Vascular Cell Adhesion Molecule-1 Mediates Lymphocyte Adherence to Cytokine-Activated Cultured Human Endothelial Cells


The expression and function of a new cytokine-induced endothelial cell adhesion protein, vascular cell adhesion molecule-1 (VCAM-1), was characterized in vitro by using a monoclonal antibody, MoAb 4B9, which recognizes a functional epitope on this protein. As determined by enzyme-linked immunosorbent assay and radioimmunoprecipitation of metabolically labeled cells, VCAM-1 was minimally expressed on unstimulated human umbilical vein endothelium (HUVE), but was rapidly induced by recombinant human tumor necrosis factor-α (rhTNF-α), rh interleukin-1, and lipopolysaccharide. In contrast to intercellular adhesion molecule-1, VCAM-1 was not induced on dermal fibroblasts or arterial smooth muscle cells after stimulation with rhTNF, or on keratinocytes after stimulation with rh interferon-γ. MoAb 4B9 significantly inhibited the adherence of peripheral blood lymphocytes (PBL) and lymphocytic cell lines, but not neutrophils, to rhTNF-activated HUVE. The inhibitory effect of MoAb 4B9 on PBL adherence to HUVE was additive to that produced by the CD18 MoAb 60.3. These results show that VCAM-1 mediates a CD18-independent pathway of peripheral blood lymphocyte adherence to cytokine-stimulated HUVE. We propose that lymphocyte binding to VCAM-1, induced on endothelium by cytokines, may be an important component of lymphocyte emigration at sites of inflammation or immune reaction.

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THE ADHERENCE of circulating lymphocytes to vascular endothelium is a critical event in the evolution of the inflammatory and immune response. Surface proteins on both lymphocytes and endothelial cells are involved in this adhesive interaction.1 The binding of lymphocyte function-associated antigen-1, LFA-1 (CD11a/CD18), on lymphocytes2 to intercellular adhesion molecule-1, (ICAM-1, CD54),3 on endothelial cells accounts for a significant portion of the binding of lymphocytes to cultured human endothelium. However, the interaction of LFA-1 with ICAM-1 is only one of several potential mechanisms by which lymphocytes adhere to vascular endothelium.4,5

Recently, using an expression-cloning technique, Osborn et al4,6 identified a novel protein that is induced on human umbilical vein endothelium (HUVE) by tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) and is involved in adherence of lymphocytic cell lines. This inducible endothelial protein has been designated vascular cell adhesion molecule-1 (VCAM-1). Independently, we generated a monoclonal antibody, MoAb 4B9, to recombinant human (rh) TNF-stimulated HUVE that inhibited the binding of several lymphocytic cell lines.

In this report we characterize the surface expression and adhesive function of VCAM-1. We demonstrate that MoAb 4B9 binds to transfected Chinese hamster ovary (CHO) cells stably expressing VCAM-1, but not to untransfected CHO cells or to CHO cells transfected with ICAM-1 or ELAM-1 cDNA. VCAM-1 is induced on HUVE by rhTNF-α, as well as by lipopolysaccharide (LPS) or rhIL-1, with kinetics of induction similar to that reported for ICAM-1.7 However, VCAM-1 differs from ICAM-1 in the pattern of cytokines that induce it and in the cells in which it can be induced. Finally, we show that MoAb 4B9 significantly inhibits the CD18-independent component of the adherence of peripheral blood lymphocytes (PBL) and lymphocytic cell lines to rhTNF-stimulated HUVE.

MATERIALS AND METHODS

Generation of CHO cell lines that stably express ELAM-1, VCAM-1, and ICAM-1. The animal cell expression vectors pBG341JOD.ELAM and .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-S.7 The resulting vector pBG341JOD contains an expression cassette for the dihydrofolate reductase cDNA gene8 and a unique NotI site downstream from the adenovirus major late promoter. CHO cells stably expressing ICAM-1 were a gift from Barbara Wallner (Biogen, Inc, Cambridge, MA). The animal cell vector pBG368 was used to generate a vector for the insertion of ICAM-1 cDNA that was provided by Dr Brian Seed.9 The cDNA sequences for either ELAM-1,10 VCAM-1, or ICAM-111 were inserted into the vector NotI site to generate the animal cell expression vectors pBG341JOD.ELAM and .VCAM, pBG368JOD.ICAM, respectively.

To generate cell lines stably expressing ELAM-1, VCAM-1, and ICAM-1, CHO-DHFR cells12 were electroporated with 20 μg pBG341JOD.ELAM or .VCAM, or pBG368JOD.ICAM as described.8 Before electroporation the plasmid was linearized using AatII. After transfection the cells were cultured in selective medium (MEM alpha minus, GibCO, Grand Island, NY) lacking ribonucleosides and deoxyribonucleotides, supplemented with 500 nmol/L methotrexate, 4 mmol/L glutamine, and 10% dialyzed 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. Clones expressing ICAM-1 were assayed by fluorescence-activated cell sorter (FACS) analysis.

**MoAbs.** MoAb 4B9, a murine immunoglobulin G, (IgG), was generated by immunizing BALB/c mice with HUVE that were nonenzymatically harvested after stimulation for 24 hours with rhTNF-α (10 ng/mL). Splenocytes were fused with the NS-1 nonsecretory murine myeloma line that was kindly provided by Dr Charles Hart (Zymogenetics, Inc, Seattle, WA). Hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for binding to rhTNF-stimulated HUVE versus unstimulated HUVE. A secondary screen assayed the inhibition of adherence of the undifferentiated leukemic cell line K562. Previous studies had shown that a major portion of the adherence of this cell line to rhTNF-stimulated HUVE was LFA-1-dependent. Initial studies used asacites (total IgG 3.2 mg/mL), while later studies used protein A (Pharmacia, Inc, Piscataway, NJ) purified IgG (20 mg/mL). MoAb RR1/1, a well-characterized murine IgG, that recognizes ICAM-1, was kindly provided by Dr Robert Rothlein (Boehringer Ingelheim Pharmaceuticals, Inc, Ridgefield, CT). MoAb 60.3, a murine IgG2b that recognizes the common β subunit of the CD11/CD18 complex, was provided by Dr Patrick Beatty (Fred Hutchinson Cancer Research Center, Seattle, WA).

**Radioimmunoprecipitation.** HUVE cells were pretreated with rhTNF-α (10 ng/mL) for 1 hour of incubation at 37°C. The medium was aspirated from all dishes and methionine-free MEM (GIBCO) was harvested, and were washed with PBS containing 2 mmol/L phenylmethylsulfonylfluoride (PMSF), and centrifuged (12,000g x 15 minutes) to remove debris. Cell lysates were precleared with nonimmune mouse IgG (Cappel Research Products, Organon Tecknika Corp, West Chester, PA) and protein A (Pharmacia). Radioimmunoprecipitation of CHO cells stably expressing VCAM-1 was performed using protein G (Pharmacia). The lysates were then incubated for 18 hours with MoAb 4B9 conjugated to Affi-Gel-10 (Bio-Rad Lab, Richmond, CA). Antibody-conjugated beads were pelleted, washed three times with PBS containing 0.1% NP-40, and placed in sample buffer with or without 5% mercaptoethanol. Samples were boiled for 2 minutes, analyzed by 3% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiographed. For N-glycosidase F treatment, immunoprecipitate samples were incubated overnight at 37°C.

**Reagents.** rhTNF-α was provided by Biogen, Inc (Cambridge, MA). Lyophilized rh cytokines (TNF-β, IL-1α/β, IL-2, IL-3, IL-4, IL-6, TGF-β) were purchased from Research & Diagnostic Systems, Inc (Minneapolis, MN) and were suspended in sterile distilled water (GIBCO). rhIFN-γ was supplied by Genentech, Inc (South San Francisco, CA). Escherichia coli 055:B5 LPS (Sigma Chemical Co, St Louis, MO) was suspended in PBS at a concentration of 1.2 mg/mL, dispersed by sonication for 5 minutes at 4°C, and stored at -80°C. N-glycanase was purchased from Genzyme Corp (Boston, MA).

**ELISA.** HUVE cells were harvested by collagenase treatment of vessels as previously described. Endothelial cells were plated in 96-well tissue culture plastic plates (Costar Corp, Cambridge, MA) and maintained until confluent. The plates were inverted and washed with RPMI (MA). Bioproducts, Walkersville, MD) containing 2% newborn calf serum (GIBCO) (RPMI/NCs) before the addition of MoAb RR1/1 or MoAb 4B9. A peroxidase-labeled, goat anti-mouse second antibody (Tago Laboratories, Burlingame, CA) was applied during a second 60-minute incubation. After washes with PBS, o-phenylenediamine-peroxide was added. Absorbance was read at 492 nm on a Tilter Multiscan MCC/340 (Flow Lab, McLean, VA). Absorbance with second antibody alone was subtracted from all absorbance values expressed.

**Adherence assay.** The human B-cell line, Ramos, was obtained from American Tissue Type Culture (Rockville, MD) and the human T-cell line, Molt 4, was provided by Dr Russell Ross (Department of Pathology, University of Washington, Seattle). The leukemic cell lines were maintained in MEM (Flow Lab) supplemented with 10% FCS (Flow Lab). Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque (Pharmacia) centrifugation. PBL were enriched by incubation of the mononuclear layer in serum-coated tissue culture Petri dishes. Nonadherent lymphocytes and the leukemia cell lines were labeled with 51Cr as described and were resuspended at a concentration of 1 x 10^6/mL in RPMI medium.

First- or second-passage HUVE were plated onto gelatin-coated 48-well tissue culture plates and allowed to reach confluence. rhTNF-α was added to some of the wells for 24 hours before the assay. The medium was decanted and the wells were washed twice with PBS. Subsequently, 0.2 mL of control medium (RPMI-2% NCS) or medium containing various MoAbs was added for a 30-minute preincubation; the medium was then decanted and 0.2 mL of labeled leukocytes was added to each well. Adherence of PBL or the leukemic cell lines to unstimulated or rhTNF-α-stimulated HUVE was measured as described. Levels of statistical significance were calculated by paired t-test using Statview (BrainPower, Inc, Calabasas, CA).

**RESULTS**

The specificity of MoAb 4B9 for VCAM-1 was established by an ELISA screen of untransfected CHO cells and transfected CHO cells stably expressing ELAM-1, VCAM-1, or ICAM-1 (Fig 1A). MoAb 4B9 reacted only with VCAM-1-transfected CHO cells. MoAb 4B9 immunoprecipitated a protein of approximately 100-kd cell from lysates of metabolically labeled CHO cells transfected with VCAM-1 cDNA (Fig 1B).

Unstimulated HUVE were metabolically labeled with 35S-methionine and 35S-cysteine during a 3-hour incubation with medium alone or medium containing rhTNF (10 ng/mL). Cell lysates were immunoprecipitated by MoAb 4B9 and subjected to SDS-PAGE and autoradiography (Fig 2). Analysis of the radioimmunoprecipitates from rhTNF-treated HUVE showed a major band at 93 Kd and a minor band at 80 Kd that migrated at slightly higher molecular weights (100 Kd and 92 Kd) under reducing conditions (Fig 2A). Analysis of radioimmunoprecipitates from unstimulated HUVE revealed faint bands at the same molecular weights. After N-glycosidase F treatment of the immunoprecipitate from rhTNF-treated HUVE, a single band was detected at 80 Kd (Fig 2B).

The expression of VCAM-1 and ICAM-1 was measured on rhTNF-stimulated HUVE by ELISA using MoAb 4B9 and an isotype-matched MoAb, RR1/1 (Fig 3). As previously reported, ICAM-1 was constitutively expressed by untreated HUVE. Surface expression of ICAM-1 peaked after 24 hours of continuous stimulation by rhTNF-α (10 ng/mL), and levels that were greater than basal were maintained for at least 48 hours. In contrast to the constitutive expression of ICAM-1, little or no VCAM-1 was
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Fig 1. MoAb 489 binds to VCAM-1-transfected CHO cells. The binding of MoAb 489 to untransfected CHO cells or to CHO cells stably expressing either ELAM-1, VCAM-1, or ICAM-1 was determined by ELISA (A). Untransfected CHO cells or CHO cells transfected with VCAM-1 cDNA were incubated with [35S]methio nine/cysteine. Cell lysates were immunoprecipitated with protein G and were subjected to SDS-PAGE (B).

expressed on unstimulated HUVE as assessed by ELISA. Maximal expression of VCAM-1 after continuous incubation with rhTNF-α occurred somewhat earlier than with ICAM-1 (4 hours vs 24 hours), but, like ICAM-1, increased expression of VCAM-1 persisted over 48 hours of incubation with rhTNF-α.

The induction of VCAM-1 on HUVE by various cytokines and inflammatory mediators was measured by ELISA. Treatment of HUVE with rhTNF-α (10 ng/mL), rhIL-1-α/-β (10 ng/mL), or LPS (120 ng/mL) for 24 hours induced surface expression of VCAM-1 (A vs 0.001 with unstimulated HUVE vs 0.091 with LPS; 0.118 with rhIL-1α; and 0.113 with rhIL-1β). No induction of VCAM-1 was seen when HUVE was treated with 10 ng/mL of rhTNF-β, rhIL-2, rhIL-3, rhIL-4, rhIL-6, or rhTGF-β (A vs 0.020). Additionally, treatment of HUVE with rhIFN-γ for 24 hours failed to induce VCAM-1 on HUVE (A vs 0.020), although it increased the expression of ICAM-1 (A vs 0.093 with unstimulated HUVE vs 0.128 with rhIFN-γ). Surface expression of VCAM-1 was not detected by ELISA when dermal fibroblasts or arterial smooth muscle cells were incubated with rhTNF-α for 24 hours (A vs 0.020) or when keratinocytes were incubated with rhIFN-γ for 24 hours (A vs 0.000), whereas ICAM-1 was induced on these cell types by these treatments (A vs 0.040 with unstimulated dermal fibroblasts vs 0.108 with rhTNF-α; A vs 0.115 with unstimulated arterial smooth muscle cells vs 0.164 with rhTNF-α; A vs 0.057 with unstimulated keratinocytes vs 0.111 with rhIFN-γ).

A series of experiments was performed to determine the role of VCAM-1 in the adherence of lymphocytic cell lines or PBL to HUVE that had been pretreated with rhTNF-α.
Fig 3. Induction of ICAM-1 and VCAM-1 by rhTNF. HUVE cells were treated with medium containing rhTNF (10 ng/mL) for varying periods of time. Surface expression of ICAM-1 (○) or VCAM-1 (●) was determined by ELISA using MoAb RR1/1 (anti-ICAM-1) or MoAb 4B9 (anti-VCAM-1). Values represent the means and SD of two experiments with four replicate wells in each experiment.

Before the adherence assay, HUVE were incubated with control medium, or medium containing MoAb RR 1/1 or MoAb 4B9. 1^5Cr-labeled leukocytes were preincubated with either control medium or medium containing MoAb 60.3 that is directed against the CD18 component of LFA-1 and inhibits ICAM-1-dependent adherence. Results are shown in Fig 4.

Adherence of the leukocytes to unstimulated HUVE varied (Ramos, 9% ± 1%; Molt-4, 25% ± 2%; and PBL, 22% ± 1%). Pretreatment of unstimulated HUVE with MoAb RR 1/1 significantly reduced the adherence of Molt-4 cells (18% ± 1%) and PBL (14% ± 1%). Similarly, treatment with MoAb 4B9 also significantly inhibited adherence of these leukocytes to unstimulated HUVE (Molt-4, 9% ± 1%; PBL, 15% ± 1%). Pretreatment with MoAb 60.3 significantly inhibited the adherence of PBL (14% ± 2%), but not Ramos or Molt-4, to unstimulated HUVE. An additive effect on inhibition of PBL, but not Molt-4, adherence to unstimulated HUVE was seen when MoAb 60.3 was used in combination with MoAb 4B9 (8% ± 1% v 15% ± 1% for MoAb 4B9 alone or 14% ± 2% for MoAb 60.3 alone). The low level of adherence of Ramos cells to unstimulated HUVE was unaffected by either MoAb 4B9 or MoAb 60.3.

Pretreatment of HUVE with rhTNF-α (10 ng/mL) for 24 hours caused a significant increase in adherence of Ramos, Molt-4, and PBL (Ramos, 28% ± 4%; Molt 4, 44% ± 3%; PBL, 35% ± 1%). Treatment of rhTNF-stimulated HUVE with MoAb RR 1/1 produced significant inhibition only of PBL adherence (27% ± 1%). Pretreatment of stimulated

Fig 4. Inhibition of leukocyte adherence to unstimulated or rhTNF-stimulated HUVE. Endothelial cells were treated with control medium (CM) or with medium containing rhTNF (10 ng/mL) for 24 hours. Before the assay, ^5Cr-labeled leukocytes were preincubated with medium alone (CM) or with medium containing MoAb 60.3 (40 μg/mL), and HUVE were preincubated with medium alone (CM) or with medium containing MoAb RR 1/1 (25 μg/mL) or MoAb 4B9 (20 μg/mL). Adherence was determined after a 30-minute incubation at 37°C. Values represent the means and SEM from three experiments with triplicate wells. Level of significance was determined by paired t-test (*P < .05; **P < .01 v CM; ***P < .05; ****P < .01 v MoAb RR 1/1 or MoAb 4B9 alone).
HUVE with MoAb 4B9 significantly inhibited the binding of each of the cell lines to rhTNF-stimulated HUVE (Ramos, 17% ± 2%; Molt-4, 20% ± 2%; PBL, 26% ± 1%). When leukocytes were preincubated in medium containing MoAb 60.3, adherence to rhTNF-stimulated HUVE was significantly reduced only for PBL (29% ± 2%). An additive effect on inhibition of adherence to rhTNF-stimulated HUVE was demonstrated when MoAb 60.3-treated PBL were added to MoAb 4B9-treated HUVE (15% ± 1% v 26% ± 1% with MoAb 4B9 alone or 29% ± 2% with MoAb 60.3 alone). The addition of MoAb 60.3 to MoAb 4B9 did not cause any further inhibition of adherence for either Ramos or Molt-4 cells.

The adherence of neutrophils to HUVE that were stimulated for 24 hours with rhTNF-α was also examined. No inhibition of neutrophil adherence to rhTNF-α–stimulated HUVE was observed. Percent adherence to unstimulated HUVE was 2% ± 1% and adherence to rhTNF-α treated HUVE was 17% ± 1% in the absence of MoAb 4B9 and 22% ± 2% in the presence of MoAb 4B9 (means ± SD of three experiments).

**DISCUSSION**

Three endothelial proteins have previously been reported to be involved in leukocyte adhesion to endothelium in humans: endothelial cell adhesion molecule-1 (ELAM-1), PAdGEM (GMP-140, CD62), and ICAM-1. Functional characterization of VCAM-1 demonstrates that it shares properties that have been reported for both ELAM-1 and ICAM-1. Like ELAM-1, VCAM-1 was not expressed or was minimally expressed on unstimulated endothelium, but was rapidly induced by LPS, rhTNF-α, or rhIL-1. However, VCAM-1 is functionally distinct from ELAM-1 in that VCAM-1 was expressed on HUVE up to 48 hours after continuous stimulation (Fig 3), and bound lymphocytes but not neutrophils. Neutrophils do not bind to VCAM-1–transfected COS cells and lack VLA-4, the leukocyte receptor for VCAM-1. VCAM-1 is similar to ICAM-1 in the duration of expression during continuous exposure to rhTNF-α (Fig 3), rhIL-1, or LPS. However, several features distinguish VCAM-1 from ICAM-1. Surface expression of VCAM-1 appears to be more limited than ICAM-1. In these experiments VCAM-1 was detected only on HUVE, whereas ICAM-1 was found on cytokine-activated fibroblasts, smooth muscle cells, and keratinocytes. ICAM-1 may also be a ligand for Mac-1 (CD11b/CD18) as well as LFA-1, and is involved in adherence of neutrophils as well as lymphocytes to HUVE.

However, VCAM-1 bound only mononuclear leukocytes. Finally, ICAM-1 is constitutively expressed on unstimulated HUVE, while VCAM-1 was absent, or minimally expressed, on unstimulated HUVE. Detectable levels of VCAM-1 on unstimulated HUVE were occasionally seen by ELISA, and a faint band was sometimes detected after immunoprecipitation of metabolically labeled, unstimulated HUVE. Also, the observation that MoAb 4B9 reduced adherence of lymphocytes to unstimulated HUVE indicates that VCAM-1 may be minimally expressed on unactivated HUVE. This low-level, constitutive expression of VCAM-1 on HUVE, however, may simply reflect activation by TNF-α or IL-1 released by residual mononuclear cells in early passage cultures.

The molecular cloning and sequencing of VCAM-1 predicts a 69-Kd core protein with six potential N-linked glycosylation sites that, if fully glycosylated, would yield a mature protein of 90 Kd. Because VCAM-1 also binds melanoma cells (Harlan JM, Kovach NL, unpublished observations, October 1989), it seems likely that INCAM-110 and VCAM-1 are the same protein.

In summary, we have generated an MoAb, 4B9, that recognizes a protein, VCAM-1, that is expressed on LPS–, rhTNF–, or rhIL-1–stimulated HUVE. VCAM-1 mediates a component of the adherence of PBL to rhTNF-stimulated HUVE. This CD18-independent mechanism of lymphocyte adherence to cytokine-stimulated endothelium may be an important pathway of lymphocyte emigration at sites of inflammation and immune reactions.

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