Human Monocytes Bind to Two Cytokine-Induced Adhesive Ligands on Cultured Human Endothelial Cells: Endothelial-Leukocyte Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1

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Vascular cell adhesion molecule-1 (VCAM-1) and endothelial-leukocyte adhesion molecule-1 (ELAM-1) are adhesive proteins induced on endothelium by cytokines. We examined the contribution of these adhesive proteins to human peripheral blood monocyte adherence to endothelium using transfected Chinese hamster ovary (CHO) cells stably expressing these proteins and monoclonal antibodies (MoAbs) to ELAM-1, VCAM-1, or CD49d/CD29 (VLA-4), the leukocyte receptor for monocytes to CHO cells transfected with cDNA of ELAM-1 or VCAM-1. Binding to ELAM-1 was inhibited by MoAb to ELAM-1 and binding to VCAM-1 was inhibited by MoAb to VCAM-1 or the α-chain of very late activation antigen-4 (VLA-4) (CD49d). Additive inhibition of adherence to unstimulated human umbilical vein endothelium (HUVE) was observed when monocytes were pre-treated with both MoAb to CD49d and MoAb to CD18, the common β-chain of the leukocyte β2 integrin receptors. Adherence of monocytes to HUVE stimulated by recombinant human tumor necrosis factor-α was not reduced by MoAbs to CD18, CD49d, or ELAM-1 when used singly, but combinations of these MoAbs produced significant inhibition. We conclude that multiple receptor-ligand systems are involved in monocyte adherence to endothelium.

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THE SEQUENCE OF EVENTS leading to monocyte emigration in response to extravasatory stimuli can be summarized as follows. Signals are generated at the inflammatory site that activate the circulating monocyte and the adjacent endothelium. As a consequence of activation, the circulating monocytes adhere to the endothelium, migrate along the endothelial surface, diapedes, and finally emigrate through subendothelial matrix to participate in the inflammatory reaction.

Neutrophils are the predominant leukocyte at inflammatory sites with the peak of emigration occurring within the first several hours after the onset of inflammation. However, within 12 to 24 hours mononuclear phagocytes become the most abundant cell in the inflammatory infiltrate. The different kinetics of emigration and subsequent accumulation in tissue of neutrophils and monocytes may be explained in part by differences in expression or configuration of adhesion proteins on the phagocyte or the endothelium.

Endothelial-leukocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are surface proteins that are induced on endothelial cells and are involved in leukocyte adhesion. Neutrophils, but not peripheral blood lymphocytes (PBL), have been reported to bind to ELAM-1. Conversely, VCAM-1 has been shown to bind PBL, but not neutrophils. Preliminary studies have suggested that monocytes also bind to VCAM-1 but the binding of monocytes to ELAM-1 has been difficult to demonstrate clearly.

In this report we demonstrate that human monocytes adhere to ELAM-1 and VCAM-1 using Chinese hamster ovary (CHO) cells that have been transfected with cDNA of ELAM-1 or VCAM-1 and are stably expressing these adhesive proteins. We show that this binding is inhibited by monoclonal antibodies (MoAbs) that are specific for ELAM-1, VCAM-1, or the leukocyte receptor for VCAM-1, very late activation antigen-4 (VLA-4, CD49d/CD29). Finally, we demonstrate that these endothelial cell ligands are involved in the adherence of human monocytes to human umbilical vein endothelium (HUVE).

MATERIALS AND METHODS

Generation of CHO cell lines that stably express ELAM-1 and VCAM-1. The animal cell expression vectors pBG341JOD.ELAM and pBG341JOD.VCAM were constructed as follows: vector pBG312 was modified by insertion of a NotI site into the polylinker sequence. The resulting plasmid, pBG341, was cleaved by NotI and AatII. The smaller of the two fragments was ligated to the larger of the two fragments generated by NotI and AatII cleavage of vector pJOD-8. The resulting vector, pBG341JOD, contains an expression cassette for the dihydrofolate reductase cDNA gene and a unique NotI site downstream from the adenovirus major late promoter. The cDNA sequences for either ELAM-1 or VCAM-1 were inserted into the vector NotI site to generate the animal cell expression vectors pBG341JOD-ELAM and pBG341JOD-VCAM, respectively.

To generate cell lines stably expressing ELAM-1 and VCAM-1, CHO-dihydrofolate reductase folato (CHO-DHFR-) cells were electroporated with 200 μg pBG341JOD.ELAM or pBG341JOD.VCAM as described. Before electroporation the plasmid was linearized using AatII. Following transfection the cells were cultured in selective medium (minimal essential medium [MEM] minus glutamine, and 10% dialysed fetal bovine serum [FBS]) to select for methotrexate-resistant cells. Individual colonies were picked, plated into 96-well cluster plates, and grown to confluence. Clones expressing sufficient ELAM-1 or VCAM-1 to bind HL60 cells or Ramos cells, respectively, were detected by adhesion assay as described.
the ELAM-1–specific MoAb BB11 and MoAb BB11 immunoprecipitates a protein of about 130 Kd from CHO.ELAM cell lines. MoAb BB11, but not MoAb 4B9, binds to CHO.ELAM-1 while MoAb 4B9, but not MoAb BB11, binds CHO.VCAM-1 (T. Carlos, unpublished observation, March, 1990). MoAb 4B9 immunoprecipitates a protein of about 100 Kd from CHO.VCAM-1 but not control CHO.11

Untransfected CHO cells were maintained in MEM α medium supplemented with 4 mmol/L glutamine and 10% FBS. Transfected CHO cells (ELAM-1 and VCAM-1) were maintained in MEM α-minus medium supplemented with 4 mmol/L glutamine, 500 nmol/L methotrexate, and 10% FBS.

Nonadherent cells. PB monocytes were purified from heparinized whole blood by the method of Recalde. Preparations were greater than 85% pure monocytes as determined by morphology, nonspecific esterase staining, and reactivity with a CD14 MoAb on fluorescence-activated cell sorter (FACS). The leukemic cell lines, HL60 and Molt 4, were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in MEM (Flow Labs, McLean, VA) supplemented with 10% Cellect fetal calf serum (FCS; Flow Labs). Leukocytes were labeled with "Cr as described and were resuspended in RPMI medium (M.A. Bioproducts, Walkersville, MD).

Cell culture. HUVE were harvested by collagenase treatment of vessels as previously published. Cells were maintained in RPMI 1640 medium supplemented with 10% adult bovine serum (Hyclone Sterile Systems, Logan, UT), 10% normal calf serum (NCS; Armour Pharmaceutical Co, Kanakee, IL), heparin (90 μg/mL; Sigma Chemical Co, St Louis, MO), and endothelial growth factor (50 μg/mL) as described by Thornton et al. Endothelial growth factor was prepared from bovine hypothalamus as described by Maciag et al.

MoAbs. MoAb 4B9 is a murine IgG, that recognizes a 100-Kd antigen on cytokine-activated endothelium. MoAb 4B9 binds to COS cells transfected with VCAM-1 CDNA and inhibits PBL adherence to cytokine-stimulated HUVE. MoAb 60.3, a murine IgG3, that recognizes the common β-subunit of the CD11/CD18 complex, and MoAb 60.5, a murine IgG2a, that recognizes class I HLA framework antigen, were provided by Dr Patrick Beatty, Fred Hutchinson Cancer Research Center, Seattle, WA. MoAb P4C2 is a murine IgG, that recognizes an epitope on the α-chain polypeptide (CD49d) of VLA-4 (CD49d/CD29). MoAb P4C2 does not recognize CHO cells or HUVE as determined by enzyme-linked immunosorbent assay (ELISA). MoAb BB11 is a murine IgG2a that binds to an epitope on ELAM-1. Anti-Leu-M3 (CD14) was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA).

Adherence assay. CHO cells were plated onto gelatin-coated 48-well tissue culture plates (Costar Corp, Cambridge, MA) and allowed to reach confluence. The medium was decanted and the wells were washed twice with RPMI containing 2% NCS (RPMI/NCS). Subsequently, 0.2 mL of control medium (RPMI/NCS) or medium containing MoAbs BB11, 4B9, or P4C2 was added to the monolayer 30 minutes before assay. MoAb P4C2 does not bind to either CHO or HUVE by ELISA assay, but binds to monocytes by FACS analysis. Untreated leukocytes or leukocytes that were pretreated for 30 minutes with MoAb 60.3 (40 μg/mL) were added to each well. Experiments involving monocyte adherence to HUVE were performed in a similar manner. For these experiments, some wells of the 48-well plates were pretreated with 10 ng/mL recombinant human tumor necrosis factor-α (rhTNF-α; Biogen Inc, Cambridge, MA) for 4 hours. Following 30 minutes of incubation at 37°C, nonadherent cells were discarded and the wells were washed once with 0.5 mL phosphate-buffered saline (PBS). Adherent cells were lysed with 1 N NaOH, and the lysate was counted in a gamma spectrophotometer. Percent adherence was calculated by the formula:

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\%\text{ adherence} = \frac{\text{[3]Cr cpm in lysate}}{\text{total [3]Cr cpm added}} \times 100
\]

Statistics. Levels of statistical significance were calculated by paired t-test using Statview (BrainPower, Inc, Calabasas, CA).

RESULTS

Monocytes bind to ELAM-1–transfected CHO cells. The adherence of the leukemic cell line HL60 to control CHO cells or CHO cells stably expressing ELAM-1 was examined in three separate experiments (Fig 1A). The adherence of HL60 to control CHO was moderate (33% ± 5%) and was not reduced by MoAb 60.3 (28% ± 9%), MoAb BB11 (31% ± 8%), MoAb P4C2 (27% ± 6%), or MoAb 4B9 (31% ± 6%). The adherence of HL60 to ELAM-1–transfected CHO was 89% ± 2%. MoAb BB11 completely blocked the binding of HL60 cells to levels observed in control CHO (26% ± 4%, P < .001). MoAb 60.3, MoAb P4C2, and MoAb 4B9 did not significantly inhibit monocyte...
binding to ELAM-1-transfected CHO cells (92% ± 9%, 84% ± 4%, and 88% ± 4%, respectively).

The binding of monocytes to control CHO cells was high (50% ± 17%), but was nearly abolished by the CD18 MoAb 60.3 (4% ± 3%, mean ± SE of three experiments). Using MoAbs specific for CD11a and CD11b, binding to CHO cells was found to be mediated by CD11a/CD18 (data not shown). To detect specific binding to ELAM-1 or VCAM-1, monocytes were pretreated with the CD18 MoAb 60.3 in subsequent experiments.

The binding of MoAb 60.3-prepertreated monocytes to ELAM-1-transfected CHO cells was 41% ± 6% (Fig 1B). Preincubation of ELAM-1-transfected CHO cells with the anti-ELAM-1 MoAb BB11 reduced binding of MoAb 60.3-prepertreated monocytes to 8% ± 4% (P < .01). No effect was observed when MoAb 60.3-prepertreated monocytes were added to ELAM-1-transfected CHO cells that had been preincubated with the CD49d MoAb P4C2 or the anti–VCAM-1 MoAb 4B9 (32% ± 9% and 39% ± 8% binding, respectively).

Monocytes bind to VCAM-1-transfected CHO cells. The binding of the Molt-4 leukemic cell line to control CHO cells and to CHO cells that stably express the adhesive protein VCAM-1 was examined (Fig 2A). The binding of Molt-4 cells to control CHO was low (1.5% ± 0.3%). The adherence of Molt-4 cells to CHO cells that stably express VCAM-1 was significantly higher (26% ± 6%). MoAbs 4B9 or P4C2 completely inhibited this binding (<1%) while the anti–ELAM-1 MoAb BB11 was without significant effect (19% ± 4%). The binding of monocytes to CHO cells that had been transfected with VCAM-1 cDNA was also examined in a series of experiments (Fig 2B). The binding of MoAb 60.3-pretreated monocytes to VCAM-1 CHO cells was 49% ± 5%. The anti–ELAM-1 MoAb BB11 did not reduce this binding (50% ± 7%). The CD49d MoAb P4C2 and the anti–VCAM-1 MoAb 4B9 significantly inhibited the adherence of MoAb 60.3-prepertreated monocytes to VCAM-1-transfected CHO cells (9% ± 2% and 19% ± 2%, respectively; P < .05). The combination of MoAb P4C2 and MoAb 4B9 produced no greater inhibition of monocyte binding than that observed with MoAb P4C2 alone (data not shown).

Monocyte adherence to unstimulated or rhTNF-α-stimulated HUVE. The binding of monocytes to untreated HUVE or HUVE that were stimulated with rhTNF-α for 4 hours was examined (Fig 3). Because the CD49d MoAb was more effective than the anti–VCAM-1 MoAb in blocking monocyte binding to VCAM-1-transfected CHO cells (Fig 2B), MoAb P4C2 rather than MoAb 4B9 was used to inhibit monocyte binding to VCAM-1 expressed on HUVE.

Monocyte adherence to unstimulated HUVE was 57% ±
While the CD49d MoAb P4C2 alone did not significantly inhibit monocyte adherence to unstimulated HUVE (49% ± 2%), a statistically significant additive inhibitory effect was seen when MoAb P4C2 was used in conjunction with MoAb 60.3 (23% ± 3%, P < .01). The further addition of the anti-ELAM-1 MoAb BBll did not affect adherence to unstimulated HUVE (25% ± 3%).

In a separate set of experiments, the addition of the CD14 MoAb Leu-M3 did not produce additive inhibition of binding of MoAb 60.3-pretreated monocytes to unstimulated HUVE. In these studies the adherence of untreated monocytes was significantly reduced by MoAb 60.3 (57% ± 2% vs 18% ± 2%, P < .05). The addition of MoAb P4C2 to MoAb 60.3-pretreated monocytes produced further significant inhibition of binding (12% ± 1%, P < .05) in contrast to the addition of Leu-M3 (19% ± 1%, P = not significant [NS]).

The adherence of monocytes to HUVE that had been treated with rhTNF-α was significantly greater than monocyte binding to unstimulated HUVE (69% ± 2%, P < .05). In contrast to the inhibitory effect of the CD18 MoAb 60.3 on binding to untreated HUVE, no inhibition of monocyte adherence to rhTNF-treated HUVE was produced by the CD18 MoAb (71% ± 2%). The addition of the CD49d MoAb P4C2 to MoAb 60.3-pretreated monocytes significantly reduced binding (53% ± 2%, P < .01). However, pretreatment of rhTNF-pretreated HUVE with the anti-ELAM-1 MoAb BBll and P4C2 followed by the addition of monocytes that were pretreated with MoAb 60.3 produced the most significant reduction of binding (38% ± 2%, P < .01 versus the combination of MoAbs 60.3 and P4C2).

In a separate set of experiments, the addition of the HLA-class I MoAb 60.5 to rhTNF-α-stimulated HUVE did not produce additive inhibition of binding of monocytes that were pretreated with MoAbs 60.3 and P4C2. In these experiments the binding of untreated monocytes to rhTNF-α-stimulated HUVE was 67% ± 2%. Pretreatment of the monocytes with MoAbs 60.3 and P4C2 significantly inhibited this binding (26% ± 6%, P < .05). Pretreatment of the rhTNF-α-pretreated HUVE with MoAb BBll produced further significant inhibition of adherence (15% ± 2%, P < .05) while pretreatment with MoAb 60.5 was ineffective (36% ± 3%, P = NS).

**DISCUSSION**

Monocytes adhere avidly to unstimulated HUVE. This basal adherence is mediated in part by the CD18 complex on the monocyte. Pretreatment of HUVE with lipopolysaccharide (LPS) produces an additional increase in monocyte adherence that is CD18-independent. In this report we demonstrate that human PB monocytes bind to two endothelial adhesion proteins, ELAM-1 and VCAM-1, that are induced on endothelial cells by LPS or cytokine treatment and that these ligands are involved in monocyte adherence to HUVE.

The capacity of monocytes to bind to ELAM-1 and VCAM-1 was first established using transfected CHO cells stably expressing these proteins. Because of the significant basal monocyte adherence to CHO cells, it was necessary to pretreat monocytes with a CD18 MoAb. By using CD11a (lymphocyte function-associated antigen-1 [LFA-1]) and CD11b (Mac-1) MoAbs in our initial studies, this basal binding to CHO cells was determined to be mediated by CD11a. CD11a is a receptor for both intercellular adhesion molecules-1 and -2, ICAM-1 (CD54) and ICAM-2. The surface expression of ICAM-1 was not detected on CHO cells by ELISA with three MoAbs to human ICAM-1. Because our human CD54 MoAbs did not bind to CHO ICAM-1, we cannot determine whether ICAM-1 or ICAM-2 is involved in this basal monocyte adherence to CHO cells. To detect specific binding to ELAM-1- or VCAM-1-transfected CHO cells, in subsequent experiments monocytes were pretreated with CD18 MoAb to eliminate CD11a/CD18-dependent binding.

Previous reports have demonstrated that ELAM-1 binds neutrophils and some leukemic cell lines (HL60, U937), but is not involved in the adherence of lymphocytes or lymphocytic cell lines. An initial report suggested that ELAM-1 also bound monocytes, but this result was questioned by the same investigators in subsequent studies. Our results demonstrate that HL60 cells and human monocytes bind to ELAM-1 expressed in CHO cells and that this binding is inhibited by the anti-ELAM-1 MoAb BBll. MoAb BBll also binds to ELAM-1 induced on HUVE and inhibits binding of HL-60 cells to TNF-treated HUVE. This result suggests that the inhibition of monocyte binding to ELAM-1-transfected CHO cells is not a selective property of ELAM-1 expressed in CHO cells.

VCAM-1 is a recently described adhesion protein that is induced on endothelium by cytokines (TNF-α or interleukin-1 [IL-1]) and LPS. The leukocyte receptor for VCAM-1 is VLA-4. COS cells transfected with VCAM-1 cDNA bind leukemic cell lines (Ramos, HL60, THP-1) that express VLA-4 but not neutrophils that lack VLA-4. MoAb 4B9 binds to CHO cells transfected with cDNA for VCAM-1 but not ELAM-1 or ICAM-1 and inhibits binding of lymphocytic cell lines, PBL, and leukocyte adhesion deficient (LAD)-lymphoblasts to rhTNF-α-treated HUVE. Our results show that the human T-cell line Molt-4 and PB monocytes bind to transfected CHO cells stably expressing VCAM-1 and that this binding is inhibited by the CD49d MoAb P4C2 and the anti-VCAM-1 MoAb 4B9.

Having established that monocytes are capable of binding to ELAM-1 and VCAM-1, we next examined the role of these endothelial ligands in monocyte adherence to unstimulated and rhTNF-α-treated HUVE using various combinations of MoAbs to the adhesion proteins. A MoAb to CD18 (60.3) was used to inhibit binding to ICAM-1 and ICAM-2, another possible ligand for CD11a/CD18. An MoAb to the α-chain polypeptide (CD49d) of VLA-4 was used to inhibit monocyte binding to VCAM-1. Binding to ELAM-1 was inhibited by an anti-ELAM-1 MoAb (BBll).

As previously described, monocyte adherence to untreated HUVE was high and a significant portion of this
adherence was CD18-dependent. The CD49d MoAb alone had little effect on monocyte adherence to untreated HUVE, but produced additive inhibition when used in combination with CD18 MoAb. The anti-ELAM-1 MoAb was without effect on monocyte binding to unstimulated HUVE either when used alone or in combination with CD18 and CD49d MoAbs.

The adherence of monocytes was significantly increased by pretreatment of the HUVE with rhTNF-α. Each of the MoAbs alone produced no significant inhibition of adherence. However, combinations of the MoAbs significantly inhibited the adherence of monocytes to rhTNF-stimulated HUVE. The need to use multiple MoAbs to detect inhibition of adherence may explain the previous difficulty in demonstrating that monocytes bind to ELAM-1. While MoAbs against one or even two adhesion proteins that are involved in the binding of monocytes to stimulated HUVE may inhibit some portion of adherence, the remaining adhesion system(s) appears to compensate so that little if any effect is observed.

The adherence of monocytes to multiple adhesive ligands induced on endothelium by cytokines or LPS may explain the continued recruitment of tissue phagocytes to the inflammatory site. Monocytes express the pan-leukocyte integrin CD11a/CD18 that is the receptor for the endothelial ligands ICAM-1 and ICAM-2. ICAM-1 is constitutively expressed in vivo and both proteins are detected on unstimulated endothelial cells in culture. An increase in the surface expression of ICAM-1 is observed for more than 48 hours after cytokine stimulation. Because the ICAMs are constitutively expressed, they may be involved in the normal transmigration of monocytes as they exit the vasculature to become tissue macrophages. An increase in surface expression of ICAM-1 at sites of inflammation could facilitate leukocyte emigration, although it would lack selectivity because its receptor, CD11a, is present on all leukocytes.

The induction of ELAM-1 and VCAM-1 on endothelium by cytokines may contribute to prolonged emigration of monocytes at sites of chronic inflammation. The induction of ELAM-1 could augment monocyte as well as neutrophil emigration during the initial period of inflammation. Cytokine-induced expression of VCAM-1, an endothelial protein that does not bind neutrophils, persists for at least 48 hours in vitro. If expression of VCAM-1 is similarly maintained at sites of inflammation, monocyte emigration, mediated by the interaction of VLA-4 and VCAM-1, may persist throughout the inflammatory response and lead to the accumulation of mononuclear leukocytes that characterizes later lesions.

We acknowledge that the adherence of isolated PB monocytes to cultured endothelium may not reproduce the sequence of events that are involved during monocyte adherence and emigration in vivo. However, these in vitro experiments have identified two inducible endothelial surface proteins that are potentially involved in this heterotypic adhesive process. In the present study we have also pointed out the complexity of this process. Clearly, the relative contribution of the various adhesion systems to monocyte emigration in inflammatory disorders can only be determined by in vivo studies. It will be important to examine the time course of expression of the endothelial adhesive ligands (ICAM-1, ELAM-1, and VCAM-1) during the inflammatory response and, particularly, to determine the effect of MoAbs to the leukocyte receptors (eg, CD18, VLA-4, MEL-14) or endothelial ligands on monocyte accumulation at inflammatory sites.

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Human monocytes bind to two cytokine-induced adhesive ligands on cultured human endothelial cells: endothelial-leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1

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