Oligospecificity of the Cellular Adhesion Receptor MAC-1 Encompasses an Inducible Recognition Specificity for Fibrinogen

Dario C. Altieri,* Rossella Bader,‡ Pier M. Mannucci,‡ and Thomas S. Edgington*

*Department of Immunology, Vascular Biology Group, Research Institute of Scripps Clinic, La Jolla, California 92037; and, ‡"A. Bianchi Bonomi," Hemophilia and Thrombosis Centre, University of Milano and Maggiore Hospital, 20122 Milano, Italy

Abstract. Mitogenesis, cellular aggregation, and motility follow upon the interaction of fibrinogen with certain defined cell surface receptors. In addition to circulating platelets and vascular endothelium, monocytes express what appears to be a receptor for fibrinogen. Evidence is presented here that the leukocyte adhesion receptor Mac-1 can be specifically induced to bind fibrinogen with characteristics immunochemically and functionally distinct from the established Arg-Gly-Asp–directed fibrinogen receptors. The competence of Mac-1 as a fibrinogen receptor is a general property of cells of monocyte and myeloid lineage acquired after maturational changes of some regions of the α subunit of Mac-1 during the process of cell differentiation. This ligand recognition specificity of Mac-1 is lacking for the resting cell. Rather, induction of fibrinogen binding capacity of Mac-1 is due to a cellular response to selected agonists characterized by inducing rapid transients of cytosolic Ca^{2+}. Although different in activation pathways and recognition specificity, Mac-1 exhibits an oligospecific ligand versatility characteristic of other homologous Arg-Gly-Asp–directed adhesion receptors.

A variety of highly specific interactions between cells, as well as between cells and extracellular matrix, guide and orientate a variety of important biological responses. Disparate processes including embryonic development (37), organ function (20), and immune and inflammatory mechanisms (32) are representative of fundamental cellular processes requiring participation of general adhesion mechanisms. A family of highly differentiated receptors modulate processes of cell–cell communication as well as cellular interaction with extracellular matrix. These receptors designated "integrins" are members of a superfamily of homologous molecules one or more of which are synthesized and expressed by most if not all diploid cells (14). These are heterodimeric, transmembrane glycoproteins (GPs)[1], composed of noncovalently linked α and β subunits and some functionally recognize the Arg-Gly-Asp (RGD) sequence in the context of contiguous residues as a ligand sequence (28). Common characteristics of the integrin family are their functional versatility and ligand multispecificity (29). High affinity saturable binding of a select few ligands, i.e., oligospecificity, is exemplified by the platelet adhesion receptor GPIIb/IIIa which binds three different and nonhomologous adhesive proteins, i.e., fibrinogen, fibronectin and von Willebrand factor (vWF), recognizing intramolecular loci within which is embedded a common RGD sequence. Specific membrane association of these proteins contributes to the complex hemostatic response of the platelet (10, 24, 26). The principle of oligomolecular specificity is conserved among other individual integrins even though expressed by histogenetically distant cell types and associated with different biological functions. Among the leukocyte subfamily of integrins, i.e., Mac-1, LFA-1, and p150,95 (30), Mac-1 participates in phagocytosis of opsonized particles (4), interacts with the complement degradation product C3bi (39), and has been implicated in a mechanism of cell migration and diapedesis (41). Further, acquired ligand binding and biological function of Mac-1 has been demonstrated after appropriate stimulation. Mac-1 then serves as a high affinity receptor for coagulation factor X (1) and locally coordinates a rapid initiation of the coagulation protease cascade (3). A more complex interface of the leukocyte integrins with diverse adhesive reactions and proteolytic responses is hypothesized. We have observed that human monocytes appear to associate with fibrinogen in a receptor mediated fashion (2). These observations have been explored further to provide evidence that association of fibrinogen with cells of monocyte/myeloid lineage is attributable to Mac-1 per se, and that this receptor function correlates with differentiation. Further, selective cellular signaling eliciting rapid perturbations of cytosolic Ca^{2+} is associated with qualitative upregulation of Mac-1 and may be a relevant event in acquisition of specific high affinity receptor function.

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1. *Abbreviations used in this paper*: GP, glycoprotein; Me_2SO, dimethylsulfoxide; vWF, von Willebrand factor.

Materials and Methods

Cells and Cell Culture

Human monocytes were prepared from EDTA-washed peripheral blood mononuclear cells by adherence to sterile plastic petri dishes precoated with autologous serum as described previously (2). Cells were resuspended at 1.5–2 × 10^7/ml in low-endotoxin, serum-free RPMI 1640 medium (Whittaker M. A. Bioproducts, Walkersville, MD) containing 20 mM Hepes (Calbiochem Behring Diagnostic, La Jolla, CA), 100 μg/ml gentamycin (Geramycin, Schering Corp., Kenilworth, NJ). The monoblast leukemia cell line U937 (27), the monocytic leukemia cell line THP-1 (35) (American Type Culture Collection, Rockville, MD), and the HL 60 promyelocytic leukemia cell line (7) were maintained in RPMI 1640, 10% FCS (HyClone, Logan, UT), 20 mM Hepes, 100 μg/ml gentamycin, 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA), and 10 μM 2-mercaptoethanol (Eastman Kodak, Rochester, NY). U937 cells were induced to a differentiated monocytic phenotype by culture for 1 or 3 d in complete media supplemented with 5 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO). Granulocytic differentiation of HL 60 cells was obtained after 4 d culture in media containing 1.25% dimethylsulfoxide (Me_2SO; Sigma Chemical Co.) as described (7). In some experiments, platelets were isolated by the method of Mustard et al. (21) from freshly collected blood anticoagulated with acid/citrate dextrose solution and resuspended in Tyrode's albumin buffer, pH 7.35, at 4 × 10^8/ml.

Monoclonal Antibodies

Monoclonal antibodies to the leukocyte adhesion receptors were: TS 1/18 and 60.3 to the common β subunit shared by Mac-1, LFA-1, and p150,95 (25, 30), and a panel specific for different regions on the α subunit of Mac-1 including 60.1 (36), M 1/70 (13), OKM1, OKM9, and OKM10 (39). The monoclonal antibodies LJP5 and LJP9 are directed against epitopes of platelet GPIIb/IIIa involved in functionally independent interactions with the adhesive proteins fibrinogen and vWF (34). The monoclonal antibodies LM609 and LM142 are specific for the vitronectin receptor expressed on endothelial cells (6). Control monoclonal antibody used in binding or inhibition experiment was the anti-class I MHC W6/32.

Binding Reactions

Purified human fibrinogen was radiolabeled with ^{125}I-sodium iodide (Amersham Corp., Arlington Heights, IL) by the Iodogen method (9) to a specific activity of 0.5–0.9 μCi/μg. The interaction of ^{125}I-fibrinogen with ADP (10 μM)-stimulated suspensions of washed platelets was studied as described by Niewiarowski et al. (22). The experimental procedures for the binding of ^{125}I-fibrinogen to monocytes have been reported elsewhere (2). Typically, 3–4 × 10^6 cells were stimulated with 10 μM ADP or ATP (Sigma Chemical Co.), 1 μM of ionomycin (Calbiochem Behring), or 0.1–1 μM of the chemotactic peptide fMLP (Sigma Chemical Co.) in the presence of 1 mM $CaCl_2$ immediately before the addition of various concentrations of ^{125}I-fibrinogen. After a 20-min incubation at room temperature, equilibrium was reached and free and monocyte-bound fibrinogen were separated by centrifugation of aliquots of the incubation reaction through silicone oil. Nonspecific binding measured in the presence of a 50-fold excess of unlabeled fibrinogen was subtracted from the total. Similar analyses were performed with aliquots of each cell line.

A synthetic peptide containing the RGD sequence (RGDV) was used in competitive inhibition analyses of ^{125}I-fibrinogen binding. Different cell types stimulated with 10 μM ADP were incubated with increasing concentrations of RGDV (2.5 × 10^{-7}M to 1 × 10^{-3}M) simultaneously with the addition of 0.14 μM ^{125}I-fibrinogen. In antibody inhibition experiments, aliquots of cell suspensions were separately preincubated with saturating concentrations of each monoclonal antibody for 30 min at room temperature in the presence of 1 mM $CaCl_2$. After stimulation with 10 μM ADP, increasing concentrations of ^{125}I-fibrinogen were added (0.017–0.44 μM). The extent of ^{125}I-fibrinogen binding in the presence or absence of inhibitor was assayed after 20 min at room temperature. Reaction of the monoclonal antibodies with the monocyte surface was analyzed by indirect staining of the cell suspension. Briefly, 1 × 10^6 monocytes or THP-1 cells were incubated in V-bottomed microtiter plates (Costar Corp., Cambridge, MA) with predetermined saturating concentrations of each antibody tested for 30 min at 4°C. After washes with FCS and RPMI 1640 containing 10% FCS, cells were incubated with fluorescein-conjugated goat $(F(ab')_2$ anti-mouse IgG for additional 30 min at 4°C. After washes in RPMI 1640, specific binding of monoclonal antibodies to the cell surface was analyzed by flow cytometry on a Becton Dickinson IV/40 fluorescence activated cell sorter.

Cell Surface Labeling and Immunoprecipitation

Platelets (4 × 10^8/ml) or monocytes (7–8 × 10^7/ml) were surface labeled with ^{125}I by the Iodogen method. After extensive washes with Hepes buffer (3.8 mM Hepes, 3.8 mM NaH_2PO_4, 0.137 M NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 0.01% dextrose, pH 7.35) the cell pellet was resuspended in 1 ml of lysis buffer containing 1% NP-40, 0.5% Triton X-100, 0.05 M Tris-HCl, 0.15 M NaCl, 0.1% BSA and 1 mM phenylmethylsulphonyl fluoride (PMSF), pH 8.3, for 30 min at 4°C. Cell nuclei and other cellular debris were removed by centrifugation at 14,000 *g* for 20 min. Aliquots of the platelet or monocyte lysate were separately incubated with LJP9 or LJP5 antibody (300 μg/ml) for 1 h at 37°C with agitation. Immune complexes were precipitated with preadsorbed purified rabbit anti-mouse immunoglobulins (Zymed Laboratories Inc., San Francisco, CA) conjugated with protein A-Sepharose CL 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) for 30 min at 4°C, extensively washed in PBS, pH 7.2, and finally resuspended in 2% SDS sample buffer, pH 6.8, containing 50 mM 2-dithiothreitol (DTT), boiled for 5 min at 100°C, clarified at 14,000 *g* for 5 min, and subjected to electrophoresis on 7.5% polyacrylamide slab gels in 0.1% SDS (16). Gels were stained with Coomassie Blue, destained, dried, and exposed for autoradiography at −70°C using a Kodak X-Omat AR X ray film and intensifying screens (Cronex, E. I. DuPont de Nemours, Wilmington, DE).

Results

Comparison Between the Monocyte Fibrinogen Receptor and Other Cell Adhesion Receptors

Preliminary experiments analyzed the relationship between the monocyte fibrinogen receptor and other cell surface adhesive receptors. Concentrations of monoclonal antibodies LJP9 and LJP5 sufficient to functionally abolish the function of platelet GPIIb/IIIa (150–200 μg/ml) (34) did not diminish the binding of fibrinogen to stimulated monocytes (Table I). Although both LJP9 and LJP5 immunoprecipitated the polypeptides corresponding to the reduced platelet GPIIb/IIIa from surface iodinated platelet lysate (Fig. 1, lanes *3* and *5*), neither antibody immunoprecipitated any identifiable molecules from surface-iodinated monocyte lysate (Fig. 1, lanes *4* and *6*).

Specific monoclonal antibody binding studies were also carried out to investigate the possible monocyte surface expression of another cell adhesion receptor structurally (11) and functionally (6) related to GPIIb/IIIa; i.e., the vitronectin receptor. Two monoclonal antibodies (LM609 and LM142) which precipitate the endothelial vitronectin receptor were used to analyze the monocytoid cell line THP-1 to avoid possible interference due to other blood cell contamination. Flow cytofluorometric analysis of the cell suspension with both antibodies was consistently negative for the expression of the vitronectin receptor (Fig. 2). In contrast, virtually all cells were positively stained by the monoclonal antibody TS 1/18 against the β subunit of the leukocyte adhesion receptors Mac-1, LFA-1, and p150,95 (30) (Fig. 2).

We previously reported that monocytes fail to bind fibronectin or vWF under the same experimental conditions in which they bind fibrinogen (2), in marked distinction with the typical adhesive function of GPIIb/IIIa (28). Therefore, the possibility that, in addition to the immunologic differences revealed by monoclonal antibodies, the monocyte fibrinogen receptor might also have a recognition specificity functionally distinct from GPIIb/IIIa–vitronectin receptors was investigated further. We examined the effect of the RGD-

Table I. Effects of Anti-Platelet Fibrinogen Receptor GPIIb/IIIa Monoclonal Antibodies LJP9 and LJP5 on ^{125}I-Fibrinogen Binding to Monocytes

^{125}I-Fibrinogen added	^{125}I-Fibrinogen bound (molecules/monocyte)		
	Control	LJP9	LJP5
μM			
0.017	4,128 ± 210	3,138 ± 289	3,522 ± 400
0.035	9,293 ± 843	7,481 ± 513	8,407 ± 1,210
0.073	17,046 ± 2,410	16,875 ± 1,538	17,330 ± 4,074
0.14	33,527 ± 4,534	35,203 ± 1,681	32,297 ± 3,708

Aliquots of monocytes were separately incubated with saturating concentrations of LJP9 or LJP5 anti-platelet GPIIb/IIIa monoclonal antibodies or control antibody in the presence of 1 mM $CaCl_2$ for 30 min at room temperature. Increasing concentrations of ^{125}I-fibrinogen 0.017–0.14 μM) were then added to the ADP (10 μM) stimulated cell suspension and specific binding was quantitated at reached equilibrium after 15 min incubation at room temperature.

containing peptide RGDV on ^{125}I-fibrinogen binding to monocytes. RGDV inhibited with high efficiency the binding of fibrinogen to ADP-stimulated platelets (IC_{50} = 5 μM) (Fig. 3). In contrast, concentrations of RGDV as high as 1 mM did not diminish fibrinogen binding to its complementary receptor on ADP-stimulated monocytes (Fig. 3).

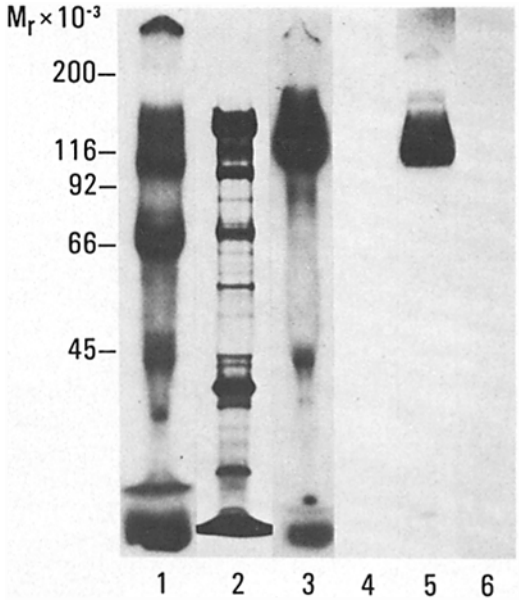

Figure 1. Immunoprecipitation of platelet or monocyte ^{125}I surface-labeled components with anti-GPIIb/IIIa monoclonal antibodies. Aliquots of iodinated platelet or monocyte lysates were separately incubated with anti-GPIIb/IIIa monoclonal antibodies LJP5 or LJP9 for 1 h at 37°C. The immune complexes were precipitated and further processed as described in Materials and Methods. Lanes *1* and *2* contain aliquots of the platelet or monocyte iodinated lysate before immunoprecipitation, respectively. Lanes *3* and *5* are the reduced antigens precipitated from the platelet lysate by LJP9 and LJP5 monoclonal antibodies and corresponding to GPIIb/IIIa. Lanes *4* and *6* are the immunoprecipitates with LJP9 and LJP5 from surface-iodinated monocyte lysate. Control experiment not shown in the figure revealed that OKM1 monoclonal antibody precipitated the polypeptides corresponding to the α and β subunit of Mac-1 from surface-iodinated monocyte lysate, under the same experimental conditions.

Identification of the Monocyte Fibrinogen Receptor in the Differentiation Molecule Mac-1

We hypothesized that the interaction of fibrinogen with monocytes might be associated with a more general mechanisms of ligand binding or adhesion of these cells, implicating the leukocyte class of adhesion receptors Mac-1, LFA-1, and p150,95 (30). Preincubation of the cell suspension with saturating concentrations of the monoclonal antibody TS 1/18 to the common β subunit shared by all three heterodimeric receptors inhibited fibrinogen binding to ADP-stimulated monocytes (Fig. 4). This implicated one or more of these molecules as responsible for the interaction with fibrinogen. However, individual receptor identity and ligand specificity is conferred by the associated α subunit. A panel of monoclonal antibodies specific for different epitopes on the α subunit of Mac-1 was analyzed for inhibition of ^{125}I-fibrinogen binding to ADP-stimulated monocytes. The results are summarized in Table II. Saturating concentrations of monoclonal antibodies OKM1 and M 1/70 inhibited fibrinogen binding to stimulated cells (Table II). However, not all intramolecular loci of Mac-1 appear to be involved in the interaction with fibrinogen as revealed by the lack of inhibi-

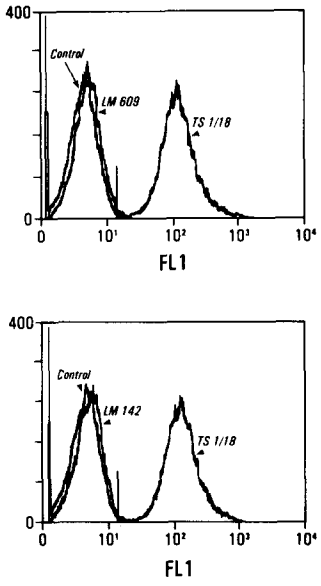

Figure 2. Indirect immunofluorescence staining of THP-1 cells with antivitronectin receptor monoclonal antibodies. 1 × 10^6 cells were separately incubated with LM609 or LM142 monoclonal antibodies for 30 min at 4°C, washed and stained with aliquots of fluorescein-conjugated goat anti-mouse immunoglobulins. The irrelevant monoclonal antibody V82A6 or the monoclonal TS 1/18 were the negative and positive controls, respectively. Fluorescence intensity (abscissa *FL1*) quantifies the surface expression of the antigen, the ordinate, and the cell number.

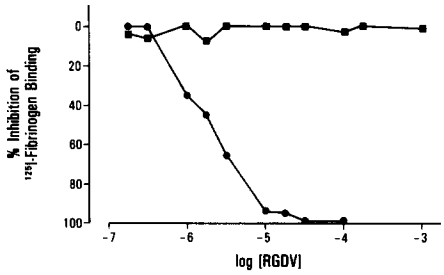

Figure 3. Effects of the synthetic peptide Arg-Gly-Asp-Val (*RGDV*) on ^{125}I-fibrinogen binding to platelets or monocytes. Various doses of RGDV (2.5×10^{-7} M to 1×10^{-3} M) were mixed at equilibrium with 10 μM ADP-stimulated suspensions of platelets or monocytes, simultaneously with a fixed concentration of ^{125}I-fibrinogen (0.14 μM). The amount of ^{125}I-fibrinogen bound in the absence of RGDV was considered 100% binding. The inhibition efficiency of RGDV was calculated by plotting the percent of inhibition of ^{125}I-fibrinogen binding to platelets (●) or monocytes (■) vs. the logarithm of peptide concentration.

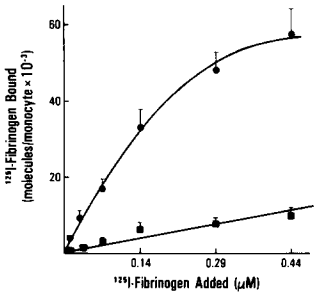

Figure 4. Effects of TS 1/18 anti-β subunit of Mac-1, LFA-1, and p150,95 monoclonal antibody on ^{125}I-fibrinogen binding to ADP-stimulated monocytes. Suspensions of monocytes were incubated with saturating concentrations of TS 1/18 or control antibody (W6/32) for 30 min at room temperature in the presence of 1 mM $CaCl_2$. After stimulation with 10 μM ADP, increasing concentrations of ^{125}I-fibrinogen were added (0.017–0.44 μM) and specific binding to control monocytes (●) or monocytes treated with TS 1/18 (■) was determined after a 20-min incubation at room temperature.

tory effect of antibodies OKM9 and OKM10 directed against different epitopes on the α subunit of Mac-1 (38).

The apparent role of Mac-1 as a cellular receptor possessing a latent recognition specificity for fibrinogen inducible by stimulation of the cells with ADP was investigated by an additional independent experimental approach. To exclude possible participation, either direct or indirect, by other blood-derived cells and also to correlate cellular differentiation with the functional acquisition of fibrinogen-binding capacity, two transformed cell lines of monocytic lineage were investigated. U937 cells were used as a monoblast precursor prototype expressing the least degree of monocytic differentiation (27); whereas THP-1 cells were used as a more mature monocytic phenotype (35). ADP-stimulated U937 bound ^{125}I-fibrinogen in a specific and saturable fashion (Fig. 5 *A*). However, the number of molecules of fibrinogen that associated with the cell surface was only 10–13% of that bound by freshly isolated monocytes under the same experimental conditions (Fig. 4). Consistently, monoclonal antibodies OKM1 (Fig. 5 *B*, upper panel, *1*) and M 1/70 (Fig. 5 *B*, lower panel, *1*) demonstrated only low density and frequency of these Mac-1 α subunit epitopes on the surface of U937 cells. After differentiation of U937 cells with PMA into a more mature monocytic phenotype, fibrinogen binding increased three- to fourfold (Fig. 5 *A*). This increased function was concordant with an increase of surface expression of the epitopes of Mac-1 recognized by OKM1 and M 1/70 (Fig. 5 *B*, *2*), which were implicated in fibrinogen binding. No further change in fibrinogen-binding capacity or surface expression of Mac-1 α subunit epitopes were observed when U937 were further cultivated for as long as 3 d in the presence of PMA (not shown).

After brief stimulation with ADP, THP-1 cells bound fibrinogen in a specific and saturable fashion (Fig. 6 *A*). Similar to freshly isolated monocytes (Table II), monoclonal antibodies OKM1 and M 1/70 blocked binding of ^{125}I-fibrinogen to THP-1 cells (Fig. 6 *A*). Consistent with a more mature phenotype than U937, THP-1 that had not been exposed to differentiation promoters constitutively expressed the epitopes of Mac-1 implicated in the binding of fibrinogen as revealed by the high density and majority of cells reactive with antibodies OKM1 (Fig. 6 *B*, upper panel) and M 1/70 (Fig. 6 *B*, lower panel) in indirect cytofluorometric analysis.

The progressive maturation of the regions of Mac-1 mediating fibrinogen binding is quantitatively analyzed in Table III. Monoclonal antibodies TS 1/18 and 60.3 detected high levels of the β subunit of Mac-1 even at early stages of cell maturation. In contrast, the epitopes of the α subunit of Mac-1 functionally involved in fibrinogen binding and recognized by monoclonal antibodies M 1/70 and OKM1 progressively increased during the cell differentiation. Other aspects of the α subunit of Mac-1 recognized by monoclonal antibody 60.1 remained quantitatively unmodified (Table III).

To determine whether inducible fibrinogen binding was a general property of cells expressing Mac-1, a relevant cell

Table II. Effects of Anti-α Subunit of Mac-1 Monoclonal Antibodies on ^{125}I-Fibrinogen Binding to Monocytes

^{125}I-Fibrinogen added	^{125}I-Fibrinogen bound (molecules/monocyte)				
	Control	OKM1	OKM9	OKM10	M 1/70
μM					
0.017	4,128 ± 210	853 ± 221	4,024 ± 682	4,000 ± 1,150	516 ± 351
0.035	9,293 ± 843	2,811 ± 859	8,648 ± 367	10,439 ± 1,876	1,069 ± 605
0.073	17,046 ± 2,410	3,920 ± 1,658	16,021 ± 1,738	18,068 ± 1,076	3,154 ± 2,307
0.147	33,527 ± 4,534	10,728 ± 2,768	30,000 ± 4,202	32,185 ± 2,690	4,465 ± 2,796

Suspensions of monocytes were separately incubated with saturating concentrations of each anti-Mac-1 antibody in the presence of 1 mM $CaCl_2$ for 30 min at room temperature. Cells were stimulated with 10 μM ADP immediately before the addition of various doses of ^{125}I-fibrinogen. Specific binding in the presence of each antibody was measured as described.

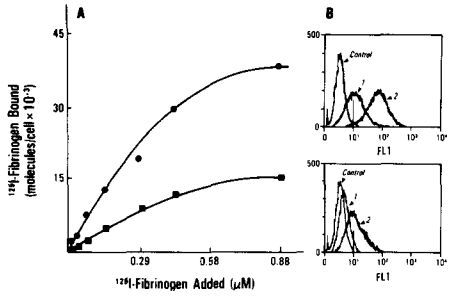

Figure 5. ^{125}I-fibrinogen binding to untreated or PMA-differentiated U937 cells. (*A*) Increasing concentrations of ^{125}I-fibrinogen (0.017–0.88 μM) were mixed with ADP-stimulated suspensions of U937 before (■) or after (●) 1 d culture in the presence of PMA. (*B*) Immunoreactivity of Mac-1 on untreated or PMA-differentiated U937 cells. Aliquots of each cell suspension were examined by flow cytometry with OKM1 (*upper panel*) or M 1/70 (*lower panel*) monoclonal antibodies, before (*1*) or after (*2*) differentiation of U937 induced by PMA.

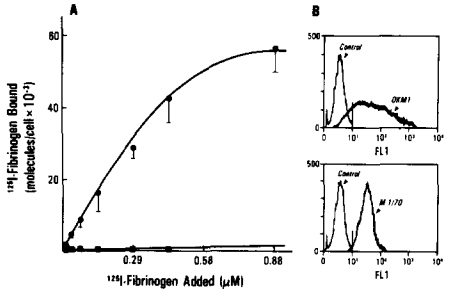

Figure 6. Effects of OKM1 anti-α subunit of Mac-1 monoclonal antibody on ^{125}I-fibrinogen binding to THP-1 cells. (*A*) Suspensions of THP-1 cells were incubated with control antibody (●) or OKM1 antibody (■) before the addition of the stimulus (ADP) and various doses of ^{125}I-fibrinogen (0.017–0.88 μM). After a 20-min incubation, specific ^{125}I-fibrinogen binding to control cells or cells treated with OKM1 was determined as described. (*B*) Immunoreactivity of Mac-1 on THP-1 cells. Monoclonal antibodies OKM1 (*upper panel*) or M 1/70 (*lower panel*) were used in flow cytofluorometric analysis of suspensions of THP-1 cells to quantitate the surface expression of the regions of Mac-1 functionally involved in fibrinogen binding at this stage of cell differentiation.

line of different differentiation was investigated. The promyelocytic leukemia cell line HL 60 was induced with Me_2SO to a granulocyte stage of differentiation (7). After stimulation with ADP, these cells bound fibrinogen avidly (Fig. 7). For comparison, the amount of ^{125}I-fibrinogen bound by monocytes is also shown in Fig. 6. Me_2SO-differentiated HL 60 bound about twofold more fibrinogen than monocytes, consistent with a quantitatively greater surface expression of Mac-1 on cells of myeloid phenotype as compared with monocytes (12).

We have observed that induction of fibrinogen binding to cells of monocyte and myeloid phenotype is rapid and induced by ADP. Initial analyses of the mechanisms of cellular signaling for this response revealed that concentrations of ADP able to induce fibrinogen binding to monocytes do not promote a mobilization of the large intracellular storage pool of Mac-1 to the plasma membrane (33), as demonstrated by the unchanged reactivity of a panel of anti-Mac-1 monoclonal antibodies before or after ADP stimulation (not shown). In addition to ADP, certain other mediators of rapid changes in the levels of intracellular cytosolic Ca^{2+} such as ionomycin (17) or the chemoattractant fMLP (15) also induced in monocytes, monocytoid cell lines, and granulocyte differentiated HL 60 cells specific fibrinogen binding to Mac-1 with characteristics indistinguishable from those observed for ADP stimulation (Table IV). Notably, the triphosphate nucleotide ATP, a known potent mobilizer of cytosolic Ca^{2+} in endothelial cells (18), neutrophils (23), and an inhibitor of fibrinogen binding to platelets (19) induced specific fibrinogen binding to monocytes and monocytoid cells (Table IV). In the absence of extracellular calcium the responses were not observed, consistent with induction of calcium flux as one of the events involved in the acquisition of fibrinogen-binding capacity of monocytic and myelocytic cells.

Discussion

In this study we have examined the interaction of fibrinogen with monocytes within the context of the adhesive responses of these cells. Either in specific forms of cellular immunity or during the complex mechanisms of inflammation, the monocyte is capable of a broad range of interactions. Among these, monocytes specifically bind fibrinogen in a reaction implicating two classes of receptors with different affinities: 1,910 high affinity sites (K_d = 4 nM) and 120,500 low affinity sites (K_d = 1.3 μM) (2). The monocyte fibrinogen receptor appeared to be only partially related to GPIIb/IIIa and in fact functional and structural differences suggested that the interaction of fibrinogen with platelets or monocytes might be mediated by different surface molecules (2). The

Table III. Maturation of the Regions of Mac-1 Involved in Fibrinogen Binding during the Process of Cell Differentiation

Monoclonal antibodies	Specificity	Surface antigen density (percentage of positive cells)		
		U937	PMA-treated U937	THP-1
TS 1/18	β subunit Mac-1	84.7 ± 1.5	88.5 ± 8.12	93.6 ± 5.6
60.3	β subunit Mac-1	83.1 ± 2	88.9 ± 7.5	87.6 ± 5.7
60.1	α subunit Mac-1	60.8 ± 1.4	63 ± 7	84.9 ± 4.8
M 1/70	α subunit Mac-1	20 ± 0.7	42 ± 4.3	73.1 ± 13.7
OKM1	α subunit Mac-1	46.3 ± 4.4	70.2 ± 10.1	60 ± 10.3

Suspensions of U937 PMA-differentiated U937 or THP-1 were separately incubated with each antibody for 30 min at 4°C before the addition of fluorescein-conjugated goat anti-mouse IgG for an additional 30 min at 4°C. After washes, cells were analyzed by flow cytometry for surface expression of the antigens.

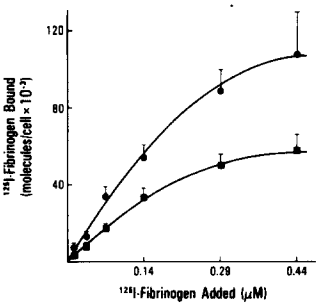

Figure 7. Comparison between Me_2SO-differentiated HL 60 cells and freshly isolated blood monocytes for the ability to bind ^{125}I-fibrinogen. Suspensions of monocytes (■) or HL 60 cells cultured for 4 d in the presence of Me_2SO to induce differentiation into mature granulocytes (●), were stimulated with 10 μM ADP in the presence of 1 mM $CaCl_2$. Increasing concentrations of ^{125}I-fibrinogen were then added (0.017–0.44 μM) and the reaction was terminated after a 20-min incubation at room temperature as described.

present study provides new and independent immunochemical evidence that the monocyte fibrinogen receptor is entirely distinct from the platelet receptor for fibrinogen, i.e., GPIIb/IIIa, and also independent from the related molecule on endothelial cells, the vitronectin receptor (11). Rather, binding of fibrinogen to monocytes is mediated by Mac-1. Inhibition of fibrinogen binding to monocytes by monoclonal antibody TS 1/18 implicates the leukocyte class of adhesion receptors Mac-1, LFA-1, and p150,95 (30). Further, monoclonal antibodies OKM1 and M 1/70 to different epitopes on the α subunit of Mac-1 inhibited fibrinogen binding to monocytes confirming the identity of Mac-1 as a fibrinogen receptor, and that as for other integrins (11) the ligand specificity for this receptor is governed by the particular α subunit. Notably, OKM10 monoclonal antibody, an efficient inhibitor of the interaction of C3bi with Mac-1 (39), failed to diminish ^{125}I-fibrinogen binding to monocytes suggesting that certain different intramolecular loci in the α subunit of Mac-1 are critical to binding of these two different ligands.

The role of Mac-1 as a leukocyte-distributed fibrinogen receptor was also confirmed and substantiated by studying a variety of transformed cell lines with monocyte–myeloid characteristics. In a system free of blood-derived cell contamination, U937, THP-1, and granulocytic differentiated HL 60 cells bound ^{125}I-fibrinogen after appropriate stimulation in a reaction consistently inhibited by monoclonal antibodies OKM1 and M 1/70. However, the different slopes of differentiation of the cells examined emphasizes how the inducible functional capacity of Mac-1 to interact with fibrinogen is related to the process of maturation and differentiation of the receptor per se, as reflected by the concordant increase of fibrinogen-binding capacity and the epitopes defined by monoclonal antibodies OKM1 and M 1/70.

Whether or not Mac-1 receptor recognition of fibrinogen might occur through recognition of a region with an embedded RGD sequence as suggested for C3bi (40) is not known. However, the short peptide RGDV, an efficient inhibitor of the interaction of fibrinogen with the platelet receptor GPIIb/IIIa was ineffective in inhibiting the association of fibrinogen with Mac-1. Short RGD peptides have also been ineffective in inhibiting C3bi association with Mac-1 (38). Further, we have observed that longer RGD peptides based on the sequence of C3bi (40) or the RGD-related peptide of the COOH terminus of the γ chain of fibrinogen (31) produce no competitive inhibition of fibrinogen binding to leukocytes for concentrations up to 0.5 mM (our unpublished observations), suggesting a specific recognition specificity of Mac-1 that is not identical to other RGD-directed receptors.

We have recently established that Mac-1 also functions as an inducible high affinity receptor for factor X in a pathway that can lead to factor X activation and initiation of thrombin generation on the cell surface (1, 3). We now demonstrate that under comparable conditions, Mac-1 binds fibrinogen. Fibrinogen, C3bi, and factor X mutually inhibit one another for binding to Mac-1, (reference 1, and our unpublished observations) and anti-Mac-1 antibodies can immunoprecipitate ^{125}I-fibrinogen chemically cross-linked to its complementary receptor on the cell surface (our unpublished observations), confirming the role of Mac-1 as an oligospecific cellular receptor. The local concentration of fibrinogen on the cell surface resulting from Mac-1 binding could facilitate more efficient conversion to fibrin, thus rendering more efficient the local generation of low levels of thrombin. Hypothetically, the high concentration of fibrinogen in plasma should competitively inhibit binding of factor X; however, sufficient factor X binding and activation occurs to support the initiation of a vigorous procoagulant response upon exposure of these cells to ADP. The basis is not yet known for the relatively greater efficiency of factor X binding, a subject of more detailed analysis of binding kinetics. As for IL-1 stimulation of cellular adhesion to endothelium, fibrinogen occupancy of Mac-1 may play a role in the localization and diapedesis of monocytes and neutrophils in the extravascular compartment in a collaborative network of interactions with endothelial cells and endothelial cell-associated fibrinogen or fibrin (3, 8). The inducibility of Mac-1 binding of fibrinogen is compara-

Table IV. Inducibility of ^{125}I-Fibrinogen Binding to THP-1 cells by Agonists Stimulating Transients in Cytosolic Ca^{2+}

^{125}I-Fibrinogen added	^{125}I-Fibrinogen bound (molecules/cell)			
	ADP	ATP	fMLP	Ionomycin
μM				
0.017	2,316 ± 280	1,787 ± 515	2,691 ± 426	2,018 ± 461
0.035	4,013 ± 828	4,142 ± 816	5,594 ± 355	4,497 ± 604
0.073	8,575 ± 1,580	7,170 ± 88	9,561 ± 2,125	7,967 ± 177
0.14	16,374 ± 4,881	14,523 ± 2,125	12,747 ± 2,130	17,564 ± 3,694
0.29	28,624 ± 2,072	32,219 ± 3,200	30,807 ± 6,847	31,250 ± 8,613
0.44	43,674 ± 8,287	46,597 ± 7,425	43,674 ± 10,146	41,608 ± 9,767

Aliquots of THP-1 cells were separately stimulated with 10 μM ADP or ATP, 1 μM fMLP, or 1 μM ionomycin in the presence of 1 mM $CaCl_2$ immediately before the addition of increasing concentrations of ^{125}I-fibrinogen (0.017–0.44 μM). Specific binding in the presence of each agonist was measured as described. Unstimulated cells or cells treated with TNFα (200 U/ml) or AMP (10 μM) did not specifically bind ^{125}I-fibrinogen.

ble to that for factor X (1). Rapid transients in the cytosolic Ca^{2+} are suggested as early events leading to transition of this receptor from a relaxed state to a reconformed high affinity state capable of avid interaction with ligand. In favor of this hypothesis, classic mobilizers of interacellular Ca^{2+} and of Ca^{2+} influx such as ionomycin (17) or fMLP (15) induce fibrinogen and factor X binding to Mac-1; and extracellular Ca^{2+} is required. Other, more physiologic agonists such as the adenine nucleotides ADP and ATP also induce fibrinogen and factor X binding to Mac-1. Both ADP and ATP produce transients in cytosolic Ca^{2+} in endothelial cells (18) and neutrophils (23) through mobilization of intracellular stores and flux from the extracellular fluid. Recent data (Altieri, D. C., and T. S. Edgington, manuscript in preparation) indicate that these agonists produce similar mobilization and flux of Ca^{2+} in cells of monocytic lineage.

In conclusion, signals involving qualitative cellular perturbations rapidly promote receptor activation of the integrin Mac-1. These reactions involve rapid acquisition of high affinity binding capacity for fibrinogen and factor X. The pattern of oligospecificity for ligands is a notable characteristic of this receptor which participates in diverse forms of molecular assembly on the surface of cells of monocyte and myeloid lineage and promote adhesive reactions.

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