Monocyte Adhesion to Activated Aortic Endothelium: Role of L-Selectin and Heparan Sulfate Proteoglycans

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Abstract. This study examines the role of L-selectin in monocyte adhesion to arterial endothelium, a key pathogenic event of atherosclerosis. Using a nonstatic (rotation) adhesion assay, we observed that monocyte binding to bovine aortic endothelium at 4°C increased four to nine times upon endothelium activation with tumor necrosis factor (TNF)-α. mAb-blocking experiments demonstrated that L-selectin mediates a major part (64 ± 18%) of monocyte adhesion. Flow microscopy experiments performed under flow indicated that monocytes abruptly halted on 8-h TNF-α–activated aortic endothelium, ~80% of monocyte attachment being mediated by L-selectin. Flow cytometric studies with a L-selectin/IgM heavy chain chimeric protein showed calcium-dependent L-selectin binding to cytokine-activated and, unexpectedly, unactivated aortic cells. Soluble L-selectin binding was completely inhibited by anti–L-selectin mAb or by aortic cell exposure to trypsin. Experiments with cycloheximide, chlorate, or neuraminidase showed that protein synthesis and sulfate groups, but not sialic acid residues, were essential for L-selectin counterreceptor function. Moreover, heparin lyases partially inhibited soluble L-selectin binding to cytokine-activated aortic cells, whereas a stronger inhibition was seen with unstimulated endothelial cells, suggesting that cytokine activation could induce the expression of additional ligand(s) for L-selectin, distinct from heparan sulfate proteoglycans. Under flow, endothelial cell treatment with heparinase inhibited by ~80% monocyte attachment to TNF-α–activated aortic endothelium, indicating a major role for heparan sulfate proteoglycans in monocyte–endothelial interactions. Thus, L-selectin mediates monocyte attachment to activated aortic endothelium, and heparan sulfate proteoglycans serve as arterial ligands for monocyte L-selectin.

L-selectin plays a major role in the regulation of the inflammatory response by mediating the initial attachment of leukocytes along endothelial cells lining postcapillary venules (4, 42, 43, 44, 85, 89–91). L-selectin shares common structural features with P- and E-selectin, including an NH2-terminal C-type lectin domain, an EGF-like domain, short consensus repeats, a transmembrane domain, and a short cytoplasmic tail (38, 39, 83, 84). L-selectin, which is expressed by most leukocytes (1, 16, 27, 39), supports leukocyte tethering and rolling along vascular endothelium by interacting with carbohydrates presented by specific endothelial cell ligands (38, 41, 42, 53, 79, 84, 89, 90). P-selectin is rapidly expressed by activated platelets and endothelial cells exposed to thrombin or histamine (26, 37, 45, 51, 52). E-selectin is expressed by endothelial cells upon activation by interleukin-1, tumor necrosis factor (TNF)α, or endotoxin (12, 13, 46, 47).

Selectins bind to various carbohydrate ligands (2, 5, 38, 53, 65, 79, 84, 88), most of them containing a lactosamine backbone and carrying sialylated, sulfated, and/or fucosylated sequences. Some complex carbohydrates, such as the tetrasaccharide sialyl Lewisα, are ligands for all three selectins; other carbohydrates interact only with one or two of them (23, 88). Selectins have also been shown to bind to complex sulfated carbohydrates that do not contain sialic acid or fucose residues, for example, heparin, sulfatide, or the HNK-1–reactive sulfoglucuronyl glycolipids (5, 55, 56, 88). Monovalent carbohydrates have low affinity for selectins, and their role in supporting leukocyte rolling is unclear (17, 33, 53). However, when oligosaccharides are presented by a protein backbone, high affinity multivalent interactions can be observed (19, 53, 65, 88). Several glycoproteins have high affinity for selectins. Most of them are sialylated or sulfated mucin-like glycoproteins with many serine and threonine residues that are potential sites for attachment of O-linked glycans. Four mucin-like ligands for L-selectin have been identified on high endothelial venules of mouse lymph nodes: GlyCAM-1, MadCAM-1, CD34, and gp 200, a glycoprotein that has not yet been

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1. Abbreviations used in this paper: BAEC, bovine aortic endothelial cells; PSLG-1, P-selectin glycoprotein ligand-1; TNF, tumor necrosis factor.
cloned (9, 11, 30, 40). GlyCAM-1 is secreted and might serve to modulate L-selectin–mediated attachment of lymphocytes to peripheral lymph node high endothelial venules (15, 40). MadCAM-1 is present on mesenteric lymph nodes as a multifunctional ligand recognized by both αβ7 integrin and L-selectin (11). CD34 is the major ligand for L-selectin in peripheral and mesenteric lymph node high endothelial venules as well as in human tonsil (9, 64). It is also expressed in larger vessels (10) and on hematopoietic cell progenitors (36). However, CD34 function in large blood vessels has not been explored. Sialic acid, fucose, and sulfate residues are required for the function of GlyCAM-1 and CD34 (30, 32). These residues as well as three NH2-terminal tyrosine sulfates have also been reported to be essential for the interaction of P-selectin glycoprotein ligand-1 (PSGL-1) with P-selectin or L-selectin (63, 68, 78, 93).

Although in vitro and in vivo studies support the existence of carbohydrate ligands for L-selectin on activated nonlymphoid vascular endothelium, the identity of these ligands has not been established (34, 35, 42, 43, 44, 48, 71, 73, 76, 77, 85, 89, 90, 92). Staining of calf pulmonary artery endothelial cell line or human umbilical vein endothelial cells with an L-selectin/IgG1 heavy chain chimera has revealed the presence of an intracellular pool of heparin-like ligands for the chimeric protein (57). Additional studies have indicated that L-selectin interacts with heparan sulfate proteoglycans associated with or secreted by cultured endothelial cells (58). However, the capacity of these proteoglycans to support leukocyte attachment to the vascular endothelium has not been examined.

Monocyte attachment to arterial endothelium is considered to be a key event of the early phase of atherosclerosis. However, little information is available on the molecular mechanisms that mediate monocyte–endothelial interactions. Earlier reports have shown that L-selectin is the major receptor for monocyte attachment to activated venous endothelium in nonstatic adhesion assay (76) and under flow conditions (48, 49). The study described here was designed to investigate the role of L-selectin and aortic ligands in mediating monocyte attachment to resting and activated arterial endothelium.

**Materials and Methods**

**Endothelial Cell Culture**

Bovine aortic endothelial cells (BAEC; provided by J.-A. Haefliger, Department of Internal Medicine, University Hospital, Lausanne), isolated by collagenase treatment of bovine aorta, were established as primary cultures (see below). Endothelial cell lines and serially passaged in RPMI 1640 medium (Gibco Laboratories, Europe) were subcloned in the pcDNAI expression vector (Invitrogen, San Diego, CA). A CD4/μ chimera was constructed by substituting the L-selectin

**Monocyte Isolation**

Human monocytes were prepared from blood buffy coats obtained from healthy blood donors. Monocytes were isolated by centrifugation on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) and adherence on gelatine

1% (Sigma Chemical Co., St. Louis, MO) at 37°C. Nonadherent cells were removed by three washes with HBSS (Gibco Laboratories). Adherent cells were then detached with PBS containing 5 mM EDTA and washed again in RPMI 1640 (Gibco Laboratories). The cell suspension obtained by this method contained >95% monocytes as determined by Giemsa stain and immunostaining with phycoerythrin-conjugated anti-CD14 mAb Leu-M3 (Becton Dickinson). L-selectin and CD14 expression by whole blood and isolated monocytes was evaluated by double immunofluorescence (see below). Monocyte isolation caused a 40–50% loss of L-selectin expression. Isolated monocytes were kept on ice and used immediately after isolation.

**mAbs**

Anti-L-selectin mAbs anti–L-AM1-3, anti-11 and anti-VCAM-1 mAb HAE2 (all IgG1) were produced as described (72, 73). mAbs were purified from hybridoma culture supernatants on Affigel protein A (Bio-Rad, Glattbrugg, Switzerland). For cell adhesion–blocking experiments, purified mAb IgG was used at 10 μg/ml. For chimeric protein–binding inhibition experiments, purified mAb IgG was used at 50 μg/ml.

**Monocyte–Endothelial Interactions under Rotation**

Cell attachment assays were carried out under rotation as previously described (73, 76, 77). BAEC, grown to confluence on tissue culture dishes, were stimulated for 8 h with 100 U/ml TNF-α (Boehringer Mannheim, Mannheim, Germany). After washing, cytokine–activated endothelial cells were preincubated for 15 min with medium alone (RPMI 1640/5% FCS) or with medium supplemented with anti-VCAM-1 mAb, L-selectin/μ, or CD4/μ chimeric proteins. Monocytes (4 × 10⁶ cells) were preincubated for 15 min on ice in 120 μl of medium (RPMI 1640/5% FCS) or in medium supplemented with mAbs. Endothelial cell monolayers were washed before adding monocytes. After 30 min of incubation at 4°C under rotation at 72 rpm, nonadherent cells were discarded. Petri dishes were then placed vertically in 2% glutaraldehyde and fixed overnight. The number of adherent monocytes was counted in six to eight microscopic fields (0.5 mm² per field), and the results were expressed as mean ± 1 SD.

**Monocyte–Endothelial Interactions under Flow**

Well-defined laminar flow was produced over confluent endothelial cell monolayers on 25-mm circular glass coverslips introduced in a parallel plate flow chamber (70). Monocytes were suspended at 0.5 × 10³/ml in RPMI 1640 medium and perfused at room temperature (18°C) through the chamber at a shear stress of 1.8 dynes/cm² via a syringe pump (model 22; Harvard Apparatus, Indulab AG, Switzerland). Monocyte–endothelial interactions were visualized using a phase-contrast videomicroscope (Axiovert; Carl Zeiss, Lausanne, Switzerland) and CCD videocamera (model XC-73CE; Sony, Japan) and videotaped (Panasonic s-VHS recorder; TSA Telecom, Lausanne, Switzerland). Endothelial cell monolayers were cultured for 8 h in medium or in medium containing 100 U/ml TNF-α and then treated for 20 min with saturating levels of chimeric proteins. To determine the involvement of endothelial glycosaminoglycans, endothelial monolayers were incubated for 45 min with heparinase I (1,200 mU/ml) or hyaluronidase (200 mU/ml) and then extensively washed with medium. Monocytes were pretreated for 15 min with saturating concentrations of anti-L-selectin mAb at 4°C and then suspended in medium. Stable adhesion was determined between 10 and 12 min of monocyte perfusion by analyzing 12–14 random fields (0.14 mm²/field, ×20 objective). Monocytes were considered as adherent after 20 s of stable contact. The rate of initial attachment was assessed by counting the number of monocytes that interacted with endothelial cell monolayers during the first 5 min of the experiments.

**Production of L-Selectin/μ Chimeric Protein**

The L-selectin/μ chimeric protein was prepared by a method described in detail elsewhere (78). Briefly, sequences encoding the lectin domain, the EGF-like domain, and the first two short consensus repeats of L-selectin were amplified by PCR using synthetic oligonucleotides. An artificial splice donor site was introduced at the 3′ end of the PCR product. The PCR product was then subcloned in a plasmid containing the HIV, CH3, and CH4 domains of IgM heavy chain (μ) in genomic configuration (kindly provided by A. Traunecker, Basel Institute for Immunology, Basel, Switzerland). After digestion with NotI and Xhol, the pl-selectin/μ fragment was subcloned in the pcDNAI expression vector (Invitrogen, San Diego, CA). A CD4/μ chimera was constructed by substituting the L-selectin
coding sequence in pcDNA I L-selectin/µ with a CD4 fragment encoding the first two NH\(^2\)-terminal domains of CD4. Chimeric molecules were produced in COS cells transiently transfected with appropriate cDNAs. Chimeras were used as concentrated COS cell conditioned media or after purification by immunoabsorption to immobilized anti-LAM1-3 mAb (77). The molecular characteristics of L-selectin/µ chimera were analyzed by SDS-PAGE. In reducing conditions, purified L-selectin/µ chimera produced decameric L-selectin/µ chimera migrated as a single band of very high molecular mass remaining at the end of the migration in the 3.75%, SDS-polyacrylamide stacking gel. No additional band of lower molecular mass was observed in the 7.5% SDS-polyacrylamide running gel. The concentration of L-selectin/µ was measured by ELISA as previously described (75, 77). The concentration of CD4/µ chimera was determined by ELISA using goat anti-human IgM heavy chain polyclonal antibody as capture antibody (Vector Laboratories, Inc., Burlingame, CA). The chimeric protein was then detected with biotinylated polyclonal goat anti-human IgM heavy chain antibody (Vector Laboratories, Inc., Burlingame, CA), avidin-HRP (Pierce, Oud-Beijerland, The Netherlands), and O-phenylenediamine (0.125%, wt/vol.; Sigma Chemical Co.) in 0.1 M citrate buffer, pH 4.5, as the substrate. E- or P-selectin/µ chimeric protein concentration was determined using purified L-selectin/µ chimera and purified human IgM as standards. Samples were run in triplicate at 1:500 to 1:5,000 dilutions. Under these conditions, a linear relationship was observed between signal intensity and protein concentration. Absorbance at 490 nm was measured using an ELISA reader (model MR 5000; Dynatech Laboratories, Inc., Chantilly, VA).

Immunofluorescence Analysis

Indirect immunofluorescence analysis was performed using suspended BAEC, which had been detached from plastic flasks with PBS/5 mM EDTA. After three washes in RPMI 1640/1% FCS medium, BAEC were incubated for 30 min at 4°C with L-selectin/µ or CD4/µ chimera. Chimeric protein binding to suspended endothelial cells was revealed using FITC-conjugated rabbit anti-human IgM heavy chain (Dako, Glostrup, Denmark). Flow cytometry was performed using a cytofluorometer (EPICS Profile; Coulter Corp., Hialeah, FL). Cells were gated by forward- and side-scatter signals. 5,000 cells were analyzed in each experiment.

Glycosaminoglycan Characterization

Endothelial cells were incubated with various enzymes for 45 min at 37°C in 25 µl RPMI 1640. Concentration curves were done for each enzyme. Optimal inhibition of L-selectin/µ binding was observed at the chosen enzyme concentrations. Heparinase I (Sigma Chemical Co.) was used at 600 µU, and heparinase II (Seikagaku Corporation, Tokyo, Japan) was used at 4 µU. In other experiments, BAEC were incubated with chondroitinase ABC (200–800 µU; Sigma Chemical Co.) or hyaluronidase (200 µU; Sigma Chemical Co.). In experiments investigating the role of sialic acid, BAEC were incubated with Vibrio cholerae neuraminidase (750 µU/ml; Boehringer Mannheim) or Arthrobacter ureafaciens neuraminidase (200 µU/ml; Oxford Glycosystems, Ltd., Abingdon, UK). At this concentration, neuraminidase completely inhibited CSLEX-1 mAb binding to KG-1 cells treated with this neuraminidase (100 U/ml). The role of sulfate was evaluated by culturing trypsinized BAEC (5 µg/ml trypsin for 30 min at 37°C) for 24 h in RPMI 1640 medium/10% FCS in the presence of 10 mM sodium chloride. In additional experiments, BAEC were cultured with cycloheximide (10 µg/ml) for 30 min before and during TNF-α treatment.

Statistical Analysis

Analysis of variance (ANOVA) and the Bonferroni multiple comparisons test were used to assess statistical significance between the different treatments versus control when three or more groups were analyzed; the Mann-Whitney test was used to compare the median of two unpaired groups, and the Wilcoxon signed rank test was used for paired groups. P values <0.05 were considered significant.

Results

Role of L-Selectin in Mediating Monocyte Adhesion to Cytokine-activated Aortic Endothelium

Monocyte adhesion assays were performed at 4°C under rotation. In these conditions, where L-selectin shedding is minimal and CD18-mediated adhesion is inactive (50, 73, 74, 76), few monocytes attached to unactivated BAEC monolayers (84 ± 20 monocytes/field, mean ± SD, n = 6). When BAEC were activated for 8 h with TNF-α (100 U/ml), a significant increase in monocyte adhesion was observed (four- to ninefold, n = 6). Thus, in the experiment illustrated in Fig. 1, the number of monocytes attached to BAEC increased from 94 ± 10 to 425 ± 33/field upon endothelium activation with TNF-α (Fig. 1, medium).

The mechanism responsible for this observation was investigated with mAbs against L-selectin or VCAM-1. Cell binding inhibition studies revealed that monocyte adhesion to cytokine-activated BAEC monolayers was inhibited by 64 ± 18% (mean ± SD, n = 6, P < 0.005) when monocytes were pretreated with the adhesion-blocking mAb anti-LAM1-3 (Fig. 1) (73, 76). Cell adhesion was not significantly inhibited in experiments with anti-LAM1-10 (not illustrated) or anti–LAM1-11 mAbs (Fig. 1), which recognize nonfunctional domains of L-selectin. A role for VCAM-1 in mediating monocyte attachment to activated BAEC was demonstrated by the capacity of the anti-VCAM-1 mAb HAE-2 to inhibit monocyte adhesion by 38 ± 6% (mean ± SD, n = 3, P < 0.01) (Fig. 1). However, the results with anti-LAM1-3 indicate that L-selectin plays a predominant role in monocyte attachment to cytokine-activated arterial endothelium under nonstatic conditions.

The notion that L-selectin could play a major role in the attachment of monocytes to cytokine-activated arterial endothelium was evaluated further in experiments comparing the effect of L-selectin/µ and CD4/µ chimera on the monocyte-binding capacity of BAEC monolayers. Whereas monocyte binding was not inhibited by pretreatment of BAEC monolayers with CD4/µ (30 µg/ml), strong inhibition (56 ± 0.005) difference in adhesion relative to control. Figure 1. Monocyte attachment to unstimulated or TNF-α-activated aortic endothelium under rotation: inhibition by mAbs. Endothelial monolayers were activated for 8 h with TNF-α (100 U/ml). BAEC were preincubated with medium or anti-VCAM-1 mAb (HAE-2). Monocytes were preincubated with medium, blocking anti-L-selectin mAb anti–LAM1-3 or control mAb anti–LAM1-11. Adhesion assays were carried out under rotation for 30 min at 4°C. Data are expressed as means ± SD. Results are representative of those obtained in six experiments. *P < 0.01. **Statistically significant (P < 0.005) difference in adhesion relative to control.

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Monocyte Adhesion to Activated Aortic Endothelial Cells: Kinetic Analysis

Monocyte adhesion to BAEC was determined under rotation before and after 2, 4, 6, and 8 h of endothelial cell incubation with TNF-α (100 U/ml). A time-dependent increase in monocyte binding was observed up to 6 h after the addition of TNF-α (Fig. 4, solid circles). At ≥2 h of activation, monocyte binding to BAEC was inhibited by 48 to 68% with anti–LAM1-3 mAb (Fig. 4, open circles). With unstimulated BAEC, the inhibition observed with monocytes pretreated with anti–LAM1-3 did not reach statistical significance.

Unstimulated and Cytokine-activated BAEC Express L-selectin Ligands

L-selectin ligand expression by suspended BAEC was detected by flow cytometry. L-selectin/μ being the probe and CD4/μ being the control. L-selectin/μ was found to bind to both unstimulated and cytokine-activated BAEC (Fig. 5, top, solid lines) whereas CD4/μ did not (Fig. 5, top, dotted lines). L-selectin/μ binding to BAEC was completely inhibited by the presence of 5 mM EDTA (Fig. 5, middle) or 100 μg/ml of function-blocking mAb anti–LAM1-3 or anti–LAM1-4, which react with epitopes located on the lectin domain of L-selectin (Fig. 5, bottom). These latter results demonstrate the calcium dependence of L-selectin binding to aortic ligands and the involvement of the L-selectin lectin domain in this reaction.

Because activation of aortic endothelium with TNF-α induced a progressive increase in L-selectin-dependent monocyte adhesion (Fig. 4), L-selectin ligand expression by BAEC was followed over a 24-h period of time. Sur-
prisingly, unstimulated BAEC or BAEC activated by TNF-α (100 U/ml) for 2, 4, 6, 8, or 24 h were found to bind L-selectin/m in a similar fashion (Fig. 6).

L-selectin Binding to BAEC: Different Ligand Characteristics on Unstimulated and Cytokine-activated Endothelial Cells

The role of proteoglycans in supporting L-selectin–endothelial interactions was investigated in experiments examining the effect of glycosidase or trypsin treatment on L-selectin binding to aortic endothelium. As illustrated in Fig. 7, L-selectin binding to unstimulated BAEC was not affected by hyaluronidase (bottom left) or chondroitinase ABC (middle right), whereas it was strongly inhibited by incubation with heparinase I (top right), heparinase I or III (not illustrated), and heparitinase II (middle left), and abrogated by cell exposure to trypsin (bottom right). Importantly, a quite different pattern was observed with BAEC activated by 8 h of incubation with TNF-α (100 U/ml) (Fig. 8). Although trypsin treatment completely inhibited the reaction (Fig. 8, bottom right), activated BAEC exposure to heparinase I, heparitinase II, or heparitinase III only had moderate inhibitory effects on L-selectin binding (Fig. 8, top right and middle). Thus, heparitinase treatment induced a significantly higher decrease in L-selectin/μ binding to unactivated BAEC (mean percentage of decrease ± SD 42 ± 17%, n = 22) than to BAEC exposed for 8 h to TNF-α (26 ± 14%, n = 14, P = 0.005). As observed with unstimulated cells, hyaluronidase and chondroitinase did not inhibit L-selectin binding to activated BAEC (bottom left and middle right).

Heparan sulfate proteoglycans are highly sulfated molecules, and sulfate residues are important for the function of several selectin ligands (30, 32, 63, 68, 78). The role of sulfate residues in L-selectin–BAEC interactions was assessed by experiments using unactivated or TNF-α–activated BAEC cultured for 24 h in the presence of 10 mM sodium chlorate, an inhibitor of sulfate synthesis (7). As shown in Fig. 9, inhibition of sulfation inhibited most L-selectin binding to both unstimulated and cytokine-activated BAEC (bottom).

Cycloheximide treatment also strongly inhibited L-selectin binding, indicating that protein synthesis is required for ligand(s) expression by both unactivated and cytokine-activated endothelium (Fig. 9, middle).

Intact sialic acid residues are required for interactions between L-selectin and mucinlike glycoproteins such as GlyCAM-1, CD34, or PSGL-1. To assess whether sialic acid residues are involved in L-selectin binding to aortic endothelium, BAEC were pretreated for 45 min with V. cholerae (750 mU/ml) or A. ureafaciens neuraminidase (200 mU/ml) before incubation with L-selectin/μ chimera (Fig. 9, Vibrio Cholerae). Endothelial cell exposure to neuraminidase did not significantly affect L-selectin/μ binding to unactivated BAEC. Thus, 63 ± 15% (n = 8) of BAEC treated with V. cholerae neuraminidase bound L-selectin/μ, whereas 47 ± 26% (n = 8) of untreated cells bound the chimera. Similarly, L-selectin/μ binding to activated BAEC was not affected by neuraminidase. L-selectin/μ,
bound to 57 ± 19% (n = 6) of untreated cells and to 59 ± 16% (n = 6) of neuraminidase-treated cells. In contrast, monocyte exposure to *V. cholerae* neuraminidase (100 mU/ml) abolished L-selectin/PSGL-1 binding to monocyte PSGL-1 (not illustrated) (78).

**Role of Heparan Sulfates in Monocyte Adhesion to Cytokine-activated Endothelium**

The role of heparan sulfates in supporting monocyte attachment to TNF-α-activated BAEC was studied by preincubating endothelial monolayers with heparinase I before monocyte addition. Adhesion assays performed under rotation after the addition of heparinase I indicated that heparan sulfates support monocyte attachment to 8-h TNF-α-activated BAEC. Monocyte adhesion to cytokine-activated aortic endothelium was reduced by 36 ± 11% (mean ± SD, n = 4, P < 0.01) using BAEC monolayers preexposed to heparinase I; BAEC pretreatment with *V. cholerae* neuraminidase (750 mU/ml, 45 min at 37°C) did not significantly inhibit monocyte binding (inhibition of L-selectin/µ binding −8 ± 6%, n = 3) (not illustrated). In control experiments in which monocytes were preincubated with anti–LAM1-3 mAb, monocyte attachment to TNF-α-activated BAEC monolayers was inhibited by 64 ± 18% (P < 0.005).

Additional experiments were performed to examine the contribution of heparan sulfate proteoglycans in mediating primary monocyte adhesion to activated endothelial monolayers under flow. Monocyte attachment was very significantly affected by the pretreatment of endothelial monolayers with heparinase I. At 1.8 dynes/cm², the total number of interacting monocytes (primary adhesion) during the first 5 min of the videotaped experiments was significantly reduced (P < 0.001). Thus, 304 ± 43 monocytes/mm² (mean ± SD, n = 3) interacted with activated endothelium, whereas 854 ± 72 interacting monocytes/mm² (mean ± SD, n = 3) were observed with untreated endothelium. The number of stably adherent monocytes was also considerably inhibited by the pretreatment of activated endothelium with heparinase I. Adherent monocytes were counted during the last 2 min of the 12-min experiments. Stable monocyte adhesion was reduced by 88 ± 6% (mean ± SD, n = 4, P < 0.001) after the pretreatment of endothelial monolayers with heparinase I (Fig. 10). Similar inhibition was obtained by treating monocytes with the function-blocking mAb anti–LAM1-3 (83 ± 8%), whereas the control anti–L-selectin mAb anti–LAM1-11 had no significant inhibitory effect.

**Discussion**

The following observations were made in this study: (a) L-selectin plays a major role in monocyte adhesion to TNF-α-activated aortic endothelial cells; and (b) heparan...
sulfate proteoglycans and possibly other protein-based ligands function as arterial counterreceptors for monocyte L-selectin. These findings provide novel information on the molecular mechanisms of monocyte attachment to activated arterial endothelium, a key cellular reaction in the initial lesion of atherosclerosis.

Cell adhesion assays performed under rotation have previously shown that L-selectin plays a major role in initiating monocyte attachment to cytokine-activated venous endothelium in vitro (76). Subsequently, experiments made with an in vitro flow system have confirmed that L-selectin has a crucial role in initiating monocyte attachment, supporting monocyte rolling, and facilitating α4β1-integrin–dependent arrest (48, 49). Thus, interactions between monocytes and venous endothelial cells seem to involve L-selectin–dependent monocyte rolling on the endothelial cell surface, followed by sequential involvement of β1 integrin, β2 integrin, and CD31 (PECAM-1) in subsequent steps of monocyte migration into tissues. In this study, we observed under rotating conditions that L-selectin plays a major role in mediating monocyte attachment to activated arterial endothelium. Involvement of L-selectin was demonstrated by experiments showing that adhesion-blocking anti–L-selectin mAbs LAM1-3 and LAM1-4 had the capacity to inhibit monocyte binding to activated aortic endothelium, whereas this reaction was not inhibited by anti–LAM1-11 and anti–LAM1-10 mAbs, which recognize domains of L-selectin not involved in cell adhesion (Fig. 1).

Further support for the notion that monocytes are attached to arterial endothelium via L-selectin was provided by experiments showing the capacity of L-selectin/μ to inhibit monocyte–endothelial interactions (Fig. 2). Equivalent inhibitions were obtained by preincubating activated aortic cell monolayers with L-selectin/μ or by treating monocytes with mAb LAM1-3, indicating that L-selectin/μ had the capacity to completely inhibit L-selectin–dependent cell adhesion. Under the same conditions, CD4/μ had no inhibitory effect on monocyte binding to activated aortic endothelium (Fig. 2).

The cellular and molecular bases of monocyte attachment were further analyzed using an in vitro flow chamber using function-blocking mAb and chimeric molecules. Observations made in videomicroscopy experiments showed that freely flowing monocytes abruptly halted on 8-h TNF-

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**Figure 8.** Interaction of L-selectin with suspended aortic endothelial cells: effect of treating TNF-α–activated BAEC (8 h, 100 U/ml) with heparinase I, heparitinase II, chondroitinase ABC, hyaluronidase, or trypsin. Unactivated BAEC were examined by indirect immunofluorescence analysis with L-selectin/μ (solid lines) and CD4/μ (dotted lines). Identical results were obtained by treating BAEC with heparinase I, II, or III. The data are representative of six experiments. Percentages of BAEC that bound to L-selectin/μ are as follows: control, 87%; heparinase I, 39%; heparitinase II, 47%; chondroitinase, 89%; hyaluronidase, 82%; trypsin, 4%.

**Figure 9.** Interaction of L-selectin with suspended aortic endothelial cells: effect of treating unstimulated or TNF-α–activated BAEC (6 h, 100 U/ml) with cycloheximide (10 μg/ml), V. cholerae neuraminidase (750 U/ml), or sodium chlorate (10 mM, 24 h). BAEC were examined by indirect immunofluorescence analysis with L-selectin/μ (solid lines) and CD4/μ (dotted lines). The data are representative of six experiments. Percentages of unactivated BAEC that bound to L-selectin/μ are as follows: control, 86%; cycloheximide, 22%; V. cholerae, 91%; chlorate, 13%. Percentages of TNF-α–activated BAEC that bound to L-selectin/μ are as follows: control, 77%; cycloheximide, 11%; V. cholerae, 89%; chlorate, 17%.
endothelium in acute rejection of rabbit cardiac allograft

lier studies reporting expression of that receptor on aortic
recruitment at the vascular endothelial cell surface.

thelial ligand(s) is an important mechanism of monocyte
TNF. Anti–VCAM-1 mAb HAE-2 (73) inhibited 38% of
port monocyte attachment to activated arterial endothe-
shown that L-selectin cooperates with VCAM-1 to sup-

Figure 10. Inhibition of monocyte adhesion to 8-h TNF-α–acti-
ated endothelium under flow (wall shear stress estimated at 1.8
dynes/cm²). Endothelial monolayers were activated for 8 h with
TNF-α (100 U/ml), washed, and then preincubated for 45 min
at 37°C with heparinase I (1,600 mU/ml) or hyaluronidase (200
mU/ml). The adhesion assay was performed in a flow chamber,
and adherent monocytes were counted as described in the legend
to Fig. 3. Data are expressed as means ± SD. Results are repre-
sentative of three experiments. **P < 0.001.

α–activated aortic endothelium primarily through L-selec-
tin. mAb blockade of L-selectin inhibited by ~80% mono-
cyte attachment to TNF-α–activated endothelium (Fig. 3).
These observations are consistent with those describing,
under flow, monocyte interactions with 6-h TNF-α–acti-
vated human umbilical vein endothelial cells (49), and
they are the first to show that L-selectin mediates mono-
cyte attachment to activated aortic endothelium. Because
recent studies reported that neutrophils can roll on al-
ready adherent neutrophils (8, 22, 78), L-selectin–mediated
monocyte primary adhesion to activated endothelium was
examined during the first 5 min of each experiment, when
the number of stably adherent monocytes is low. Careful
analysis of the video records showed that flowing mono-
cytes occasionally slowed down and arrested on adherent
monocytes, facilitating their attachment to activated endo-
thelium. These interactions were discarded for quantita-
tive analysis. Only single-cell interactions with activated
endothelium were taken into consideration. The strong in-
hibition of monocyte attachment to activated endothelium
induced by the pretreatment of endothelium with L-selec-
tin/μ further indicated that L-selectin interaction with endo-
thelial ligand(s) is an important mechanism of monocyte
recruitment at the vascular endothelial cell surface.

Interestingly, studies with adhesion-blocking mAbs have
shown that L-selectin cooperates with VCAM-1 to sup-
port monocyte attachment to activated arterial endothe-
lium. Anti–VCAM-1 mAb HAE-2 (73) inhibited 38% of
monocyte adhesion (Fig. 1). The induction of VCAM-1 by
TNF-α observed here is consistent with results from ear-
lier studies reporting expression of that receptor on aortic
endothelium in acute rejection of rabbit cardiac allograft
(82) or after balloon injury of the aorta (81). In addition,
several studies have reported that VCAM-1 is expressed
on atherosclerotic lesions, suggesting that VCAM-1 could
play a critical role in regulating monocyte entry into the
arterial wall (20, 59). Because this study has identified
L-selectin as a major mediator of monocyte attachment to
cytokine-activated arterial endothelium, it will be important
to assess in subsequent work the extent to which L-selectin
is also involved in regulating monocyte entry into ather-
sclerotic lesions. Clearly, a detailed elucidation of the mo-
lecular mechanisms involved in monocyte attachment to
the arterial wall will be required to understand how ath-
erosclerotic plaques are formed and to generate drugs that
may have the capacity to inhibit the formation of these
lesions.

Adhesion-blocking mAb studies have previously sug-
gested that an inducible ligand for monocytes is expressed
on human umbilical vein endothelial cells upon activation
with TNF-α (34, 48, 49, 76). Other investigators have re-
ported that additional endothelia can also express cyto-
kine-inducible ligands (14). Here, the progressive increase
in L-selectin–mediated monocyte adhesion observed after
activation of endothelial cells with TNF-α suggested again
that inducible ligands for L-selectin are expressed on acti-
vated aortic endothelial cells (Fig. 4). The nature of these
ligands was probed by experiments examining the binding
of soluble recombinant L-selectin/μ to live endothelial
cells. Considering that multivalency could be an important
factor in selectin function (65), we used a decameric form
of L-selectin instead of a dimeric chimera to improve the
detection of L-selectin ligands. Surprisingly, soluble L-selec-
tin/μ was also found to bind to unactivated aortic endothe-
ial cells (Fig. 5). This result was unexpected because un-
activated endothelium supported only little monocyte
binding (Figs. 1–3). The specificity of L-selectin/μ binding
to aortic cells was established using EDTA or function-
blocking mAb anti–LAM1-3 or anti–LAM1-4, which com-
pletely inhibited L-selectin binding, whereas control anti–
L-selectin mAbs had no effect on this reaction. Activation
of endothelial cells by TNF-α had little influence on L-selec-
tin/μ binding (Figs. 5 and 6). Endothelial cell treatment
with various glycosaminoglycan-cleaving enzymes demon-
strated that ligands expressed on both unactivated and ac-
tivated aortic endothelium were sensitive to heparinase I
and heparitinase II (Figs. 7 and 8). In addition, binding of
L-selectin to aortic endothelium was completely abolished
by trypsin, which indicates that L-selectin binds to heparan
sulfate chains attached to protein in the form of proteogly-
cans. Importantly, the reactivity of L-selectin with cytokine-
activated aortic cells was only partially susceptible to hepa-
rinase I and heparitinase II digestion. This latter observation
suggests that cytokine activation could induce the expres-
sion of additional ligands, distinct from heparan sulfate
proteoglycans that interact with L-selectin to support mono-
cyte adhesion. Alternatively, TNF-α could increase mono-
cyte adhesion to endothelium by modifying heparan sulfate
proteoglycan glycosylation or sulfation. This mechanism
could induce expression of L-selectin–binding sequences
responsible for high affinity interactions between L-selectin
t and cytokine-activated aortic cells; these sequences
would not be expressed on unstimulated arterial endothe-
lum. An additional option is that the arterial endothelial
ies demonstrated that a major part of leukocyte–leukocyte already adherent leukocytes (3, 8, 25, 60, 73). Several studies have shown that additional ligands distinct from E-selectin could be involved in initiating monocyte attachment through L-selectin. E-selectin and heparan sulfate could cooperate with heparan sulfate proteoglycans to mediate monocyte attachment to endothelial cells (6, 61, 78). In addition, L-selectin expressed by monocyte attachment to activated endothelial cells. Expression of this adhesion molecule has been observed on monocyte rolling along endothelium, whereas less abundant high affinity ligands could be required to allow monocyte arrest. The increase in L-selectin–dependent monocyte adhesion observed after activation of BAEC with TNF-α (Fig. 4) could be explained by the expression of ligands not present on unstimulated BAEC.

E-selectin is an inducible high affinity ligand that could cooperate with heparan sulfate proteoglycans to mediate monocyte attachment to activated endothelial cells. Expression of this adhesion molecule has been observed on endothelial cells lining atherosclerotic lesions and in rabbits fed a hypercholesterolemic diet (24). PSGL-1 interacts with E-selectin to mediate monocyte attachment to endothelial cells (6, 61, 78). In addition, L-selectin expressed by human neutrophils binds to E-selectin through a carbohydrate ligand expressed by the lectin domain of L-selectin (62). This latter interaction was studied in a control shear adhesion assay by Lawrence et al. (41) and others (61), who observed an L-selectin–dependent neutrophil tethering to E-selectin. E-selectin and heparan sulfate could cooperate to mediate monocyte attachment to activated endothelium. Further studies will be required to determine if additional ligands distinct from E-selectin could be involved in initiating monocyte attachment through L-selectin to activated endothelium. Finally, leukocyte recruitment in inflammatory lesions is not only dependent on the interaction of neutrophils with endothelial cells but could be considerably increased by the rolling of leukocytes on already adherent leukocytes (3, 8, 25, 60, 73). Several studies demonstrated that a major part of leukocyte–leukocyte interactions is regulated by L-selectin and its ligand PSGL-1 (8, 28, 60, 78, 87). Thus, L-selectin is critically involved in promoting leukocyte recruitment at the site of inflammation by its capacity to regulate leukocyte interactions with endothelial cell surface and leukocyte attachment to already adherent leukocytes.

The strong inhibition by cycloheximide of L-selectin ligand expression by unstimulated and cytokine-activated aortic endothelium indicated that protein synthesis is required for L-selectin binding (Fig. 9). Heparan sulfate proteoglycans involved in L-selectin binding are probably renewed in a continuous fashion. Earlier reports on heparan sulfate proteoglycans have indicated that these species have half-lives of between 3 and 8 h at the endothelial cell surface, removal from the cell surface resulting from proteoglycan endocytosis and shedding into the extracellular space (94).

The role of sulfates on L-selectin binding to aortic endothelium was evaluated because sulfate residues were found to be necessary for the function of several selectin ligands (9, 11, 29, 30, 40, 63, 68, 88, 93). Inhibition of ATP–sulfurylase by chlorate (7) prevented most soluble L-selectin binding, demonstrating that sulfation is critical for the interaction of L-selectin with arterial endothelial cell ligands (Fig. 9). Inhibition of sulfation could abolish the interaction of L-selectin with highly sulfated molecules, like heparan sulfate, thereby inhibiting most L-selectin reactivity with BAEC. It is also possible that other unidentified sulfated ligands interact with L-selectin to support monocyte adhesion to activated aortic endothelium.

Several glycoprotein ligands for selectins require sialic acid residues for function. In the present study, digestion of activated and unactivated aortic endothelium with neuraminidase did not affect significantly L-selectin binding or monocyte attachment, under rotation, to aortic endothelium. In this regard, aortic endothelium L-selectin ligands behave quite differently from GlyCAM-1, CD34, or PSGL-1 (9, 18, 31, 40, 54, 67, 86). The lack of effect of neuraminidase treatment on monocyte adhesion and on L-selectin/μ binding to BAEC suggests that sialic acid residues could not be essential for L-selectin ligand function. However, this result must be cautiously interpreted because we cannot exclude that a subset of sialic acid residues resistant to enzymatic cleavage could play a role in L-selectin binding.

Norgard-Sumnicht et al. (57, 58) have previously reported the presence of heparan sulfate in a calf pulmonary artery endothelial cell line (American Type Culture Collection CCL 209). However, staining of this cell line with an L-selectin/IgG1 heavy chain chimera revealed the presence of an intracellular pool of heparan sulfate but no significant surface expression of the ligand (57). Here, using a decameric L-selectin chimera, we show that heparan sulfate proteoglycans are expressed at the surface of aortic endothelial cells and play a major role in L-selectin–dependent attachment of monocytes to TNF-α–activated aortic endothelium (Fig. 10). Moreover, endothelial monolayer treatment with heparinase I inhibited monocyte adhesion to activated endothelial monolayers. Future studies will be aimed at identifying and characterizing heparan sulfate proteoglycans involved in L-selectin endothelial cell interactions and the additional ligand(s) that may cooperate with heparan sulfate proteoglycans to support monocyte adhesion. It is possible that heparan sulfate expressed by arte-
rial endothelium has L-selectin–specific recognition sequences that are not present on heparan sulfate extracted from bovine intestinal mucosa. Indeed, Diamond et al. (21), using a flow system, did not observe interactions between L-selectin and bovine intestinal mucosa heparan sulfate.

The in vitro observation that heparan sulfate proteoglycans are ligands for L-selectin and mediate monocyte attachment to activated aortic endothelium has to be extended by in vivo studies. The identification of specific sequences responsible for the interaction of L-selectin with sulfated glycosaminoglycans may lead to the preparation of heparan sulfate analogues with the potential of inhibiting pathological leukocyte recruitment in inflammatory diseases. The ability of some heparin oligosaccharides to inhibit leukocyte migration at sites of inflammation suggests that this approach might have therapeutic potential (56, 58).

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