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 attachment. Videomicroscopy 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 NH_2-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)[1]-α, or endotoxin (12, 13, 46, 47).

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1. *Abbreviations used in this paper*: BAEC, bovine aortic endothelial cells; PSGL-1, P-selectin glycoprotein ligand-1; TNF, tumor necrosis factor.

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 Lewis^x, 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 mucinlike 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

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 α4β7 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 NH_2-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. Haeffliger, Department of Internal Medicine, University Hospital, Lausanne), isolated by collagenase treatment of bovine aorta, were established as primary culture and serially passaged in RPMI 1640 medium (Gibco Laboratories, Paisley, Scotland) supplemented with 10% FCS (Gibco Laboratories). For adhesion studies, BAEC (passages 3–5) were plated on 60 × 15-mm tissue culture dishes (Becton Dickinson, Basel, Switzerland) and grown within a 2-cm-diam circle delineated by a ring of 12 M polysiloxane as described elsewhere (72, 73). For use in the parallel flow chamber, BAEC were plated on 25-mm circular glass coverslips (Bellco Glass, Inc., Vineland, NJ). Because a lower reactivity of BAEC with L-selectin/μ chimera was observed with endothelial passages >8, only passages 3–5 were used to perform immunofluorescence analysis or adhesion assays.

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 adhesion 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 and flow cytometry (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–LAM1-3, -4, and -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 (4×10^6 cells) were preincubated for 15 min on ice in 120 μl of medium (RPMI 1640/5% FCS) or in medium supplemented with mAb. 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% glutaraldhehyde and fixed overnight. The number of adherent monocytes was counted in six to eight microscopic fields (0.5 mm^2 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 0.5×10^6/ml in RPMI 1640 medium and perfused at room temperature (18°C) through the chamber at a shear stress of 1.8 dynes/cm^2 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 mm^2/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 CH2, 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 XhoI, 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 immunoadsorption 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 migrated with molecular masses ranging from 95,000 to 110,000 daltons. In nonreducing conditions, the 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.), avidin–HRP (Pierce, Oud-Beijerland, The Netherlands), and *O*-phenylendiamine (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 cytofluorimeter (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 mU, and heparitinase II (Seikagaku Corporation, Tokyo, Japan) was used at 4 mU. In other experiments, BAEC were incubated with chondroitinase ABC (200–800 mU; Sigma Chemical Co.) or hyaluronidase (200 mU; Sigma Chemical Co.). In experiments investigating the role of sialic acid, BAEC were incubated with *Vibrio cholerae* neuraminidase (750 mU/ml; Boehringer Mannheim) or *Arthrobacter ureafaciens* neuraminidase (200 mU/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 chlorate. 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 ±

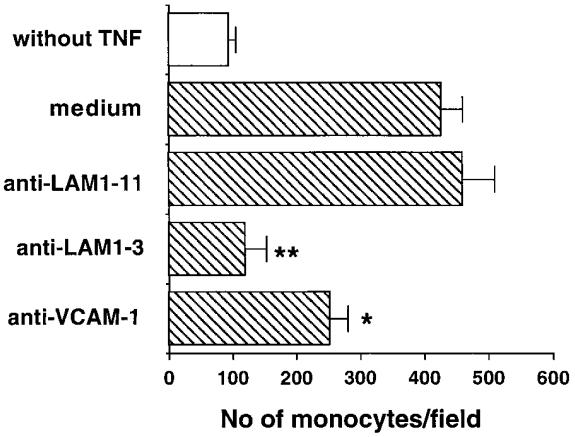

Figure 1. Monocyte attachment to unstimulated or TNF-α–activated aortic endothelium under rotation: inhibition by mAbs. Endothelial monolayers were activated for 8 h with TNF-α (100 U/ml). BAEC were preincubated with medium or anti–VCAM-1 mAb (HAE-2). Monocytes were preincubated with medium, blocking 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.

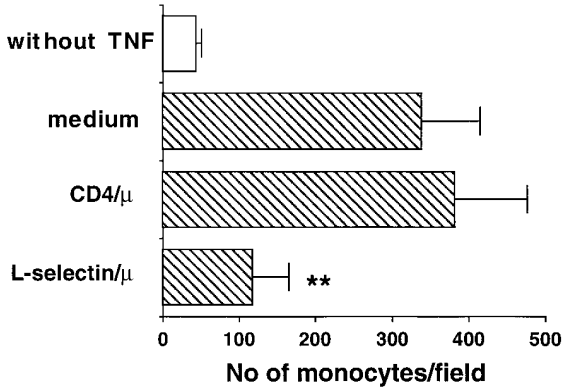

Figure 2. Monocyte attachment to unstimulated or TNF-α–activated aortic endothelium under rotation: inhibition by chimeric proteins. Endothelial monolayers were activated for 8 h with TNF-α (100 U/ml). Unstimulated and TNF-activated BAEC were then preincubated with medium, L-selectin/μ, or CD4/μ. Adhesion assays were carried out under rotation for 30 min at 4°C. Data are expressed as means ± SD. Results are representative of three experiments. $^{**}P < 0.005$.

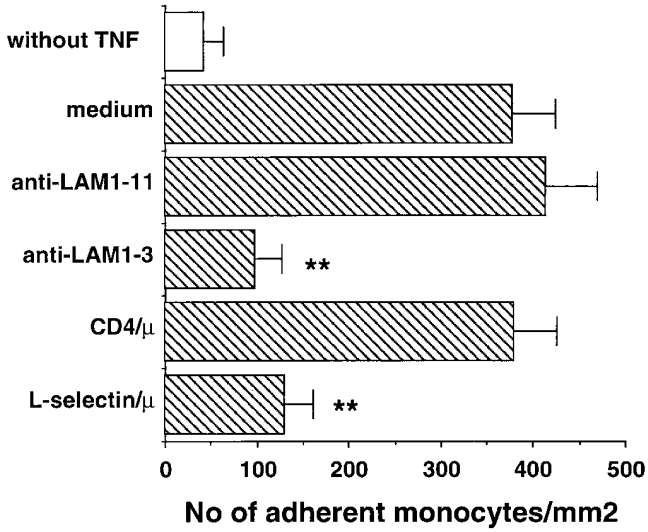

Figure 3. Monocyte adhesion to 8-h TNF-α–activated aortic endothelium under flow: inhibition by anti–L-selectin mAb LAM1-3 or L-selectin/μ chimera. After 20 min of preincubation with L-selectin/μ or CD4/μ chimera, BAEC monolayers were washed and inserted into the flow chamber. Untreated monocytes or monocytes preincubated in medium or with anti–LAM1-3 or anti–LAM1-11 mAb at 4°C were perfused across the monolayers at 1.8 dynes/cm^2 wall shear stress. Data are expressed as means ± SD. Stably attached cells (>20 s) were counted in 13 random fields after 10 to 12 min of perfusion (rolling and transiently interacting cells were not included). Results are representative of four experiments. **Statistically significant ($P < 0.001$) difference in adhesion relative to control.

9%, $n = 3$, $P < 0.005$) was observed when monolayers were preincubated with L-selectin/μ (50 μg/ml) (Fig. 2).

The role of L-selectin in mediating monocyte primary adhesion was further examined in a parallel flow chamber at a shear stress of 1.8 dynes/cm^2 (70). All monocytes interacting with endothelial monolayers during the first 5 min of the experiment were counted. Most of these cells were rolling before being abruptly halted and becoming stably adherent or detaching. The inhibition of L-selectin with the function-blocking mAb anti–LAM1-3 reduced monocyte primary adhesion to 8-h TNF-α–activated endothelium by 78 ± 12% (mean ± SD, $n = 4$). Thus, after pretreatment with anti–LAM1-3 mAb, only 209 ± 44 monocytes/mm^2 (mean ± SD, $n = 4$) interacted with activated endothelium, whereas 970 ± 84/mm^2 interacting monocytes/mm2 were observed after pretreatment with the nonblocking anti–LAM1-11 mAb (not illustrated). Pretreatment of endothelial cells with L-selectin/μ similarly reduced monocyte primary adhesion by 65 ± 14% (mean ± SD, $n = 3$). In contrast, no inhibition was observed when endothelium was pretreated with the control chimeric protein CD4/μ. The number of stably adherent monocytes at the end of the 12-min flow experiments was strongly reduced by pretreating monocytes with the anti–LAM1-3 mAb (83 ± 8%; mean ± SD, $n = 4$) or endothelial cell monolayers with L-selectin/μ chimera (71 ± 9%; mean ± SD, $n = 3$). In the experiment illustrated in Fig. 3, 97 ± 30 (mean ± SD, $n = 13$) adherent monocytes/mm^2 were observed after monocyte preincubation with anti–LAM1-3, and 413 ± 56 monocytes/mm^2 were observed after pretreatment with anti–LAM1-11 mAb; in the same experiment, 130 ± 31 (mean ± SD, $n = 13$) monocytes/mm^2 adhered to activated endothelium pretreated with L-selectin/μ, whereas 380 ± 45 monocytes adhered to monolayers pretreated with CD4/μ (50 μg/ml).

Monocyte Adhesion to Cytokine-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-

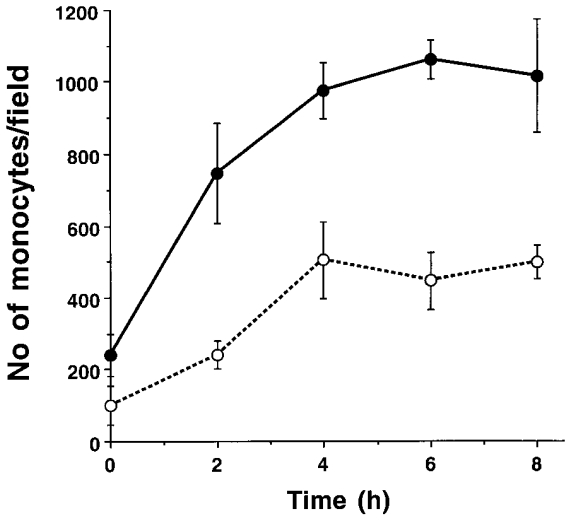

Figure 4. Kinetics of monocyte attachment to TNF-α–activated aortic endothelium under rotation. Endothelial monolayers were stimulated with TNF-α (100 U/ml) for 0–8 h at 37°C before the addition of monocytes. The L-selectin–dependent component of monocyte adhesion was determined using function-blocking mAb anti–LAM1-3. Adhesion assays were performed under rotation for 30 min at 4°C. Solid circles represent adhesion of untreated monocytes. Open circles represent adhesion of monocytes pretreated with anti–LAM1-3 mAb. Data are expressed as means ± SD. Results are representative of two experiments.

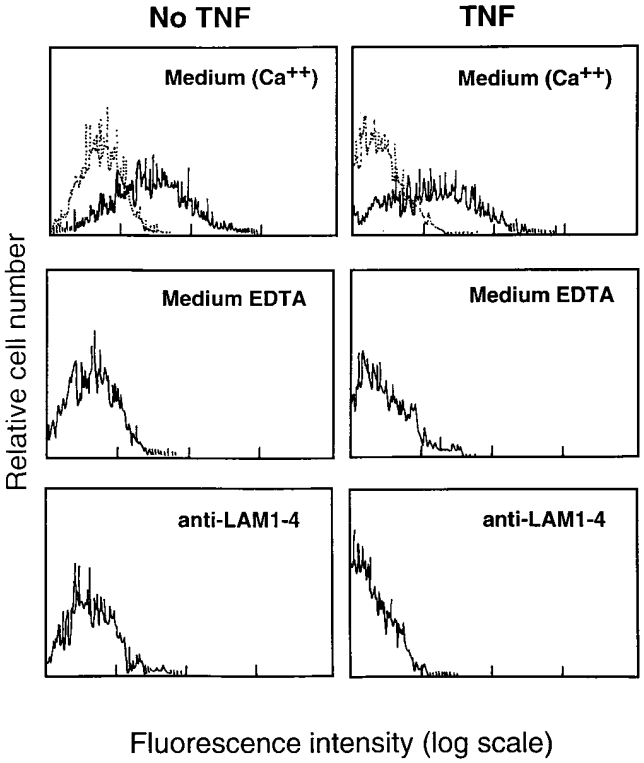

Figure 5. Interaction of L-selectin with suspended aortic endothelial cells. Unactivated or TNF-α–activated BAEC (8 h, 100 U/ml) were examined by indirect immunofluorescence analysis for the expression of L-selectin ligands. L-selectin/μ was used as the probe (*solid lines*), and CD4/μ, an isotype-matched chimeric protein, was used as the control (*dotted lines*). The data are representative of three experiments.

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/μ 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, heparinase 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

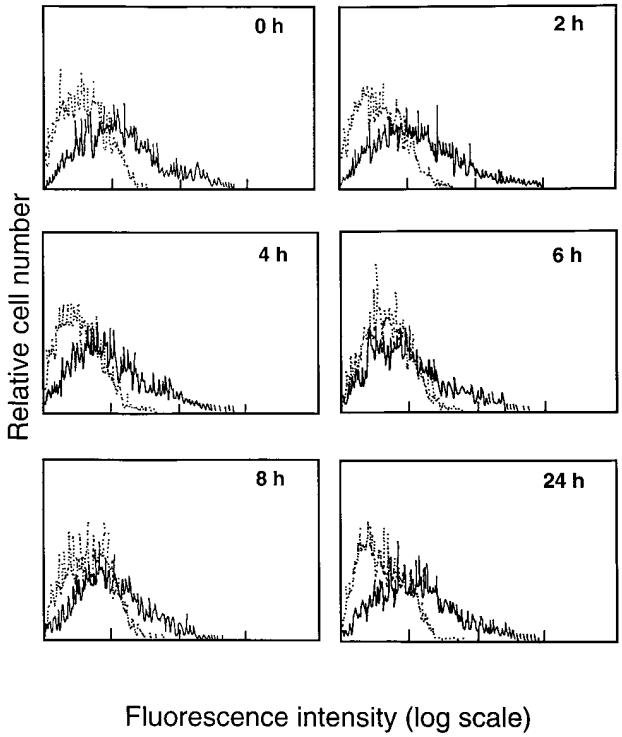

Figure 6. Time course of aortic endothelial cell activation. Endothelial cells were activated with TNF-α (100 U/ml). At the indicated times, indirect immunofluorescence analysis was performed with L-selectin/μ (*solid lines*) and CD4/μ (*dotted lines*). The data are representative of three experiments.

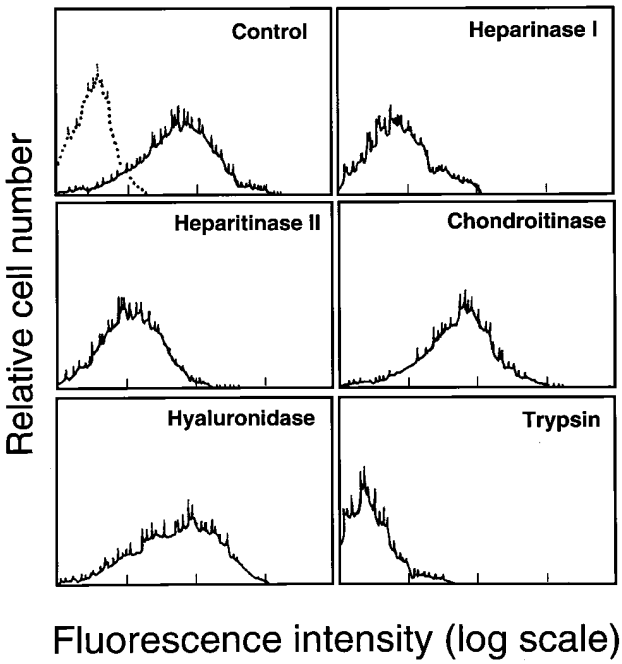

Figure 7. Interaction of L-selectin with suspended aortic endothelial cells: effect of treating unstimulated BAEC with heparinase I, heparitinase II, chondroitinase ABC, hyaluronidase, or trypsin. Unactivated BAEC were examined by indirect immunofluorescence analysis with L-selectin/μ (*solid lines*) 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 were as follows: control, 86%; heparinase I, 54%; heparitinase II, 56%; chondroitinase, 89%; hyaluronidase, 90%; trypsin, 7%. The background staining with CD4/μ chimera was <1%.

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/μ 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/cm^2, 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/mm^2 (mean ± SD, n = 3) interacted with activated endothelial monolayers during this time, whereas 854 ± 72 interacting monocytes/mm^2 (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

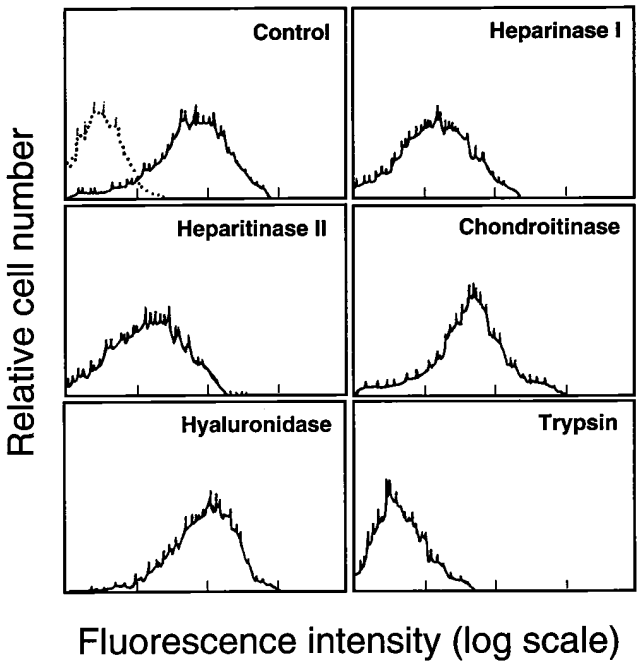

Figure 8. Interaction of L-selectin with suspended aortic endothelial cells: effect of treating TNF-α–activated BAEC (8 h, 100 U/ml) with heparinase I, heparitinase II, chondroitinase ABC, hyaluronidase, or trypsin. Unactivated BAEC were examined by indirect immunofluorescence analysis with L-selectin/μ (*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%.

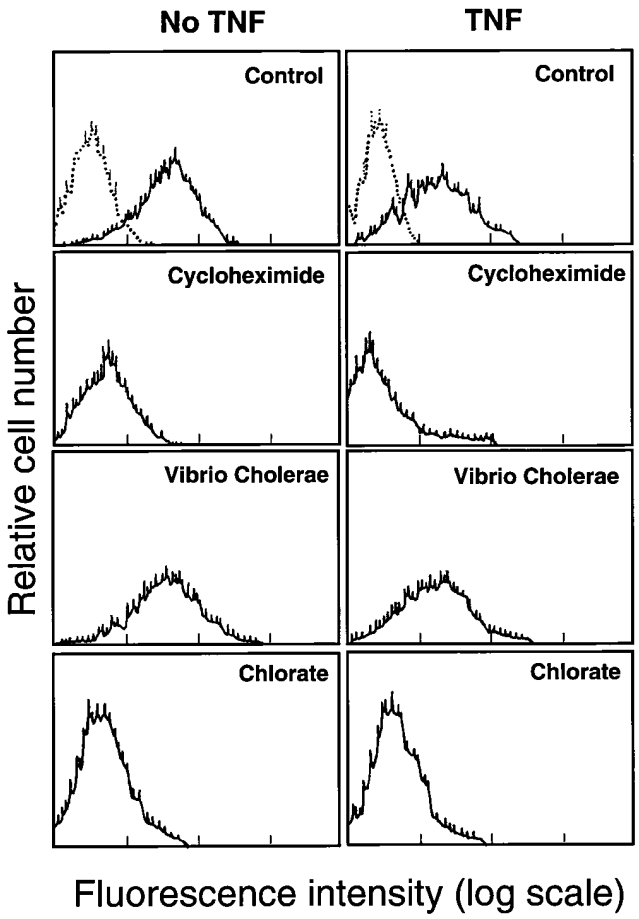

Figure 9. Interaction of L-selectin with suspended aortic endothelial cells: effect of treating unstimulated or TNF-α–activated BAEC (6 h, 100 U/ml) with cycloheximide (10 μg/ml), *V. cholerae* neuraminidase (750 U/ml), or sodium chlorate (10 mM, 24 h). BAEC were examined by indirect immunofluorescence 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%.

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-

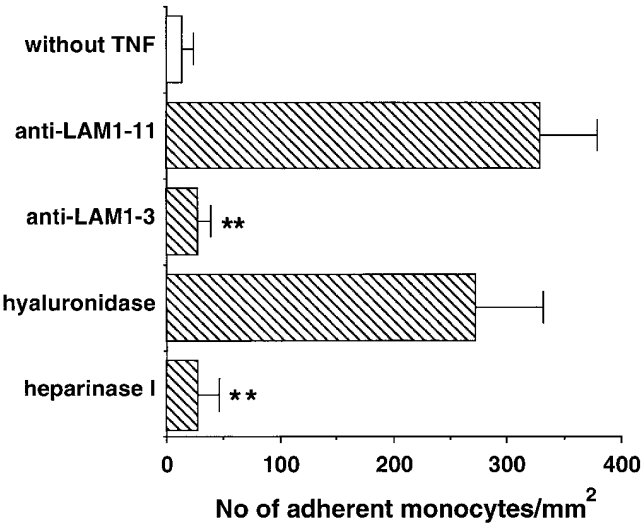

Figure 10. Inhibition of monocyte adhesion to 8-h TNF-α–activated endothelium under flow (wall shear stress estimated at 1.8 dynes/cm^2). Endothelial monolayers were activated for 8 h with TNF-α (100 U/ml), washed, and then preincubated for 45 min at 37°C with heparinase I (1,600 mU/ml) or hyaluronidase (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 representative of three experiments. $^{**}P < 0.001$.

α–activated aortic endothelium primarily through L-selectin. mAb blockade of L-selectin inhibited by ~80% monocyte attachment to TNF-α–activated endothelium (Fig. 3). These observations are consistent with those describing, under flow, monocyte interactions with 6-h TNF-α–activated human umbilical vein endothelial cells (49), and they are the first to show that L-selectin mediates monocyte attachment to activated aortic endothelium. Because recent studies reported that neutrophils can roll on already 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 monocytes occasionally slowed down and arrested on adherent monocytes, facilitating their attachment to activated endothelium. These interactions were discarded for quantitative analysis. Only single-cell interactions with activated endothelium were taken into consideration. The strong inhibition of monocyte attachment to activated endothelium induced by the pretreatment of endothelium with L-selectin/μ further indicated that L-selectin interaction with endothelial 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 support monocyte attachment to activated arterial endothelium. 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 earlier 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 atherosclerotic lesions. Clearly, a detailed elucidation of the molecular mechanisms involved in monocyte attachment to the arterial wall will be required to understand how atherosclerotic 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 suggested 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 reported that additional endothelia can also express cytokine-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 activated 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-selectin/μ was also found to bind to unactivated aortic endothelial cells (Fig. 5). This result was unexpected because unactivated 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 completely 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-selectin/μ binding (Figs. 5 and 6). Endothelial cell treatment with various glycosaminoglycan-cleaving enzymes demonstrated that ligands expressed on both unactivated and activated 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 proteoglycans. Importantly, the reactivity of L-selectin with cytokine-activated aortic cells was only partially susceptible to heparinase I and heparitinase II digestion. This latter observation suggests that cytokine activation could induce the expression of additional ligands, distinct from heparan sulfate proteoglycans that interact with L-selectin to support monocyte adhesion. Alternatively, TNF-α could increase monocyte 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 and cytokine-activated aortic cells; these sequences would not be expressed on unstimulated arterial endothelium. An additional option is that the arterial endothelial

response observed after TNF-α activation occurs with preexisting L-selectin ligands. In this scenario, unstimulated aortic endothelial cells have dispersed L-selectin ligands on their surface, which can bind L-selectin/μ but cannot support monocyte adhesion; in contrast, on the surface of activated endothelial cells, L-selectin ligands could form patches capable of both L-selectin/μ and monocyte binding. Further studies will be needed to investigate these possibilities.

The lack of significant change in L-selectin/μ binding to aortic endothelial cells after TNF-α activation does not preclude the existence of inducible ligands for L-selectin (Figs. 5 and 6). Thus, the increase in L-selectin–dependent binding of monocytes observed after BAEC activation could be mediated by ligands that react with high affinity with L-selectin but are expressed at low density at the cell surface. One can speculate that heparan sulfate proteoglycans function as low affinity L-selectin ligands that attract monocytes at the vascular cell surface (coreceptor function) and direct them to less abundant high affinity receptors. This process could be analogous to the one that regulates the presentation of FGF by multimeric heparan sulfate proteoglycans to high affinity FGF receptor (69). In addition, the capacity of heparan sulfate proteoglycans to present cytokines to attracted monocytes may provide adhesion-inducing signals that regulate subsequent steps of adhesion (66, 79, 80). As discussed above, the partial susceptibility of L-selectin ligands to heparin lyases suggests that additional ligands for L-selectin could be expressed by TNF-α–activated endothelium. Heparan sulfate proteoglycans could have an important role in supporting 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 unactivated 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 needs 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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