Brief Definitive Report

Monocyte Attachment to Activated Human Vascular Endothelium In Vitro Is Mediated by Leukocyte Adhesion Molecule-1 (L-Selectin) under Nonstatic Conditions

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Summary

The receptors that mediate monocyte adhesion to cytokine-stimulated endothelial monolayers were assessed using a nonstatic (rotating) cell-attachment assay. In this system, leukocyte adhesion molecule-1 (LAM-1) (L-selectin) mediated a major portion (87 ± 15% at 37°C) of monocyte attachment to activated endothelium. mAb blocking of endothelial leukocyte adhesion molecule-1 (41% inhibition), CD18 (36%), and vascular cell adhesion molecule-1 (25%) function had lesser effects on attachment. These results suggest that LAM-1 may serve an important role in monocyte attachment to endothelium at sites of inflammation.

The initial event in the emigration of monocytes from the blood stream into inflamed tissues is adhesion to the endothelial blood vessel lining (1). Monocyte adhesion to cytokine-activated cultured human umbilical vein endothelial cells (HUVEC) is partially inhibited by mAb binding to CD18, CD11b, CD11c, intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1) (E-selectin), and vascular cell adhesion molecule-1 (VCAM-1) (inducible cell adhesion molecule-110 [INCAM-110]), or combinations of these mAbs (2–4). However, these in vitro assays were carried out under static conditions, while monocyte interactions with endothelium in vivo occur under conditions of blood flow. When examined under nonstatic (stirred) conditions in vitro, monocyte adherence to endothelial cells appeared to involve both CD18-independent and unknown, CD18-independent mechanisms (5). In the present study, a nonstatic (rotation) assay (6) was used to examine the adhesion molecules involved in the attachment of peripheral blood monocytes to cytokine-activated HUVEC.

Materials and Methods

Monocytes. Blood monocytes were isolated by density gradient centrifugation followed by counterflow centrifugation elutriation as described (7). Preparations contained 87 ± 5% monocytes as determined by light scatter, Wright-Giemsa stain and surface antigen analysis. Monocytes (5×10^5) were incubated in 1 ml of RPMI 1640 (Gibco, Gaithersburg, MD) containing 1% human serum albumin alone or containing PMA (100 ng/ml; Sigma Chemical Co., St. Louis, MO), GM-CSF (Genetics Institute, Cambridge, MA; 20 ng/ml; ~2×10^9 U/mg), A23187 (3 μM; Sigma Chemical Co.), TNF-α (200 U/ml; Genzyme Corp., Cambridge, MA), or LPS (1 μg/ml; *Escherichia coli* strain 0111:B4; Sigma Chemical Co.) for 20 min at 37°C with gentle mixing to prevent cell attachment. All buffers and reagents contained <1 ng/ml of endotoxin.

mAb. The anti-leukocyte adhesion molecule-1 (LAM-1) mAb (all IgG1) (8), anti-CD18 (H52, IgG1) (9), and anti-VCAM-1 mAb (HAE-2, IgG1) (6) were used as diluted ascites fluid (1:100). The anti-ELAM-1 mAb (H18/7; IgG2a) (10) and anti-HLA class I mAb (W6/32, IgG2a) were used as purified F(ab')$_2$ fragments (25 μg/ml).

Endothelial-leukocyte Attachment Assay. Assays were as described (6). Briefly HUVEC (passage 2 to 3) were grown to confluence on gelatin-coated glass slides, stimulated with TNF-α (100 U/ml; 6 h), washed, and incubated for 15 min with media (RPMI 1640/5% FCS) alone or containing mAb. In parallel, monocytes (4×10^6) were incubated in media (100 μl, 10 min, 4°C) containing monocyte- and/or endothelial-directed mAb before addition to the endothelial monolayers that were kept static or rotated at 64 rpm. After 8 min at 37°C or 30 min at 4°C, the medium was gently removed and the slides were fixed overnight. The number of adherent leukocytes in 4–12 microscopic fields (0.09 mm^2) were counted and the results were expressed as means ± SD. Inhibition of monocyte adhesion was calculated using the level of attachment to unstimulated HUVEC as the baseline and the level of monocyte attachment to stimulated HUVEC obtained in the presence of control mAb as the maximal value.

Statistics. Significance was determined using the paired Student's *t* test.

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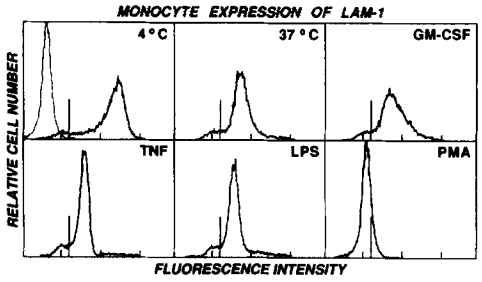

Figure 1. Monocyte expression of LAM-1. Monocytes were isolated by elutriation, kept at 4°C, or incubated for 20 min at 37°C in media alone or with various activating agents. LAM-1 expression was then assessed by indirect immunofluorescence staining using the anti-LAM1-1 mAb with flow cytometry analysis as described (16). The fluorescence intensity (four decade log scale) of cells stained with an isotype-matched unreactive control mAb is shown as a thin line (4°C panel).

Results

Monocyte Expression of LAM-1. Isolation of monocytes by elutriation only resulted in a 40% loss of cell surface LAM-1 expression when compared with monocytes in whole blood by indirect immunofluorescence analysis. In three experiments, incubation of monocytes at 37°C for 20 min resulted in a further 53 ± 10% loss of LAM-1 compared with cells kept at 4°C for 20 min (Fig. 1). Treatment of the cells with GM-CSF, TNF-α, LPS, PMA, and calcium ionophore, resulted in LAM-1 loss of 73 ± 10%, 63 ± 11%, 80 ± 5%, 94 ± 1%, and 89 ± 7%, respectively. Therefore, after elutriation, the monocytes responded to inflammatory stimuli and shed LAM-1.

LAM-1 Mediates Monocyte Attachment to Activated Endothelium. Monocyte binding to endothelium under static and rotating conditions was examined to determine whether different receptors function during the different phases of adhesion. Under static conditions at 37°C, monocytes avidly bound (479 ± 27/field) to unactivated HUVEC monolayers (Fig. 2). Although few monocytes (79 ± 4) attached to the endothelium under rotating conditions, a significant increase in monocyte attachment occurred under both static (676 ± 36) and rotating (671 ± 32) conditions when endothelial cells were cultured with TNF-α for 6 h. Treatment of monocytes with an anti-HLA class I mAb had no effect on attachment (Fig. 2). In contrast, treatment of monocytes with anti-LAM1-3 mAb inhibited a significant proportion (87 ± 15% inhibition, $n = 3$, $p < 0.005$) of cytokine-induced adhesion

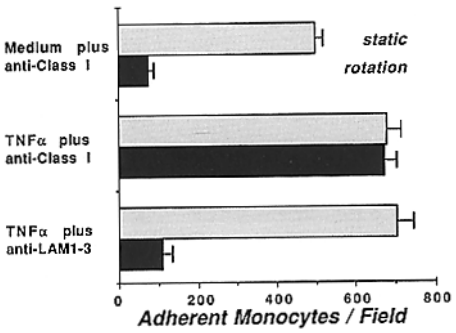

Figure 2. LAM-1 mediates monocyte attachment to endothelium under nonstatic conditions. Endothelial monolayers were cultured in medium or TNF-α and the monocytes were incubated with anti-HLA class I or anti-LAM1-3 mAb before and during the assay at 37°C. During the attachment assay, the slides were kept static (*shaded bars*) or were rotated (*filled bars*) at 64 rpm. Results are representative of three experiments.

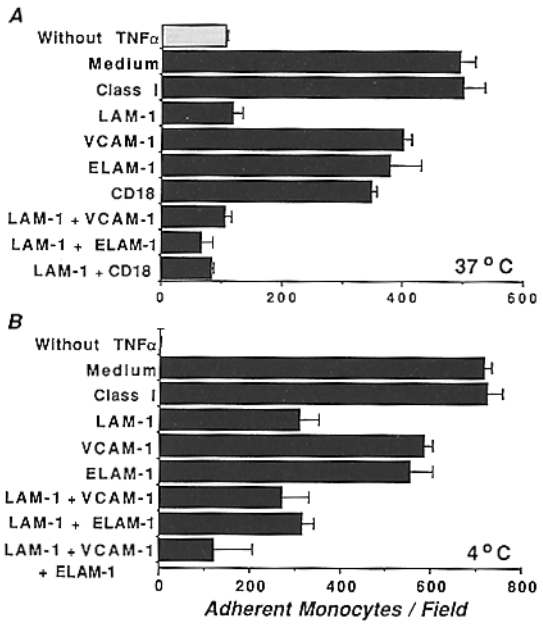

Figure 3. LAM-1 primarily mediates monocyte attachment to endothelial cells under nonstatic conditions. Assays were carried out at 37°C for 8 min (*A*), and 4°C for 30 min (*B*). Confluent endothelial monolayers were cultured with medium (*shaded bars*) or with TNF-α (*filled bars*). Monocytes and endothelial cells were treated with medium or mAb reactive with HLA class I (W6/32), LAM-1 (anti-LAM1-3), VCAM-1 (HAE-2), ELAM-1 (H18/7), and CD18 (H52) before and during the assays. Results are representative of three experiments.

Table 1. *Inhibition of Monocyte Binding to Activated HUVEC at 4°C*

mAb	No. bound/field	Percent inhibition
Medium	725 ± 56	
LAM1-1	421 ± 52	42‡*
LAM1-3	230 ± 50	68§
LAM1-4	213 ± 68	71§
LAM1-5	655 ± 52	10
LAM1-6	356 ± 38	51§
LAM1-7	323 ± 45	55§
LAM1-10	613 ± 40	15
LAM1-11	709 ± 56	2

* Statistical significance of inhibition.
‡ $p < 0.01$.
§ $p < 0.005$.

under rotating conditions, but failed to inhibit attachment to activated endothelium in static assays (Fig. 2). When viewed by phase contrast microscopy, adherent monocytes rapidly underwent shape change at 37°C and spread on the activated endothelium. Although the anti-LAM1-3 mAb blocked most monocyte attachment, it failed to inhibit spreading of the adherent monocytes, suggesting that different adhesion molecules mediated these distinct events. Treatment of monocytes with PMA as in Fig. 1, which promotes loss of LAM-1 with concomitant activation of CD18, inhibited 89% of monocyte attachment to TNF-activated endothelium under nonstatic conditions (data not shown). However, lack of attachment may not be solely due to loss of LAM-1 since monocyte activation in general may cause decreased attachment with LAM-1 loss serving as a marker for this event.

LAM-1 is the Principal Adhesion Receptor for Monocyte Attachment to Activated Endothelium under Nonstatic Conditions. In three experiments, antibody binding to VCAM-1 inhibited cytokine-induced increases in monocyte adhesion by 25 ± 1% (p <0.025), ELAM-1 mAb inhibited 41 ± 16% (p <0.05), and CD18 mAb inhibited 36 ± 3% (p <0.01), indicating that these receptors participate in monocyte adhesion to activated endothelium at 37°C (Fig. 3 *A*). The combination of anti-LAM1-3 mAb with either anti-ELAM-1 (97 ± 5% inhibition), anti-VCAM-1 (87 ± 18%) or anti-CD18 (88 ± 18%) mAb only minimally increased the level of inhibition of activation-dependent adhesion (~10%) indicating that these receptors may function in processes subsequent to LAM-1-mediated attachment.

At 4°C, where LAM-1 shedding is minimal and CD18-mediated adhesion is inactive, anti-LAM1-3 mAb inhibited a majority of monocyte attachment (60 ± 8%, n = 3, p <0.005) to activated endothelium, while anti-ELAM-1 and anti-VCAM-1 mAb only inhibited by 19 ± 7% and 28 ± 7%, respectively (Fig. 3 *B*). Anti-HLA class I mAb was without effect (~4% inhibition). The combination of anti-LAM1-3 mAb with anti-VCAM-1 or ELAM-1 mAb inhibited attachment by 63 ± 1% and 56 ± 1%, respectively, which was not significantly different from anti-LAM1-3 alone. The combination of anti-LAM1-3, anti-ELAM-1, and VCAM-1 mAb inhibited attachment by 83 ± 12%. At 4°C, the attached monocytes remained spherical and did not spread on the activated endothelium. However, these assays were carried out for 30 min since the cellular activation-dependent change of LAM-1 affinity for ligand (11) appeared to result in a temperature-dependent decrease in the attachment of monocytes. Nonetheless, under nonstatic conditions LAM-1 was the principal adhesion mechanism that mediated monocyte attachment to cytokine-activated endothelium.

The Same Epitopes of LAM-1 Mediate Monocyte and Lymphocyte Attachment to Endothelium. mAb that identify spatially and functionally distinct epitopes on LAM-1 (8) were used to identify the epitopes involved in monocyte attachment to endothelium. The anti-LAM1-1, -3, -4, -6, and -7 mAb, which inhibit lymphocyte binding to high endothelial venules and to stimulated HUVEC (6), also inhibited monocyte binding (Table 1). Anti-LAM-1 mAb (anti-LAM1-5, -10, and -11) that do not inhibit lymphocyte attachment, did not inhibit monocyte attachment. Thus, identical epitopes of LAM-1 mediate monocyte and lymphocyte attachment.

Discussion

Although multiple adhesion mechanisms have been implicated in monocyte adhesion to endothelium, mAb binding to functional epitopes on LAM-1 (L-selectin) inhibited a major portion of monocyte attachment to cytokine-activated endothelium under nonstatic conditions (Figs. 2 and 3). The finding that LAM-1-mediated binding was only detectable under nonstatic conditions, suggests that LAM-1 serves as the primary receptor that initiates attachment of monocytes to activated endothelium. Similarly, in vitro studies have demonstrated that P-selectin mediates neutrophil rolling at physiologic flow rates (12), while other studies have demonstrated that LAM-1 mediates neutrophil rolling in vivo (13, 14). VCAM-1, ELAM-1, and CD18 also participated in monocyte adhesion to activated endothelium, but their relative contributions were modest in this assay system. LAM-1 also mediates lymphocyte and neutrophil attachment to activated endothelium under nonstatic conditions in vitro (6), but LAM-1 contributes less to lymphocyte (56%) and neutrophil (27%) attachment when compared with the current results for monocyte attachment (87%).

A central role for LAM-1 in monocyte attachment to activated vascular endothelium in vivo may partially explain the selective recruitment of different leukocyte subsets during different phases of inflammation. Neutrophils accumulate very rapidly in acute inflammatory lesions whereas lymphocytes and monocytes become the predominant cell type with more chronic inflammation. In vitro, expression of the LAM-1 ligand on cytokine-activated endothelium is sustained (6), in contrast to ELAM-1 expression (10), and is similar to the pattern of VCAM-1 and ICAM-1 expression (15). Thus, sustained expression of the LAM-1 ligand in combination with other adhesion molecules in vivo could induce preferential recruitment of monocytes and lymphocytes during late phases of inflammation.

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