin-mediated adhesion.[12] Flow cytometric analysis using a P-selectin-IgG chimera provided additional evidence that P-selectin recognized moieties on the surface of KM12-L4 cells. Taken together, these findings suggest that KM12-L4 cells exhibit specific adhesive interactions with P-selectin under flow.

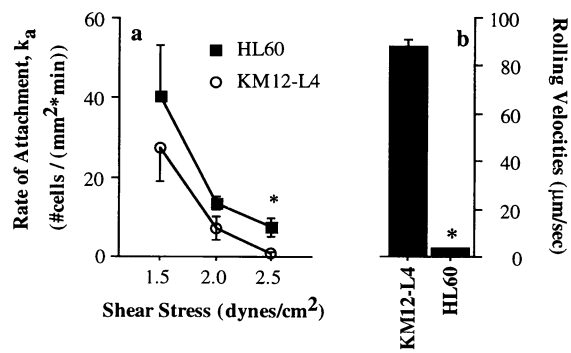

Figure 8. *Attachment and rolling of KM12-L4 cells and HL-60 cells on P-selectin.* a: *The rate of attachment of KM12-L4 cells and HL-60 cells to P-selectin was determined at three different shear stresses. At 1.5 and 2.0 dynes/cm², there was no apparent difference between the rates of attachment for the two cell types. However, at 2.5 dynes/cm², HL-60 cells appeared to have a higher rate of attachment compared with the rate exhibited by the KM12-L4 cells. *P < 0.05; n = 4.* b: *The average rolling velocity for the KM12-L4 and HL-60 cells was determined for >30 cells taken from three separate experiments. The KM12-L4 cells rolled at a significantly higher velocity compared with the rolling velocity observed for HL-60 cells. Shear stress = 1.5 dynes/cm²; *P < 0.05; n = 3.*

PSGL-1 is the only well characterized counter-receptor for P-selectin that has been shown to mediate leukocyte adhesion to P-selectin under fluid flow.[17,19] We present several lines of evidence that demonstrate that PSGL-1 is not involved in the adhesive interactions between KM12-L4 cells and P-selectin. First, PSGL-1-mediated adhesion to P-selectin has been reported to be OSGE sensitive.[19] However, OSGE treatment of the KM12-L4 cells did not significantly alter the level of KM12-L4 cell binding to P-selectin, whereas an identical treatment diminished HL-60 interactions with P-selectin. Second, PSGL-1 was not detected on KM12-L4 cells by flow cytometric analysis using a well characterized polyclonal antibody and MAb to PSGL-1. Third, a 2.5-kb transcript for PSGL-1 was not detected by Northern blot analyses of total cellular RNA or poly-$(A)^+$-purified mRNA derived from KM12-L4 cells. Finally, a MAb to PSGL-1, PL1, known to inhibit PSGL-1-mediated myeloid cell adhesion to P-selectin under flow[17,19] had no effect on the interaction of KM12-L4 cells with P-selectin under flow. These results strongly suggest that KM12-L4 cells express a counter-receptor(s) for P-selectin that is not PSGL-1 but is capable of mediating KM12-L4 cell adhesion to P-selectin under flow.

The neuraminidase used in these studies, derived from *Vibrio cholerae*, cleaves α2-3, α2-6, and α2-8 sialyl linkages and abolished the adhesive interactions between KM12-L4 cells and P-selectin. Hence, it appears that the KM12-L4 cell P-selectin counter-receptor expresses α2-3, α2-6, and/or α2-8 sialyl linkages. However, the specific sialylated structures, ie, SLe^x, SLe^a, or other sialylated moieties, that are actually involved in the binding to P-selectin remain to be determined. As OSGE, an enzyme shown to cleave mucins with many closely spaced *O*-linked oligosaccharides,[42] did not appear to diminish the KM12-L4 cell adhesive interactions with P-selectin, the counter-receptor is probably not such a glycoprotein. Other molecular structures have been reported to bind to P-selectin in equilibrium binding assays including CD24[49] and sulfatides, a class of heterogeneous 3-sulfated galactosyl ceramides.[50] The results with OSGE and neuraminidase presented in this work indicate that these molecules are not good candidates for the KM12-L4 cell P-selectin counter-receptor as CD24 is a mucin[49] and the sulfatides are nonsialylated.[50]

To gain further insight into P-selectin-mediated adhesion under flow, we compared the rate of attachment and rolling velocity of KM12-L4 cells and HL-60 cells under identical conditions of flow. The HL-60 cells rolled at a much lower velocity compared with the KM12-L4 cells and also exhibited a higher rate of attachment at 2.5 dynes/cm^2. Additional calculations that account for the size difference between the HL-60 cells (~12 μm) and KM12-L4 cells (~15 μm) yield similar conclusions. Several parameters may account for the observed differences in dynamic adhesion including the number[29] and the topological distribution of the counter-receptors.[51] In addition, differences in the intrinsic properties of the bonds that mediate the attachment and rolling of the two cell types may lead to the observed differences. For example, if the KM12-L4 counter-receptor-P-selectin bond had a rapid dissociation rate, one would predict that this would result in a high rolling velocity.[28,29]

Intrinsic kinetic properties of the P-selectin-counter-receptor bond will be influenced by the structure of the counter-receptor. It has recently been observed that the high equilibrium binding avidity (ability to survive stringent washing conditions) of PSGL-1 to P-selectin is afforded by SLe^x-type glycosyl structures and an anionic polypeptide segment containing at least one sulfated tyrosine residue present on the amino-terminal portion of PSGL-1.[35,52] In this vein, Zhou et al[53] observed that CHO cells expressing SLe^x exhibited low affinity binding to fluid-phase soluble P-selectin whereas HL-60 cells, which express PSGL-1, exhibited high affinity binding to fluid-phase soluble P-selectin. Hence, the unique structure of PSGL-1 appears to yield high equilibrium binding avidity and affinity. Under fluid flow conditions, perhaps this unique structure affords a high dynamic binding avidity between P-selectin and PSGL-1, which yields high rates of attachment and low rolling velocities as was observed for the HL-60 cells (Figure 8). In contrast, carbohydrate structures presented on a neutral or unevolved counter-receptor may afford a relatively low dynamic binding avidity to P-selectin and hence yield relatively low rates of attachment and high rolling velocities as was observed for the KM12-L4 cells (Figure 8).

Acknowledgments

We thank Drs. Roger McEver and Kevin Moore (University of Oklahoma) for MAb PL1, Drs. Tucker Collins, Margaret Read, Amy Williams, and Jackie Pierce (Brigham and Women's Hospital) for technical assistance in Northern blot analysis, and Dr. Michael Gimbrone for helpful discussions. We thank Dr. Francis Sullivan (Genetics Institute) for the full-length cDNA to PSGL-1 and Drs. Jennifer R. Allport and Daniel M. Greif for review of the manuscript.

References

1. Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994, 76:301–314
2. von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC: Two-step model of leukocyte-endothelial cell interactions in inflammation: distinct roles for LECAM-1 and the leukocyte β_2 integrins *in vivo*. Proc Natl Acad Sci USA 1991, 88:7538–7542
3. Luscinskas FW, Lawler J: Integrins as dynamic regulators of vascular function. FASEB J 1994, 8:929–938
4. Butcher EC: Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. Cell 1991, 67:1033–1036
5. Lawrence MB, Springer TA: Leukocytes roll on a selectin at physiologic flow rates: distinct from and prerequisite for adhesion through integrins. Cell 1991, 65: 1–20
6. Lawrence MB, Springer TA: Neutrophils roll on E-selectin. J Immunol 1993, 151:6338–6346
7. Mayadas TN, Johnson RC, Rayburn H, Hynes RO, Wagner DD: Leukocyte rolling and extravasation are severely compromised in P-selectin deficient mice. Cell 1993, 74:541–554
8. Luscinskas FW, Ding H, Lichtman AH: P-selectin and VCAM-1 mediate rolling and arrest of CD4$^+$ T-lymphocytes on TNF-α-activated vacsular endothelium under flow. J Exp Med 1995, 181:1179–1186
9. Tedder TF, Steeber DA, Pizcueta P: L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. J Exp Med 1995, 181:2259–2264
10. Spertini O, Luscinskas FW, Kansas GS, Griffin JD, Gimbrone MA Jr, Tedder TF: Leukocyte adhesion molecule (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. J Immunol 1991, 147:2565–2573
11. Luscinskas FW, Kansas GS, Ding H, Pizcueta P, Schleiffenbaum BE, Tedder TF, Gimbrone MA Jr: Monocyte rolling, arrest, and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, β_1-integrins, and β_2-integrins. J Cell Biol 1994, 125:1417–1427
12. Carlos TM, Harlan JM: Leukocyte-endothelial adhesion molecules. Blood 1994, 84:2068–2101
13. Walz G, Aruffo A, Kolanus W, Bevilacqua MP, Seed B: Recognition by ELAM-1 of the sialyl-Le^x determinant on myeloid and tumor cells. Science 1990, 250:1132–1135
14. Handa K, Neudelman E, Stroud M, Shiozawa T, Ha-

komori S: Selectin GMP-140 (CD62;PADGEM) binds to sialosyl-Le^x, and sialosyl-Le^a, and sulfated glycans modulate the binding. Biochem Biophys Res Commun 1991, 181:1223–1230
15. Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S, Paulson JC: CD62 and endothelial cell-leukocyte adhesion molecule 1 (ELAM-1) recognize the same carbohydrate ligand, sialyl-Lewis x: Proc Natl Acad Sci USA 1991, 88:6224–6228
16. Moore KL, Eaton SF, Lyons DE, Lichenstein HS, Cummings RD, McEver RP: The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, *O*-linked poly-*N*-acetyllactosamine. J Biol Chem 1994, 269:23318–23327
17. Moore KL, Patel KD, Bruehl RE, Fugang L, Johnson DA, Lichenstein HS, Cummings RD, Dainton DF, McEver RP: P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J Cell Biol 1995, 128:661–671
18. Sako D, Chang X-J, Barone KM, Vachino G, White HM, Shaw G, Veldman GM, Bean KM, Ahern TJ, Furie B: Expression cloning of a functional glycoprotein ligand for P-selectin. Cell 1993, 75:1–20
19. Patel KD, Moore KL, Nollert MU, McEver RP: Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. J Clin Invest 1995, 96:1887–1896
20. Majuri M, Mattila P, Renkonen R: Recombinant E-selectin-protein mediates tumor cell adhesion via sialyl-Le^a, and sialyl-Le^x. Biochem Biophys Res Commun 1992, 182:1376–1382
21. Sawada R, Lowe JB, Fukuda M: E-selectin-dependent adhesion efficiency of colonic carcinoma cells is increased by genetic manipulation of their cell surface lysosomal membrane glycoprotein-1 expression levels. J Biol Chem 1993, 268:12675–12681
22. Goetz DJ, Brandley BK, Hammer DA: An E-selectin-IgG chimera supports sialylated moiety dependent adhesion of colon carcinoma cells under fluid flow. Ann Biomed Eng 1995, 24:87–98
23. Aruffo A, Dietsch MT, Wan H, Hellstrom KE, Hellstrom I: Granule membrane protein 140 (GMP140) binds to carcinomas and carcinoma-derived cell lines. Proc Natl Acad Sci USA 1992, 89:2292–2296
24. Mannori G, Crottet P, Cecconi O, Hanasaki K, Aruffo A, Nelson RM, Varki A, Bevilacqua MP: Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. Cancer Res 1995, 55:4425–4431
25. Stone JP, Wagner DD: P-selectin mediates adhesion of platelets to neuroblastoma and small cell lung cancer. J Clin Invest 1993, 92:804–813
26. Giavazzi R, Foppolo M, Dossi R, Remuzzi A: Rolling and adhesion of human tumor cells on vascular endothelium under physiological flow conditions. J Clin Invest 1993, 92:3038–3044
27. Tozeren A, Kleinman H, Grant D, Morales D, Mercurio A, Byers S: E-Selectin-mediated dynamic interactions of breast-, and colon-cancer cells with endothelial-cell monolayers. Int J Cancer 1995, 60:426–431
28. Alon R, Hammer DA, Springer TA: Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. Nature 1995, 374:539–542
29. Hammer DA, Apte SM: Simulation of cell rolling and adhesion on surfaces in shear flow: general results and analysis of selectin-mediated neutrophil adhesion. Biophys J 1992, 63:35–57
30. Varki A: Selectin ligands. Proc Natl Acad Sci USA 1994, 91:7390–7397
31. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr: Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 1987, 84:9238–9242
32. Vachino G, Chang X-J, Veldman GM, Kumar R, Fouser LA, Berndt MC, Cumming DA: P-selectin glycoprotein ligand-1 is the major counter-receptor for P-selectin on stimulated T cells and is widely distributed in non-functional form on many lymphocytic cells. J Biol Chem 1995, 270:21966–21974
33. Spertini O, Kansas GS, Reimann KA, Mackay CR, Tedder TF: Function and evolutionary conservation of distinct epitopes on the leukocyte adhesion molecule-1 (LAM-1) that regulate leukocyte migration. J Immunol 1991, 147:942–949
34. Hanai N, Shitara K, Furuya A, Yoshida H, Dohi T, Nudelman E, Hakomori S-I, Satoh S: Detailed characterization of reactivities of anti-gastric cancer monoclonal antibodies to carbohydrate antigen. Anticancer Res 1990, 10:1579–1586
35. Sako D, Comess KM, Barone KM, Camphausen RT, Cumming DA, Shaw GD: A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell 1995, 83:323–331
36. Morikawa K, Walker SM, Jessup JM, Fidler IJ: In vivo selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice: Cancer Res 1988, 48:1943–1948
37. Read MA, Neish AS, Luscinskas FW, Palombella VJ, Maniatis T, Collins T: The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity 1995, 2:493–506
38. Lawrence MB, Smith CW, Eskin SG, McIntire LV: Effect of venous shear stress on CD18-mediated neutrophil adhesion to cultured endothelium. Blood 1990, 75: 227–237
39. Goldman AJ, Cox RG, Brenner H: Slow viscous motion of a sphere parallel to a plane wall-II Couette flow. Chem Eng Sci 1967, 22:653–660
40. Abbassi O, Kishimoto TK, McIntire LV, Anderson DC, Smith CW: E-selectin supports neutrophil rolling *in vitro* under conditions of flow. J Clin Invest 1993, 92:2719–2730
41. Alon R, Rossiter H, Springer TA, Kupper TS: Distinct cell surface ligands mediate T lymphocyte attachment

and rolling on P- and E-selectin under physiological flow. J Cell Biol 1994, 127:1485–1495

42. Norgard KE, Moore KL, Diaz S, Stults NL, Ushiyama S, McEver RP, Cummings RD, Varki A: Characterization of a specific ligand for P-selectin on myeloid cells. J Biol Chem 1993, 268:12764–12774
43. Moore KL, Stults NL, Diaz S, Smith DF, Cummings RD, Varki A, McEver RP: Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J Cell Biol 1992, 118:445–456
44. Moore KL, Varki A, McEver RP: GMP-140 binds to a glycoprotein receptor on human neutrophils: evidence for a lectin-like interaction. J Cell Biol 1991, 112:491–499
45. Baumhueter S, Singer MS, Hensel WJ, Hemmerich S, Renz M, Rosen SD, Lasky LA: Binding of L-selectin to the vascular sialomucin CD34. Science 1993, 262: 436–438
46. Sutherland DR, Abdullah KM, Cyopick P, Mellors A: Cleavage of the cell-surface *O*-sialoglycoproteins CD34, CD43, CD44,and CD45 by a novel glycoprotease from *Pasteurella haemolytica*. J Immunol 1992, 148:1458–1464
47. Picker LJ, Warnock RA, Burns AR, Doerschuk CM, Berg EL, Butcher EC: The neutrophil selectin LECAM-1 presents carbohydrate ligands to the vascular selectins ELAM-1 and GMP-140. Cell 1991, 66:921–933
48. Tempelman LA, Hammer DA: Receptor-mediated binding of IgE-sensitized rat basophilic leukemia cells to antigen-coated substrates under hydrodynamic flow. Biophys J 1994, 66:1–13
49. Sammer M, Aigner S, Hubbe M, Schirrmacher V, Schachner M, Vestweber D, Altevogt P: Heat-stable antigen (CD24) as ligand for mouse P-selectin. Int Immunol 1994, 6:1027–1036
50. Aruffo A, Kolanus W, Walz G, Fredman P, Seed B: CD62/P-selectin recognition of myeloid and tumor cell sulfatides. Cell 1991, 67:35–44
51. von Andrian UH, Hassien SR, Nelson RD, Erlandsen SL, Butcher EC: A central role for microvillous receptor presentation in leukocyte adhesion under flow. Cell 1995, 82:989–999
52. Pouyani T, Seed B: PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell 1995, 83:333–343
53. Zhou Q, Moore KL, Smith DF, Varki A, McEver RP, Cummings RD: The selectin GMP-140 binds to sialylated, fucosylated lactosaminoglycans on both myeloid and nonmyeloid cells. J Cell Biol 1991, 115:557–564