Molecular Identification of a Novel Fibrinogen Binding Site on the First Domain of ICAM-1 Regulating Leukocyte-Endothelium Bridging*

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Binding of fibrinogen to intercellular adhesion molecule 1 (ICAM-1) enhances leukocyte adhesion to endothelium by acting as a bridging molecule between the two cell types. Here, a panel of four monoclonal antibodies (mAbs) to ICAM-1 was used to dissect the structure-function requirements of this recognition. All four mAbs bound to ICAM-1 transfectants and immunoprecipitated and immunoblotted ICAM-1 from detergent-solubilized JY lymphocyte extracts. Functionally, mAbs 1G12 and 2D5 inhibited binding of 125I-fibrinogen to ICAM-1-transfectants and abrogated the enhancing effect of fibrinogen on mononuclear cell endothelial cell adhesion to endothelium and transendothelial migration. In contrast, mAbs 3D6 and 6E6 did not affect ICAM-1 recognition of fibrinogen. With respect to other ligands, mAbs 1G12 and 2D5 completely inhibited attachment of Plasmodium falciparum-infected erythrocytes to immobilized recombinant ICAM-1-Fc, whereas they had no effect on LFA-1-dependent T cell binding to ICAM-1-Fc. Conversely, mAbs 3D6 and 6E6 completely abolished LFA-1 binding to ICAM-1-Fc. Epitope assignment using ICAM-1 chimeras and receptor mutants revealed that the fibrinogen-blocking mAbs 1G12 and 2D5 reacted with domain 1 of ICAM-1, and their binding was disrupted by 97 and 70% by mutations of D26 and P70, respectively, whereas mAbs 3D6 and 6E6 bound to domain 2 of ICAM-1. By recognizing a site distinct from that of β2 integrins Mac-1 or LFA-1, fibrinogen binding to ICAM-1 may provide an alternative pathway of intercellular adhesion and/or modulate integrin-dependent adherence during inflammation and vascular injury.

The regulated adhesion of leukocytes to endothelium followed by their extravascular emigration and tissue homing form the basis of host defense mechanisms and immune-inflammatory responses. These processes depend on a stepwise adhesion cascade coordinated by the sequential ligand recog-

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1 The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; PRBC, parasitized red blood cell; TNFα, tumor necrosis factor α; PBS, phosphate-buffered saline.

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gest that ICAM-1 recognition of fibrinogen may directly contribute to the pathogenesis of vascular injury in vivo.

In order to dissect the structure-function relationship of the ICAM-1-fibrinogen interaction and to begin to elucidate its potential role in vascular cell responses we have generated a panel of monoclonal antibodies (mAbs) to ICAM-1. Using these mAbs to probe the ligand repertoire of ICAM-1, we have identified a discrete region in the first domain (22), which functions as a fibrinogen binding site and is distinct from previously recognized ICAM-1 ligand binding sites (23–26).

**EXPERIMENTAL PROCEDURES**

*Cells and Cell Culture—*Human umbilical vein endothelial cells (HUVECs) were established from umbilical cords or purchased from Clonetics (San Diego, CA). Cells were maintained in growth medium (Clonetics) containing 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker, Walkersville, MD), 2 mM L-glutamine (Irving Scientific, Santa Ana, CA), 10 mM HEPES, and endothelial cell growth factor (Biomedical Technologies, Stoughton, MA). In some experiments, HUVECs were stimulated with 100 units/ml tumor necrosis factor α (TNFα, Genzyme Corp., Cambridge, MA) for 4–6 h at 37°C (11). The prolylcytolytic cell line HL-60 (American Type Culture Collection, Rockville, MD) was transformed by infection with Moloney murine leukemia (American Type Culture Collection) plasmid and JY were grown in RPMI 1640 (BioWhittaker) containing 10% FBS (BioWhittaker), 2 mM L-glutamine (Irving), 10 mM HEPES, and 10 μM 2-mercaptoethanol (Eastman Kodak Co.). HL-60 cells were typically differentiated to a monocytic phenotype by culture with 0.1 μM 1,25-dihydroxy vitamin D3 (Biomol, Plymouth Meeting, PA) and 17.8 μg/ml indomethacin (Calbiochem), as described (12). Peripherally blood mononuclear cells were isolated by Ficoll-Hypaque gradient (Biomedical Technologies, Stoughton, MA) and drawn from normal informed volunteers by differential centrifugation. Cell extracts were centrifuged at 15,000 × g for 15 min at 4°C, and the supernatant was solubilized with 20 mM Tris-HCl, pH 8.3, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone for 20 min at 37°C, and the supernatant was solubilized with 20% SDS. Solubilized proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized using nitro blue tetrazolium (Sigma) as a substrate.

**Binding Studies—**The experimental procedures for the binding of 125I-fibrinogen to ICAM-1-expressing cells have been described (11). Briefly, serum-free suspensions of JY lymphocytes (1.5 × 10^7/ml) were mixed with 0.44 μM 125I-fibrinogen in the presence of 2.5 mM CaCl_2 for 20 min at 22°C. At the end of the incubation, cell surface-associated radioactivity was separated from unbound material by centrifugation of 300-μl aliquots of the JY incubation reaction through a mixture of silicone oil (Dow Corning, New Bedford, MA) at 15,000 × g for 5 min, and radioactivity was determined in a gamma counter. Alternatively, confluent monolayers of ICAM-1 transfectants were incubated with 0.44 μM 125I-fibrinogen for 20 min at 22°C as described above, washed three times in serum-free RPMI 1640, solubilized in 20% SDS, and counted in a gamma counter. Non specific binding (10–30%) was assessed in the presence of a 50-fold molar excess of unlabeled fibrinogen added at the start of the incubation and was subtracted from the total to calculate net specific binding. In mAb inhibition experiments, JY lymphocytes or ICAM-1 transfectants were incubated with 25 μg/ml control mAb 6A11 or the various anti-ICAM-1 mAbs for 45 min at 37°C, washed, and incubated with 0.44 μM 125I-fibrinogen as described above before determination of specific binding.

**Fibrinogen-dependent Leukocyte-Endothelium Interaction—**Serum-free suspensions of JY lymphocytes at 5 × 10^7/ml were labeled with 0.5 μCi/ml [125I]fibrinogen (Na2CrO4, specific activity, 487 mCi/mg; DuPont NEN) for 2 h at 37°C with incorporation of 2–4 cpm/ml. After washing the cells in PBS, pH 7.4, cells were equilibrated with 0.44 μM fibrinogen or control protein transferrin in the presence of 2.5 mM CaCl_2 and 100 mM D-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone for 20 min at 22°C before addition of 1.5 × 10^6 cells/ml to resting or TNFα-stimulated HUVEC monolayers. After a 30-min incubation at 22°C, cells were washed and attached cells were solubilized in 20% SDS with determination of radioactivity in a beta counter. For mAb inhibition experiments, resting or TNFα-stimulated HUVECs were incubated with 25 μg/ml control mAb 6A11 or the various anti-ICAM-1 mAbs for 45 min at 37°C, washed, and mixed with 51Cr-labeled JY lymphocytes or control monolayers were incubated with 25 μg/ml control mAb 6A11 or the various anti-ICAM-1 mAbs for 45 min at 37°C, washed, and incubated with 0.44 μM 125I-fibrinogen as described above before determination of specific binding.

**Immunoprecipitation and Immunoblotting—**JY lymphocytes (10^7/ml) were surface labeled with 125I-labeled sodium by the IODO-GEN method (30), washed to remove nonincorporated radioactivity, and lysed in 50 mM Tris-HCl, pH 8.3, 150 mM NaCl, 0.5% Triton X-100, 0.5% Nonidet P-40, 50 mM leupeptin, 100,000 IU/ml Trasylol, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM phenylalanyl-l-prolyl-l-arginine chloromethyl ketone (Boehringer Mannheim), and 100 μg/ml soybean trypsin inhibitor (Sigma) for 30 min on ice. Cell extracts were centrifuged at 15,000 × g for 20 min at 4°C to remove nuclei and other detergent-insoluble materials, preclarified, and incubated with the various primary mAbs for 16 h at 4°C with agitation. The immune complexes were precipitated by addition of 0.2 ml of Sepharose CL4B-conjugated protein A (0.2 g/ml) (Pharmacia) for 4 h at 4°C, washed in lysis buffer, boiled for 5 min at 100°C, and electrophoresed on a 7.5% SDS-polyacrylamide gel followed by autoradiography using a Kodak X-Omat AR x-ray film and intensifying screens (DuPont NEN, Wilmington, DE). For immunoprecipitation, detergent-solubilized JY extracts were electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore Corp., Bedford, MA) for 2 h at 450 mAmp, blocked in 5% nonfat dry milk for 1 h at 4°C, and incubated with 25 μg/ml aliquots of the various primary mAbs for 1 h at 4°C in 5% dry milk. Membrane filters were incubated with alkaline phosphate-conjugated goat anti-mouse IgG (Promega, Madison, WI) for 30 min at 22°C and washed, and protein bands were visualized using nitro blue tetrazolium (Sigma) as a substrate.
the wells were washed, radioactivity associated with the wells under the various conditions tested was determined in a beta counter.

In another series of experiments, ICAM-1-Fc was spotted onto plastic microtiter plates at a concentration of 50 μg/ml and allowed to adsorb for 2 h at 37 °C. Spots were aspirated to dryness and wells were postcoated with PBS, pH 7.4, plus 1% bovine serum albumin. Plates were incubated with 10 μg/ml of control or the various anti-ICAM-1 mAbs for 30–60 min before addition of aliquots of a suspension of trophozoite-infected erythrocytes at 8% parasitemia, 2% hematocrit, for 1 h at 37 °C, resuspending the mixture every 10 min, as described previously (23). Plates were washed with binding medium containing 1% glutaraldehyde to fix cells to the plate, prior to Giemsa staining and drying. The number of parasitized red blood cells (PRBCs) adherent per square millimeter was determined under high power light microscopy.

Epitope Mapping—A panel of domain expression and homolog-scanning mutants of ICAM-1 was utilized as described (23). The panel permits the assignment of mAb epitopes to individual domains or combinations of domains and sublocalization within domain 1 of ICAM-1 (23). cDNAs encoding ICAM-1 mutants into the pCDM8 expression vector were transfected into COS7 cells using the DEAE-dextran method. Transfection was allowed to proceed for 2–4 h in the presence of chloroquine before washes and a 2-min treatment with 10% DMSO (23). After culture in fresh Dulbecco’s modified Eagle’s medium plus 1% glutaraldehyde to fix cells to the plate, prior to Giemsa staining and drying. The number of parasitized red blood cells (PRBCs) adherent per square millimeter was determined under high power light microscopy.

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Resting HUVECs (A), TNFα-stimulated HUVECs (B), or stable ICAM-1 transfectants (C) were blocked in 20% human serum and analyzed for their reactivity by flow cytometry with control anti-CD11b mAb OKM1, anti-ICAM-1 mAb LB-2, or the novel anti-ICAM-1 mAbs 1G12 and 2D5 in A and B or with 1G12, 2D5, 3D6, and 6E6 in C. Background fluorescence obtained with mAb OKM1 is shown for each cell type as a negative control. Horizontal and vertical axes measure fluorescence intensity on a 4-log scale and the cell number, respectively.

Results

Establishment of Novel Anti-ICAM-1 mAbs—Four murine mAbs raised against ICAM-1+ Daudi cells were analyzed for reactivity with ICAM-1-expressing cells by flow cytometry. The newly generated mAbs 1G12 and 2D5 bound to resting HUVECs with a broad and heterogeneous pattern of reactivity, indistinguishable from that observed with anti-ICAM-1 mAbs for 5 min on ice before being washed and incubated with fluorescein-conjugated polyclonal goat anti-mouse IgG (Sigma) for 30 min on ice. Cells were washed, fixed in PBS plus 1% FBS and 1% formalin, and stored at 4 °C before flow cytometric analysis on a Coulter or a FACScan cell sorter as described (23). In another series of experiments, aliquots (1 × 10^6/ml) of resting or TNFα-stimulated HUVECs or wild-type ICAM-1 transfectants were harvested, washed once in PBS/EDTA and twice in PBS, pH 7.4, blocked in 20% human serum for 30 min at 0 °C, and incubated with 25 μg/ml of the various primary mAbs in PBS, pH 7.4, plus 2% bovine serum albumin for 1 h on ice. After the washes, the cells were stained with a 1:20 dilution of fluorescein-conjugated goat F(ab’)_2, anti-mouse IgG (Tago Inc., Burlingame, CA) for 45 min on ice, washed, and immediately analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Figure 2. Characterization of anti-ICAM-1 mAbs by immunoprecipitation and immunoblotting. A, 125I-labeled detergent-solubilized extracts of CD11b/CD18+ JT lymphocytes were immunoprecipitated with control mAb OKM1 or anti-ICAM-1 mAb 1G12, 2D5, 3D6, 6E6, or LB-2. After addition of protein A-conjugated Sepharose CLA, immunoprecipitated proteins were washed, electrophoresed on a 7.5% SDS-polyacrylamide gel under reducing conditions, and visualized by autoradiography. B, detergent-solubilized JT lymphocyte extracts were electrophoresed on a 7.5% SDS-polyacrylamide gel under reducing conditions, transferred to Immobilon membranes, and incubated with control mAb OKM1 or anti-ICAM-1 mAb 1G12 or 2D5 for 1 h at 4 °C. The membrane was washed, incubated with alkaline-phosphatase-conjugated goat anti-mouse IgG, and washed again, and protein bands were visualized by using tetrazolium salts.
Novel Anti-ICAM-1 mAbs—The effect of the new mAb panel on ICAM-1 recognition was investigated. Saturating concentrations of anti-ICAM-1 mAb 2D5 inhibited the binding of 125I-fibrinogen to JY lymphocytes (Fig. 3A) or ICAM-1 transfectants (Fig. 3B) by 70–90%, whereas control mAb 6A11 and anti-ICAM-1 mAbs 3D6 and 6E6 were ineffective under the same experimental conditions (Fig. 3). Anti-ICAM-1 mAb 1G12 partially inhibited 125I-fibrinogen binding to JY lymphocytes by 60% and to ICAM-1 transfectants by 30–45% (Fig. 3). In parallel experiments, anti-ICAM-1 mAb 1G12 or 2D5 completely inhibited the fibrinogen-dependent increase in JY lymphocyte adhesion to resting or TNFα-stimulated HUVEC (Fig. 4A) and abrogated transendothelial cell migration of vitamin D3-differentiated HL-60 cells mediated by fibrinogen (Fig. 4B), in agreement with previous observations (12). In contrast, control mAb 6A11 and anti-ICAM-1 mAbs 3D6 and 6E6 did not reduce fibrinogen-dependent adhesion of JY lymphocytes to resting or TNFα-stimulated HUVEC (Fig. 4A) or transendothelial migration of differentiated HL-60 cells mediated by fibrinogen (Fig. 4B) under the same experimental conditions. No disruption of the HUVEC monolayer was microscopically observed under the various conditions tested (not shown).

The effect of the new mAb panel on ICAM-1 recognition of β2 integrin LFA-1 (32) or P. falciparum-infected erythrocytes (7) was next investigated. Consistent with the inability of mAbs 1G12 and 2D5 to diminish binding of vitamin D3-differentiated HL-60 cells to ICAM-1 transfectants (12), these mAbs did not significantly reduce the binding of PHA-activated human T cells to immobilized recombinant ICAM-1–Fc at any concentration tested (Fig. 5A). In contrast, increasing concentrations of...
mAb 3D6 or 6E6 completely blocked T cell adhesion to ICAM-1-Fc-coated plates in a dose-dependent manner (Fig. 5A). In control experiments, anti-CD11a mAb 38 and anti-ICAM-1 mAbs 15.2 and 7.5C2 also inhibited T cell attachment to immobilized ICAM-1-Fc in a dose-dependent fashion (Fig. 5A), in agreement with previous observations (23). Finally, the fibrinogen binding site on ICAM-1 Domain 1

**Fig. 5.** Effect of anti-ICAM-1 mAbs on ICAM-1 recognition of \( \beta_2 \) integrin LFA-1 (A) or *P. falciparum*-infected erythrocytes (B). Ninety-six-well plastic microtiter plates were coated with recombinant ICAM-1-Fc, washed, and postcoated with assay buffer containing 2.5% albumin. A, ICAM-1-Fc-coated plates (0.24 \( \mu \)g/well) were incubated with the indicated increasing concentrations of the various anti-ICAM-1 mAbs or control anti-CD11a mAb 38 and further mixed with \( [3H] \)-labeled PHA-activated human T cells (2 \( \times 10^5 \)/well) for 30 min at 37 °C before washing and quantitation of specific cell attachment. B, the experimental conditions were essentially as in A, except that immobilized ICAM-1-Fc (50 \( \mu \)g/ml) was incubated with the various mAbs (10 \( \mu \)g/ml) for 30–60 min at 22 °C before addition of a suspension of trophozoite-infected erythrocytes at 8% parasitemia, 2% hematocrit for 1 h at 37 °C, and quantitation of PRBC adhesion by Giemsa staining. For both panels, data are the mean ± S.D. of 2 (A) or 4 (B) determinations.
Novel Fibrinogen Binding Site on ICAM-1 Domain 1

The experimental conditions are the same as in Fig. 4A. Control JY lymphocyte adhesion to HUVECs was 18.5% and 32.1% in the absence and in the presence of fibrinogen, respectively. The epitope assignment (23, 28) of the various anti-ICAM-1 mAbs used is as follows: mAbs 7.5C2 and CBR-IC1/4, domain 1, D26-sensitive; mAb RR1/1, domain 1, P70-sensitive; mAb 7F7, domain 1, L42-sensitive; mAb 8.4A6, domain 2. Data are expressed as the mean ± S.D. of two independent determinations.

Table I

| mAb       | Inhibition of adhesion % of control |
|-----------|------------------------------------|
| 2D5       | 93.2 ± 14.2                        |
| 7.5C2     | 95.6 ± 18.5                        |
| CBR-IC1/4 | 43.0 ± 10.2                        |
| RR1/1     | 85.3 ± 13.3                        |
| 8.4A6     | 11.2 ± 9.2                         |
| 7F7       | 3.5 ± 7.2                          |

DISCUSSION

In this study, we used a novel mAb panel specific for distinct ICAM-1 epitopes to dissect the ICAM-1 recognition for fibrinogen and its relationship with the binding site(s) for LFA-1 integrin and *P. falciparum*-infected erythrocytes. Based on inhibition studies and epitope mapping with recombinant ICAM-1 chimeras and receptor mutants, we have identified a novel fibrinogen binding site on the first Ig-like domain of ICAM-1, completely disrupted by mutation of D26 and partially affected by mutation of P70. This region was distinct from the LFA-1 interacting site (26), whereas it more completely overlapped the ICAM-1 recognition of *P. falciparum*-infected erythrocytes (23). Interestingly, the same residues, D26 and P70, have been previously shown to contribute part of the binding site of the major *Rhinovirus* serogroup on domain 1 of ICAM-1 (25).

The initial suggestion that the association of fibrinogen with ICAM-1 involved structurally distinct requirements from LFA-1 recognition (26) came from the limited inhibition of ligand binding obtained with mAb LB-2 (11), which maps to the LFA-1 binding site at K40 and L43 on domain 1 (23). Consistent with this prediction, the novel anti-ICAM-1 mAbs 2D5 and 1G12 described here completely suppressed the recognition of fibrinogen without affecting the LFA-1 binding site, and conversely, mAbs 3D6 and 6E6 failed to reduce fibrinogen binding but completely abolished the ICAM-1-LFA-1 interaction. Previous studies demonstrated that the binding sites for PRBCs and LFA-1 are spatially distinct (23) and could be located on essentially opposite sides of domain 1 of ICAM-1 by molecular modeling (23). The fact that mAbs 1G12 and 2D5 inhibit equally well both fibrinogen and PRBC binding to ICAM-1 places the fibrinogen binding site in a structural region separate from the LFA-1 site (23, 32). Three of the four described mAbs sensitive to the mutation D26PKL/KEQDS (1G12, 2D5, and 7.5C2) block fibrinogen binding, whereas the fourth mAb (CBR-IC1/4) has a partial inhibitory effect. Intriguingly, mAbs 7.5C2 and CBR-IC1/4 also block LFA-1 access to ICAM-1 but not PRBC binding (23, 28). Thus, four mAbs have now been shown to recognize a very limited region of the domain but have quite distinct functional effects. The simplest explanation for this finding, which has previously been demonstrated for CD4 (33), is that the two sets of mAbs bind to a closely related region of the molecule but approach it in quite different directions. This may also mean that the direct binding site(s) for fibrinogen, PRBCs, or LFA-1 may not involve these specific residues, but an adjacent region, dependent on the precise footprint of...
the mAb and the positioning of the Fc portion once it is bound. We were unable to test this possibility using direct binding of 125I-fibrinogen to human/murine ICAM-1 chimeras because it has been previously demonstrated that murine ICAM-1 recognizes human fibrinogen (11).

The main implication of these observations is that the ICAM-1-fibrinogen pathway of intercellular bridging (11–13, 15) operates structurally independently of $\beta_2$ integrins LFA-1 and Mac-1, whose binding sites on ICAM-1 have been previously localized to domains 1 (see above), and 3 (34), respectively. This suggests that leukocyte-endothelium interaction mediated by fibrinogen may be also independently regulated from the stepwise adhesion cascade contributed by selectins and $\beta_2$ integrins (1, 2). In this context, fibrinogen supported firm adhesion of monocytic cells to rabbit mesentery endothelium in vivo, even in the absence of an initial selectin-dependent component of leukocyte tethering and rolling (15). Alternatively, the membrane-distal location of the fibrinogen-recognition site on ICAM-1, at the apex of the protein, makes it ideally positioned to act in an accessory fashion to cooperatively potentiate LFA-1- or Mac-1-dependent leukocyte adherence, bridging the distance between cells while the steric inhibition of the cellular glyocalyx is overcome. Along these lines, it is noteworthy that although the fibrinogen binding site may have an impact on the LFA-1 site, as judged by the effect of cross-blocking mAbs and P70 mutations, the ICAM-1-LFA-1 interaction, conversely, can be entirely abrogated by mAbs to domain 2, which have no effect on fibrinogen recognition, including the novel mAbs 3D6 and 6E6 and mAbs 8.4A6 and R6.5D6, characterized in previous studies (23). This opens the attractive possibility that fibrinogen occupancy, while initiating intercellular bridging per se, may shift the adhesive balance between $\beta_2$ integrins and ICAM-1 by primarily cooperating with Mac-1-dependent interactions. The potential pathophysiological implications of these observations are underscored by the prominent role of fibrinogen as a major risk factor for atherosclerosis and vascular diseases, invariably characterized by increased leukocyte adhesion to endothelium and infiltration of the arterial intima (19). Indeed, elevated plasma concentrations of fibrinogen have been shown to correlate with an increased adherence of leukocytes to endothelium in patients with advanced atherosclerosis (35).

In summary, the identification of a novel fibrinogen binding site on ICAM-1 may help in elucidating the pleiotropic contribution of this adhesive receptor to inflammation and vascular injury (36). The differential inhibitory properties of the mAb panel described here may be beneficial to selectively targeting specific aspects of ICAM-1-dependent adherence and leukocyte recruitment at inflammatory sites in vivo.

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