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FIBRINOGEN ASSOCIATION WITH HUMAN MONOCYTES: EVIDENCE FOR CONSTITUTIVE EXPRESSION OF FIBRINOGEN RECEPTORS AND FOR INVOLVEMENT OF MAC-1 (CD18, CR3) IN THE BINDING

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Radiolabeled fibrinogen (Fg) specifically binds to mononuclear leukocytes (MNL) and to purified monocytes, but not to nylon-nonadherent lymphocytes. The association is rapid, Ca^{++}-dependent and reversible. MNL containing Fg-binding monocytes had not been exposed to endotoxin (<4 pg/mL) during the isolation and the binding test, and Fg binding was not altered by preincubation of MNL with lipopolysaccharide. The binding of Fg was inhibited by anti-Mac-1 antibodies (OKM1). Antibodies to surface-bound Fg were able to induce luminol-dependent chemiluminescence, indicating that Fg binding sites have receptor function. Emission of a signal depended on MNL exposure to Fg, on specific, divalent antibodies, but not on the antibody Fc portion. These data show that human monocytes constitutively express specific Fg receptors and suggest that Mac-1, a member of the integrin superfamily, is involved in Fg recognition.

Mononuclear phagocytes (MP) are not only of key importance in host defense, tissue repair and regeneration; they also have a role in coagulation and hemostasis (1-7). Activated MP express strong procoagulant activity (1); MP may be a focus of deposition of fibrinogen (Fg) and/or fibrin (Fb) in vivo (2) and in vitro (3,4) and are involved in Fb degradation and clearance (5). A role for Fg has recently been implicated in the differentiation of monocytes into macrophages (6). It may be assumed that such responses involve specific binding sites for Fg or Fb on the MP membrane. However, a search for binding sites to the unique amino acid sequence exposed on monomeric Fb after thrombin cleavage, glycyl-prolyl-arginine (GPR), provided no evidence for high-affinity binding sites on human MP for either the GPR sequence or the Fb fragment E (C.

Abbreviations: MP, mononuclear phagocytes; Fg, fibrinogen; Fb, fibrin; Fn, fibronectin; SDS-PAGE, sodium dodecyl sulfate polyacrylamid gel electrophoresis; PBS-A, phosphate-buffered saline containing azide; LAL, limulus amoebocyte lysate; fMLP, formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetae; MNL, mononuclear leukocytes; HBSS, Hanks' balanced salt solution; IgG-agg, heat-aggregated IgG; CL, chemiluminescence; LPS, lipopolysaccharide.

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Trezzini, unpublished). In contrast, the present report shows that specific binding sites for intact Fg are constitutively expressed on human peripheral blood monocytes, that crosslinking of bound Fg transduces a signal and that the Mac-1 antigen is involved in Fg binding.

MATERIALS AND METHODS

Reagents: Fibrinogen was purchased from Sigma, St. Louis, Mo (Cat. Nr. 4883) and from KabiVitrum (Stockholm, Sweden) and iodinated by use of chloramine T (8) to a specific activity of 10^5 cpm/ng, followed by dialysis against phosphate-buffered (10 mM, pH 7.4) saline containing 0.02 % sodium azide (PBS-A). It was nondegraded as assessed by sodium dodecyl sulfate polyacrylamid gel electrophoresis (SDS-PAGE) (9) both before and after iodination. Fibronectin (Fn) was from Serva (Heidelberg, F.R.G.), human serum albumin (HSA) from Behringwerke, goat anti-Fn and anti-Fg IgG were from Calbiochem (Lucerne). F(ab')2 and Fab fragments were prepared according to standard methods (10) and dialysed against PBS. Purity was checked by SDS-PAGE (9). RPMI 1640 was provided by Seromed (Munich). OKM1 monoclonal antibodies (Ortho, Raritan. N.J.), were kindly provided by Dr. M. Spycher, Central Laboratory of the S.R.C. Blood Transfusion Service, Berne. Also from the Central Laboratory was standard human gammaglobulin (IgG). The colorimetric limulus amoebocyte lysate (LAL) test kit for endotoxin quantitation (11) (KabiVitrum) was used in a microtiter plate assay adapted to semiautomated photometry. The following reagents were from Sigma: ADP, E. coli lipopolysaccharide (B111:04) (LPS), formyl-methionyl-leucyl-phenylalanine (fMLP), several arginyl-glycyl-asparagyl- (RGD) containing oligopeptides, cytochalasin B, hirudin, luminol, phorbol 12-myristate-13 acetate (PMA) and zymosan. Bovine thrombin (61 NIH units/mg) was from Hoffmann-La Roche, Basel, all other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Monocyte isolation: Mononuclear leukocytes (MNL) were isolated from buffy coats of whole blood donations by a ficoll-hypaque procedure (7), aimed at minimizing platelet contamination and using pyrogen-free ficoll-hypaque. Monocytes were purified from MNL by centrifugation-elutriation as described (12). Pooled monocyte fractions were between 92 and 98 % pure, as assessed by morphology and by nonspecific esterase staining (7); viability was 99 %.

Radioligand binding test: Cell aliquots (2-4 x 10^6 monocytes per ml) were incubated with ^{125}I-Fg either in the presence or absence of excess (≥100fold) cold Fg in a volume of 200 µl. ^{125}I-Fg concentration varied between 73.5 and 0.14 nM, but was usually 36.7 nM. The medium was RPMI 1640 containing HEPES (30 mM), HSA (0.5 % w/v) and azide (0.02 %). Incubation was performed for 60 min in a shaking 37°C waterbath, unless noted otherwise. Then, 80 µl aliquots were layered onto phthalate oil in 400 µl polypropylene tubes (Beckman) as described (13). Tubes were spun for 3 min at 9'000xg, and aliquots of supernatants and cellular pellets were counted separately in a gamma counter.

Chemiluminescence assay: MNL were preincubated for 60 min at 37°C, in Hanks' balanced salt solution (HBSS) either in the absence or presence (3 µM) of Fg. They were resuspended in HBSS at 4°C after 3 washes and kept on ice. These cells, or MNL prepared without pretreatment, were diluted to 0.4 x 10^6 monocytes/ml with cold HBSS containing luminol (5 µM/ml), distributed to polystyrene tubes (0.25 ml/tube), dark-adapted for 25-40 min on ice and warmed up for 4 min in a 37°C waterbath. They then were stimulated with 50 µl of antibodies (0.2 mg /ml) or with heat-aggregated (11 min 63°C) human IgG (1 mg/ml; IgG-agg.), prior to recording of chemiluminescence (CL) for 3 min in a Biolumat LB 950 luminometer (Berthold, Wildbad, F.R.G.) interphased to an IBM XT 286 personal computer. CL stimulated with PMA (10^{-7} M) or opsonized zymosan (200 µg/ml) was determined as described elsewhere (14).

RESULTS AND DISCUSSION

Peripheral blood MNL specifically bind ^{125}I-labeled Fg: Fig. 1 shows that human MNL specifically bind radiolabeled Fg, that the association occurs rapidly and can be reversed by the addition of either excess unlabeled Fg or EDTA. Studies with varying concentrations of Ca^{++} and Mg^{++} suggested that Ca^{++} but not $Mg^{++}$was required, having its optimum at physiological Ca^{++} concentrations (2.5 mM). Similar amounts of Fg were bound in the presence of cytochalasin B (12 ug/ml) or hirudin (0.2 U/ml). MNL bound similar amounts of Fg as did elutriation-purified monocytes, on a per-monocyte basis, whereas nylon wool-nonadherent cells bound little, if any, Fg (not shown). This suggests that the monocyte is the major cell within MNL which specifically binds Fg in a Ca^{++} dependent and reversible manner.

Monocytes require no exogenous stimulation for binding Fg: In earlier studies (15, 16) monocytes did not specifically bind Fg unless stimulated either with ADP, with thrombin or with fMLP (15,16). In our hands, none of these stimuli increased the amount of Fg binding to elutriated monocytes (not shown) or to unseparated MNL (Table 1). It was therefore considered that monocytes were inadvertently stimulated by LPS contaminating either monocyte or Fg preparations, thereby inducing expression of Fg binding sites. Using a colorimetric LAL-test detecting as little as 4 pg/ml standard endotoxin activity, it was assured that iodination of Fg and isolation of MNL was performed under strictly pyrogen-free conditions. We so far did not achieve elutriating monocytes in the absence of endotoxin in the high-pg range, a problem to be addressed in another study. However, MNL exposed to LPS prior to or during interaction with

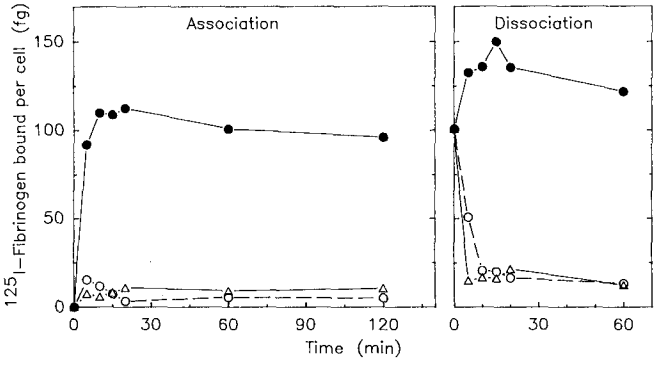

Figure 1: Kinetics of association with and dissociation from monocytes of ^{125}I-Fg. Association: MNL were exposed to labeled Fg, either alone (●) or together with cold Fg (□) or with EDTA (△), and free from bound activity was separated at the indicated time. Dissociation: To MNL pre-exposed to labeled Fg, either medium (●) or excess unlabeled Fg (□) or EDTA (△) was added, and bound Fg was determined at the indicated time. In this figure and the tables the amount of ^{125}I-Fg was calculated per monocyte, since lymphocytes also present in MNL were found to bind negligible amounts of Fg.

Table 1: Expression of Fg binding sites does not require prestimulation of monocytes

Agent used for prestimulation	^{125}I-Fg bound (fg per monocyte)	
	Total binding	Nonspecific binding
-	71.9	11.2
ADP (10 uM, 10 min)	96.2	26.7
fMLP (0.1 ug/ml, 10 min)	60.2	6.1
Thrombin (1 U/ml, 10 min) then hirudin (5 U/ml, 10 min)	68.9	17.8
LPS (10 ug/ml, 1 hr)	68.9	18.0
" (10 ug/ml, 2 hr)	80.2	6.8
Polymyxin B (5 ug/ml, added during binding test	60.9	16.5

After stimulation whith each agent, MNL were washed twice with PBS.

Fg bound similar amounts of Fg as did unexposed control cells (Table 1 and data not shown). Polymyxin B, an antibiotic neutralizing some of the effects of endotoxin (17), did not influence Fg binding to monocytes (Table 1). Moreover, the MNL used were shown to express no detectable procoagulant activity and to secrete no measurable IL-1 (P. Kuhnert and T.W. Jungi, unpublished) which would be sensitive hallmarks for priming by endotoxin (1,18). This argues against a requirement of LPS or any other stimulatory agent for expression of Fg binding sites on monocytes. Altieri et al. used monocytes purified by selective adherence and subsequent dislodgement (15) for determining Fg binding to monocytes. This procedure alters monocyte functions (19) and impairs the capacity to bind Fg (C. Trezzini, unpublished).

Involvement of Mac-1 in monocyte Fg receptor function: Other reports have previously described Fg binding sites on hematopoietic cells. These include an Fg receptor expressed on certain cell lines (20), the platelet Fg receptor, GPIIb/IIIa (21), a monocyte Fg receptor related to GPIIb/IIIA (15) and other related molecules referred to as integrins (22). Mac-1 (CR3, CD18), a member of the integrin family, is expressed on monocytes (23). The binding of integrins to their ligands often involves a conserved amino acid sequence RGD (23, 24). The effect of synthetic peptides containing RGD, and of anti-Mac-1 antibodies, on the binding of ^{125}I-Fg were therefore investigated. Table 2 shows that OKM1, an anti-Mac-1 antibody, significantly reduces binding of Fg to monocytes, thus confirming a recent abstract (16). This suggests that Mac-1 has either a Fg binding site itself, or is closely associated with a Fg binding site. Although the binding of another physiological ligand of Mac-1, C3bi, involves the RGD sequence (23), Fg binding to monocytes was not prevented by any of the RGD peptides (RGD, RGDS, GRGDS, GRGDSPK, and fibronectin-related peptide) tested at concentrations up to 1 mM. This leaves open

Table 2: Inhibition by OKM1 andibodies of Fg binding to monocytes

Exp.	OKM1[a]	^{125}I-Fg bound (fg per monocyte) after incubation with ^{125}I-Fg (37 nM)	^{125}I-Fg + EDTA (5 mM)
1	-	270.5	11.3
	10 ug/ml	63.9 (79 %)[b]	11.0
2	-	95.0	12.0
	25 ug/ml	28.6 (82 %)	13.6

a) Cells were preincubated with or without OKM1 for 30 min at 37°C before ^{125}I-Fg (either alone or with EDTA) was added for a 60 min incubation.
b) Percentages in brackets refer to inhibition of specific binding.

whether the RGD sequence within Fg is involved in Fg association with its binding site on monocytes. It has been reported that a Fg receptor is absent from monocytes of thrombasthenia Glanzmann patients which also lack GPIIb/IIIa on platelets (15). However, our preliminary evidence suggests that Fg normally binds to monocytes in these subjects (C. Trezzini et al., in preparation).

Crosslinking of bound Fg induces a respiratory burst: It was next determined whether Fg binding sites have receptor properties, capable of transducing a signal. As an effector system, triggering of a respiratory burst, as indicated by luminol-enhanced CL, was chosen for several reasons. Firstly, CL emission requires protein kinase C activation, phospholipase A2 activation, calcium mobilisation and other biochemical events associated with the triggering of many receptor systems of leukocytes (25). Secondly, the signal is specific for monocytes within MNL as lymphocytes fail to express luminol-enhanced CL (14). Thirdly, CL is observed within minutes after stimulation, thus establishing a close temporal relationship between stimulation and response. Fig. 2 shows that Fg-pretreated MNL responded within $<$1 min to stimulation by goat anti-Fg IgG, and the signal was even stronger than that induced by non-immune IgG-agg which stimulated cells via Fc receptors and served as a positive control. Anti-Fg Fab'2 also mediated a CL signal, albeit of lower magnitude than complete antibodies. This ruled out an obligatory role for Fc receptors in signal transduction although antibodies stimulating the cells by interacting with both antigen and Fc receptor induced more CL than by interacting with the FcR or the recognized ligand only. Specific antibodies were required, since anti-Fn IgG (Fig. 2) and goat IgG (not shown) did not induce a CL response. MNL preincubated in medium did not respond to anti-Fg, but did respond to IgG-agg (Fig. 2). Monovalent Fab fragments failed to elicit a respiratory burst (Fig. 2). The magnitude of the CL peak depended on the Fg concentration used for preincubation, 1 mg/ml being

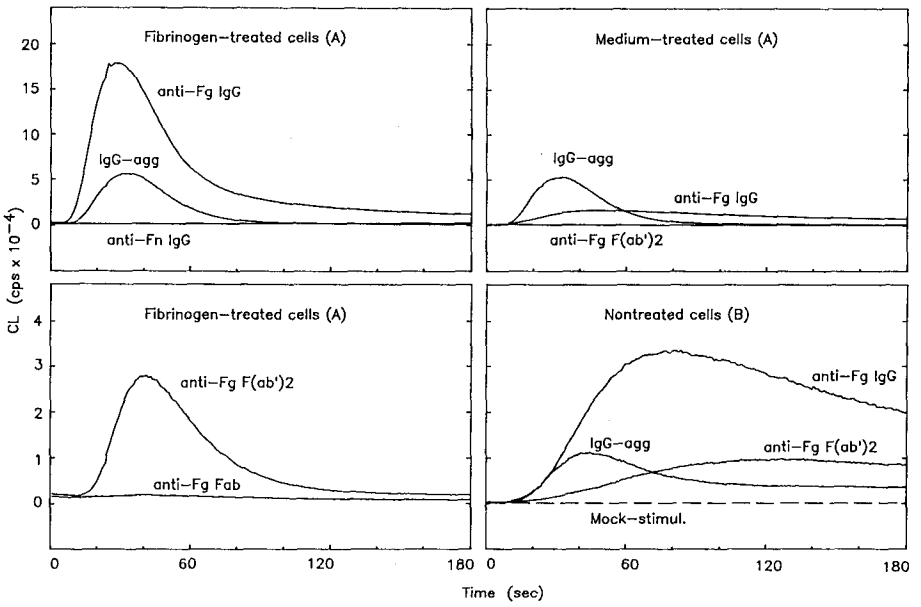

Figure 2: Temporal traces of chemiluminescence recordings from MNL stimulated with antibodies. MNL had been preincubated for 1 hr in the presence of HBSS alone or of of 1 mg/ml Fg. Cells referred to as nontreated had been isolated in the cold under pyrogen-free conditions, dark-adapted, warmed up (4 min) and then stimulated with antibodies or IgG-agg. The other cells had been incubated for 60 min with or without Fg prior to washing and dark-adaption at 4°C. Representative experiments are shown. (A) and (B) allude to two MNL donors.

optimal. After removal of Fg by washing, the intensity of the CL signal was inversely related to the time for which cells had been incubated at 37°C prior to stimulation. Warming up cells for 3-5 min was optimal However, when keeping them for 1 hr at this temperature, the response to anti-Fg F(ab')2 was lost, that to anti-Fg IgG markedly reduced, but that to IgG-agg only lightly reduced. MNL not exposed to Fg in vitro, but isolated in the cold also responded to triggering with either anti-Fg IgG and anti-Fg F(ab')2 (Fig. 2), although much weaker than Fg-exposed counterparts. Taken together, these results suggest that two requirements had to be met: (i) Monocytes had to be covered with Fg; (ii) divalent, Fg-specific antibodies were necessary. That the stimulation is mediated by complexes forming between anti-Fg Fab'2 and Fg shed from the cells is unlikely, since antibody-inducible CL disappears after prolonged incubation of Fg-covered cells at 37°C. It thus appears that CL is induced by antibody-mediated crosslinking of receptor-bound Fg on the monocyte surface. While our studies monitor for an immediate consequence of receptor stimulation, later sequelae of cell exposure to Fg have been reported. These include enhancement of procoagulant activity in monocytes (15), induction of proliferation in hematopoietic cells (20) and promotion of differentiation

from monocytes to macrophages (6). Future studies have to search for the physiological function(s) of monocyte Fg receptors.

Preincubation of MNL with Fg in the presence of anti-Mac-1 prevented a CL signal upon antibody-crosslinking (not shown). This could be due to an inhibition by anti-Mac-1 of Fg binding to its receptor. However, OKM1 pretreatment interfered also with the generation of CL induced by IgG-agg (reduction 50-90 %), zymosan and PMA (reduction 20-60 %). This is compatible with the anti-Mac-1-induced inhibition of Fc receptor-mediated phagocytosis (26,27) and with the down-modulation of the respiratory burst upon ingestion of C3b-coated Leishmania parasites (28). The exact relationship of Mac-1 and the constitutive Fg receptor on monocytes remains to be determined.

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