High Molecular Weight Kininogen Inhibits Fibrinogen Binding to Cytoadhesins of Neutrophils and Platelets

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Abstract. Fibrinogen inhibited 125I-high molecular weight kininogen (HMWK) binding and displaced bound 125I-HMWK from neutrophils. Studies were performed to determine whether fibrinogen could bind to human neutrophils and to describe the HMWK–fibrinogen interaction on cellular surfaces. At 4°C, the binding of 125I-fibrinogen to neutrophils reached a plateau by 30 min and did not decrease. At 23 and 37°C, the amount of 125I-fibrinogen bound peaked by 4 min and then decreased over time because of proteolysis of fibrinogen by human neutrophil elastase (HNE). Zn++ (50 μM) was required for binding of 125I-fibrinogen to neutrophils at 4°C and the addition of Ca++ (2 mM) increased the binding twofold. Excess unlabeled fibrinogen or HMWK completely inhibited binding of 125I-fibrinogen. Fibronectin degradation products (FNDP) partially inhibited binding, but prekallikrein and factor XII did not. The binding of 125I-fibrinogen at 4°C was reversible with a 50-fold molar excess of fibrinogen or HMWK. Binding of 125I-fibrinogen, at a concentration range of 5–200 μg/ml of added radio-ligand, was saturable with an apparent $K_d$ of 0.17 μM and 140,000 sites/cell. The binding of 125I-fibrinogen to neutrophils was not inhibited by the peptide RGDS derived from the α chain of fibrinogen or by the mAb 10E5 to the platelet glycoprotein Ib/IIa heterodimer. Fibrinogen binding was inhibited by a γ-chain peptide CYGHHLGGAKQAGDV and by mAb OKM1 but was not inhibited by OKM10, an mAb to a different domain of the adhesion glycoprotein Mac-1 (complement receptor type 3 [CR3]). HMWK binding to neutrophils was not inhibited by OKM1. These observations were consistent with a further finding that fibrinogen is a noncompetitive inhibitor of 125I-HMWK binding to neutrophils. Fibrinogen binding to ADP-stimulated platelets was increased twofold by Zn++ (50 μM) and was inhibited by HMWK. These studies indicate that fibrinogen specifically binds to the C3R receptor on the neutrophil surface through the carboxy terminal of the α-chain and that HMWK interferes with the binding of fibrinogen to integrins on both neutrophils and activated platelets.

Human kininogens are multifunctional proteins (48) coded for by a gene containing 11 exons (58). The first nine are expressed as a heavy chain–containing domain with cysteine protease inhibitory activity (43). Exon 10 codes for bradykinin and 12 additional amino acids which, like domains 1–9, are common to both high (120,000) and low (67,000) molecular weight kininogen (HMWK and LMWK, respectively). By differential splicing of the mRNA one obtains HMWK with the rest of domain 10, specifying a light chain (56 kD) with surface binding (51) and prekallikrein and factor XII binding sites (57). Together, these domains confer on HMWK the ability to accelerate activation of the contact phase of blood coagulation. Alternatively, a different splicing site allows attachment of domain 11, supplying an alternate light chain which has no known biologic activity and is contained within LMWK.

Vroman et al. (60) noted that HMWK can displace fibrinogen from artificial hydrophilic surfaces such as glass. This effect is specifically due to the light chain of HMWK since purified LMWK did not alter the surface expression of fibrinogen (47). To date no evidence exists to indicate whether such a phenomenon exists on biologic surfaces. Fibrinogen has been shown to specifically bind to platelets (7, 31) as well as to integrins on endothelial cells (14) and monocytes (1). Similarly, HMWK has been demonstrated to bind to platelets (19, 20), neutrophils (21), and endothelial cells (49). The binding of fibrinogen to platelet membrane receptors (7, 28, 35) and to neutrophil surfaces (8) may result in the aggregation of these cells. Since neutrophils can ingest fibrin (5), accumulate within thrombi, and penetrate preformed blood clots (22), we sought to determine whether 125I-fibrinogen...
binds to the neutrophil surface and, if so, to which receptor. We also investigated whether HMWK can modify the interaction of fibrinogen with neutrophils and platelets. These studies demonstrate that 125I-fibrinogen binds to complement receptor type 3 (CR3) on human neutrophils in a specific, reversible, and saturable manner. Furthermore, HMWK and fibrinogen reciprocally inhibit binding of the other protein to both the neutrophil and activated platelet surface.

Materials and Methods

Materials

Iodogen (chloramid, 1,3,4,6-tetrachloro-3 α, 6 di-phenyl-glycoluril) was obtained from Pierce Chemical Co. (Rockford, IL). 125I-Na (50 mCi/ mmol) was obtained from ICN Pharmaceuticals (Irvine, CA). N-Butylphthalate was obtained from Fisher Scientific Co. (Pittsburgh, PA). Aplieion oil (a mixture of silicon oils) was obtained from Apiex Products Limited (London, England). Hanks' balanced salt solution (HBSS) free of calcium chloride, magnesium sulfate, and magnesium chloride was obtained from Gibco Laboratories (Grand Island, NY). Ficoll-Paque was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Methoxyxycinyl-ala-alapro-val p-nitroanilide and phorbol myristate acetate were purchased from Sigma Chemical Co. (St. Louis, MO).

Plasma and Neutrophils

Pooled normal plasma (lot 6130) was purchased from George King Biomedical Inc. (Overland Park, KS). Total kininogen-deficient plasma (plasma deficient in both HMWK and LMWK) and neutrophils deficient in HMWK were donated by Mrs. Williams (12). Normal donors were young males and females (age 21-45 yr) who were not on any medication and had given their written, informed consent.

Neutrophil Isolation

Human neutrophils were isolated from whole blood anticoagulated with 0.1 vol acid-citrate-dextrose by sedimentation at 1 g in dextran (1.5%). After sedimentation, the upper leukocyte-enriched plasma was gently layered over 15 ml of Ficoll-Paque (each 100 ml contained 5.7 g Ficoll 400 and 9 g sodium diatrizoate sodium) and centrifuged at 400 g for 45 min at 23°C. The cell pellet was resuspended in an erythrocyte lysis buffer composed of 155 mM NaHCl, 2.7 mM KHCO3, and 37 mM EDTA, pH 7.4. The suspension was centrifuged at 400 g for 20 min, and the cell pellet was washed three times in excess saline, pH 7.4 (8). After the final saline wash, the cells were resuspended in HBSS without magnesium or calcium (107-109 cells/ml). Cell count and purity were determined after dilution in Turk’s solution (3% glacial acetic acid and 1% crystal violet). This procedure yielded ~8 x 10⁹ neutrophils/U whole blood, and the cells were isolated to 96% purity. Contaminating cells were platelets, not monocytes, and could not contribute >1.5% of the fibrinogen binding sites. In certain experiments the isolated neutrophils were stimulated as previously described (61) by incubating 10⁷ cells/ml with PMA (1 μM) at 25°C for 10 min. Activation was followed by measuring the release of human neutrophil elastase (HNE) into the supernatant (61).

Platelet Isolation

Platelets were prepared from freshly collected blood in acid-citrate-dextrose by the method of Mustard et al. (33). The final platelet suspension was made in a Tyrode’s solution (12 mM NaHCO3, 0.3 mM Na2H2PO4, 2.65 mM KCl, 137 mM NaCl, 12.5 mM glucose, and 3.5 mg/ml BSA, pH 7.35, containing 0.3% albumin in the absence and presence of 50 μM ZnCl2). The cell pellet was resuspended in an erythrocyte lysis buffer composed of 155 mM NaHCl, 2.7 mM KHCO3, and 37 mM EDTA, pH 7.4. The suspension was centrifuged at 400 g for 20 min, and the cell pellet was washed three times in excess saline, pH 7.4 (8). After the final saline wash, the cells were resuspended in HBSS without magnesium or calcium (107-109 cells/ml). Cell count and purity were determined after dilution in Turk’s solution (3% glacial acetic acid and 1% crystal violet). This procedure yielded ~8 x 10⁹ neutrophils/U whole blood, and the cells were isolated to 96% purity. Contaminating cells were platelets, not monocytes, and could not contribute >1.5% of the fibrinogen binding sites. In certain experiments the isolated neutrophils were stimulated as previously described (61) by incubating 10⁷ cells/ml with PMA (1 μM) at 25°C for 10 min. Activation was followed by measuring the release of human neutrophil elastase (HNE) into the supernatant (61).

Proteins

HMWK was purified using a modified method (20) of Kerbiriou and Griffin (26). Under reducing conditions, this preparation of HMWK on 7.5% polyacrylamide with SDS was primarily a single band with a molecular mass of 120 kD, >98% purity, and a specific activity of 12-20 U/ml. Purified HMWK was radiolabeled with 125I-Na using Iodogen by the method of Fraker and Speck (16) under conditions previously described (46). The specific radioactivity of the protein varied from 1-3.5 μCi/μg with >75% of the molecules of HMWK being iodinated. The radiolabeled protein retained >95% of its procoagulant activity as well as its antigenic properties, as previously reported (20). Purified factor XII (70 μg/ml) and prekallikrein (1 mg/ml) were provided by Dr. Robin Pixon (Temple University, Philadelphia, PA). Foy, an inhibitor of cathepsin G but not HNE (18), was kindly provided by Dr. Andrei Budzynski (Temple University, Philadelphia, PA). HMWK was purified to homogeneity by the procedure of Baugh and Travis (6). Human fibrinogen (Kabi AB, Stockholm, Sweden) was further purified by ammonium sulfate precipitation (34), radiolabeled with 125I-Na with the aid of Enzymo beads (Pierce Chemical Co.) or Bio-Rad-beads (Bio-Rad Laboratories, Richmond, CA), and separated from free iodine by gel filtration using a Sephadex G25 column. The radiolabeled fibrinogen demonstrated 95% clottability. Fibronec toin degradation products (FNDPs) were obtained from Dr. Andrei Budzynski (Temple University, Philadelphia, PA) and have been characterized previously (61). A peptide, C1YQQHGIL-GAQGMDV, modeled from a portion of the y chain of fibrinogen, was purchased from Peninsula Laboratories, Inc. (Belmont, CA).

Antibodies

A monoclonal polyclonal neutralizing antibody to HNE (24) was generously provided by Dr. Frederick Kueppers (Temple University, Philadelphia, PA). The mAb 10E5 was a generous gift of Dr. Barry Coller (State University of New York at Stony Brook, Stony Brook, NY) (11). The mAb OKMI was purchased from Ortho Diagnostic Systems Inc. (Westwood, MA) (59). The mAb IMY8 was purchased from Coulter Electronics Inc. (Hialeah, FL). The mAb OKM10 was obtained from Ortho Diagnostic Systems Inc. (courtesy of Dr. Pat Ralao) in the form of IgG purified using protein A.

Effect of Zn⁺⁺ on the Elution of Fibrinogen from HPLC Column

To investigate the effect of Zn⁺⁺ on the physical state of fibrinogen, the elution patterns of fibrinogen from HPLC column equilibrated either with Zn⁺⁺-free Tyrode's buffer or with Tyrode's buffer containing Zn⁺⁺ were compared. Zn⁺⁺-free Tyrode's solution was obtained by passage over a Chelex-100 resin (Bio-Rad Laboratories). A TSK4000 size exclusion HPLC column (Pharmacia Fine Chemicals) was equilibrated with normal Tyrode's solution containing 0.3% albumin in the absence and presence of 50 μM ZnSO₄. The column in Tyrode's buffer in the presence and absence was calibrated with molecular mass standards: thyroglobulin (664 kD), ferritin (440 kD), catalase (232 kD), and aldolase (158 kD). Purified fibrinogen (Kabi AB) was applied to the column with each buffer, and the elution profiles were recorded.

Assays

HMWK procoagulant activity was measured by a one-stage kaolin activation assay (12) using total kininogen-deficient plasma as substrate. Samples were compared with a simultaneously performed standard curve from pooled normal human plasma diluted 1:10 to 1:1,000 with 0.01 M Tris, 0.15 M NaCl, pH 7.4. One unit was defined as that amount of procoagulant released from 10 units of fibrinogen in 0.1 ml of pooled normal plasma diluted 1:10 to 1:1,000 with 0.01 M Tris, 0.15 M NaCl, pH 7.4. One unit was defined as that amount of procoagulant activity in 1 ml of pooled normal plasma. HNE activity was measured by a chromogenic assay using the substrate, methoxyxycinyl-ala-alapro-val-p-nitro-anilide (61).

Binding Experiments

In all binding experiments, neutrophils were at a final concentration of 10⁷/ml. In a typical binding experiment, 300-400 μl of washed neutrophils in HBSS without added calcium or magnesium, pH 7.4, were incubated at 4°C without stirring in a 1.5 ml conical polystyrene centrifuge tube (Sarstedt, Inc., Princeton, NJ). 400 μl 125I-fibrinogen or 1 μg 125I-HMWK as well as additions were added to yield a total volume of 350-450 μl. After various incubations for different experiments, 50-μl aliquots were removed (in triplicate) for each experimental point. Each sample was centrifuged at 9,650 g at 23°C in a microfuge (model B; Beckman Instruments, Inc., Fullerton, CA) for 2 min through a 200-μl mixture of silicon oils (1:9 Apiezon/A-butyl-phthalate) in polystyrene microsedimentation tubes with narrow bore extended tips (Sarstedt, Inc.) After the supernatant was removed, the tips containing the pellet were amputated and counted in a Rack gamma counter (LKB Instruments, Inc., Gaithersburg, MD).
The binding of $^{125}$I-fibrinogen to ADP-stimulated platelets was performed as previously described (28). All fibrinogen binding experiments were performed in the presence of Ca$^{++}$ (2 mM) and Mg$^{++}$ (1 mM). $^{125}$I-fibrinogen (3,000 cpm/ug) at a concentration range of 10–500 µg/ml was incubated with the platelet suspension and additions for 5 min at 23°C. The platelet pellet was separated from the supernate by centrifugation through a silicone oil gradient as previously described (28).

**Calculation of Binding Experiments**

Calculation of bound fibrinogen was based on the specific activities of the radiolabeled ligand, while nonspecific binding was the amount of $^{125}$I-fibrinogen bound in the presence of a 50-fold molar excess of unlabeled ligand. Specific binding was obtained by subtracting the nonspecific binding from the total binding.

In competition-inhibition binding experiments (50) with unlabeled fibrinogen, the binding affinity of $^{125}$I-fibrinogen was calculated from the IC$_{50}$ using a computer program to determine the 50% inhibition point (10) as previously reported (20, 46). In concentration-dependent binding experiments, the binding of $^{125}$I-fibrinogen to neutrophils was analyzed by the graphical method of Scatchard (45) as well as the computer programs of Munson and Rodbard (32) and Brass and Shattil (9) using an Apple Ile Computer (Apple Computer Corporation, Cupertino, CA). Experimental results at each concentration of ligand (both labeled and unlabeled) and bound radioligand were entered into a preanalysis program (32) to calculate the amount of specifically bound ligand. The values then were fit to a Scatchard plot by a computer program developed by Brass and Shattil (9). Comparisons of experimental groups in the binding studies were performed by the t test (paired). A difference was considered significant at p < 0.05. Binding of $^{125}$I-fibrinogen and HMWK to platelets was analyzed by the methods of Scatchard (45) and Segal (54).

**Results**

**Displacement of Bound $^{125}$I-HMWK by Fibrinogen from the Neutrophil Surface**

While investigating the binding of $^{125}$I-HMWK to neutrophils, it was found that a 50-fold molar excess of fibrinogen could inhibit HMWK binding. Studies were conducted to determine if fibrinogen could displace $^{125}$I-HMWK already bound to the surface of the neutrophil (Fig. 1). $^{125}$I-HMWK binding to neutrophils increased over time and reached a plateau by 20 min. A 50-fold molar excess of fibrinogen added at either 8 or 12 min was able to displace at least 86% of $^{125}$I-HMWK bound to the neutrophil surface.

**Binding of $^{125}$I-labeled Fibrinogen to Isolated Human Neutrophils and Effect of Neutrophil Proteases**

Investigations were conducted to determine whether $^{125}$I-
fibrinogen was able to bind directly to the external membrane of neutrophils. Binding experiments of \(^{125}I\)-fibrinogen to washed neutrophils were performed at 4, 23, and 37°C (Fig. 2). The binding of \(^{125}I\)-fibrinogen to neutrophils at 4°C increased over time reaching a plateau by 40-45 min (data not shown). However, \(^{125}I\)-fibrinogen binding at both 23 and 37°C peaked within 1-4 min and then decreased over the next 30 min to a level approaching that of nonspecific binding.

The finding that the level of \(^{125}I\)-fibrinogen binding to the surface of neutrophils at 23 and 37°C decreased further after 4 min suggested that the ligand was proteolysed. To determine if the decrease in neutrophil-bound fibrinogen at later time points was due to proteolysis, binding studies were performed at 23 and 37°C in the presence of eglin, which inhibits both of the major neutrophil proteases, cathepsin G and HNE. Eglin prevented the decrease in neutrophil-bound fibrinogen at both temperatures (Fig. 2). This finding confirmed that proteolysis of bound fibrinogen was occurring. All subsequent binding experiments were performed at 4°C.

Studies were then performed to ascertain which neutrophil enzyme was responsible for the radioligand's proteolysis (Fig. 3). Proteolysis of the bound fibrinogen was evident by an absent \(\alpha\) chain, as well as minimal cleavage of the \(\beta\) chain (Fig. 3, lanes 1, 2, and 4-7). This proteolysis of neutrophil-associated fibrinogen was only prevented by a monospecific polyclonal neutralizing antibody directed toward HNE (lane 3) and eglin (lane 8). Leupeptin (lanes 2 and 7) and soy bean trypsin inhibitor (lane 4), cysteine and serine protease inhibitors, respectively, and Foy (lane 5), a specific cathepsin G inhibitor, failed to block the proteolysis of the chains of bound \(^{125}I\)-fibrinogen. These data indicated that HNE was responsible for the proteolysis of neutrophil-bound \(^{125}I\)-fibrinogen.

**Role of Divalent Cations in Fibrinogen Binding**

The divalent cations required for the interaction of \(^{125}I\)-fibrinogen with neutrophils were determined. Since binding of \(^{125}I\)-fibrinogen to platelets required extracellular Ca\(^{++}\) (7) and binding of \(^{125}I\)-HMWK to platelets (19, 20), neutrophils (21), and endothelial cells (49) required Zn\(^{++}\), binding studies were performed in the presence of these divalent cations (Fig. 4). Binding of \(^{125}I\)-fibrinogen to neutrophils was maximal in the presence of plasma concentrations of both Ca\(^{++}\) (2 mM) and Zn\(^{++}\) (50 \(\mu\)M). Ca\(^{++}\) alone could not support \(^{125}I\)-fibrinogen binding to neutrophils. In the presence of Zn\(^{++}\) alone, binding was half that of the maximal level obtained when both Zn\(^{++}\) and Ca\(^{++}\) were present. These studies indicated that both Zn\(^{++}\) and Ca\(^{++}\) were required for optimal binding of \(^{125}I\)-fibrinogen to neutrophils. Non-specific binding was the same regardless of the absence or presence of any one or more divalent cations.

To preclude the possible formation of fibrinogen aggregates in the presence of Zn\(^{++}\), we assessed the elution pat-
terns of fibrinogen from a size exclusion HPLC column in the presence and absence of this cation (Fig. 5). The elution patterns and predicted molecular weights were identical under both experimental conditions. Furthermore, the fibrinogen peak from the Zn\(^{2+}\)-free Tyrode's solution, reapplied to the column with Zn\(^{2+}\) buffer, eluted identically (not shown).

**Specificity of Binding of 125I-Fibrinogen to Neutrophils**

To ascertain whether the binding of 125I-fibrinogen to neutrophils was specific, we first tested the capacity of other proteins besides unlabeled fibrinogen to inhibit binding of 125I-fibrinogen to neutrophils (Table I). The binding of 125I-fibrinogen to neutrophils was not inhibited by a 50-fold molar excess of factor XII or prekallikrein. FNDPs at a 50-fold molar excess inhibited fibrinogen binding by 26\% (Table I), while a 50-fold molar excess of HMWK was able to inhibit the binding by 94\% (Table I). The ability of fibrinogen to inhibit the binding of 125I-fibrinogen to neutrophils was concentration dependent (Fig. 6). Using the mean ± SEM for each point from four experiments, unlabeled fibrinogen inhibited the binding of 125I-fibrinogen to neutrophils 50\% at a concentration of 2.8 ± 1.3 \(\mu\)M, which gave a calculated apparent \(K_I\) of 0.49 ± 0.30 \(\mu\)M. This value was not significantly different from the calculated apparent \(K_I\) obtained from the IC\(_{50}\) for each individual experiment.

**Reversibility of Binding of 125I-Fibrinogen to Neutrophils**

Binding of 125I-fibrinogen to neutrophils was reversible at 4°C (Fig. 7). When a 50-fold molar excess of unlabeled fibrinogen was added to the binding reaction at 10 and 28 min, rapid dissociation of the bound ligand occurred with 94 and 88\% of the bound ligand, respectively, displaced within 1 min. Neutrophil-bound 125I-fibrinogen also was displaced by a 50-fold molar excess of HMWK when added at 5 or 10 min (Fig. 7). At 5 and 10 min, 82 and 77\%, respectively, of the bound 125I-fibrinogen was displaced by HMWK.

![Figure 5. Effect of Zn\(^{2+}\) on the elution of fibrinogen from HPLC column.](image)

**Table I. Specificity of Binding of 125I-Fibrinogen to Neutrophils**

<table>
<thead>
<tr>
<th>Protein competitor*</th>
<th>125I-Fibrinogen binding inhibition† %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>100</td>
</tr>
<tr>
<td>FNDPs</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>HMWK</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>Factor XII</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>10 ± 4</td>
</tr>
</tbody>
</table>

* Each competitor was added in a 50-fold molar excess of fibrinogen.
† Values present are the mean ± SEM of three experiments.

Since at low concentrations of added fibrinogen the binding of 125I-fibrinogen to neutrophils was specific and reversible, studies were performed under equilibrium conditions to determine if binding was saturable. Increasing concentrations of 125I-fibrinogen were added to neutrophils in the absence or presence of a 50-fold molar excess of unlabeled ligand (Fig. 8). As the concentration of 125I-fibrinogen increased, the level of specific binding increased until it leveled off at ~120 \(\mu\)g/ml of added 125I-fibrinogen (Fig. 8 B). Using the graphical method of Scatchard (45), a single saturable binding site was characterized with an apparent \(K_d\) of 0.17 \(\mu\)M and 140,000 sites/cell (Fig. 8 A). Confirmation of this interpretation of the graphical representation of the experimental data was obtained by computer analysis of the same experimental data (32). A plot of the computer-fitted points from the three individual experiments showed (Fig. 8 C) a sigmoid curve with a plateau at ~0.2 \(\mu\)M added fibrinogen. This result characterizes one saturable binding site with an apparent \(K_d\) of 0.15 \(\mu\)M. This \(K_d\) is not significantly different from the \(K_d\) of 0.49 \(\mu\)M obtained by competition inhibition analysis (Fig. 6).

**Characterization of Fibrinogen/HMWK Interaction on the Neutrophil Surface**

Since the binding of fibrinogen to platelets is inhibited by certain mAbs to the glycoprotein (GP) IIb/IIIa complex (11), the tetrapeptide RGDS (41), and a dodecapeptide from the \(\gamma\) chain of fibrinogen (27), the effect of these agents on fibrinogen binding to neutrophils was investigated. The binding of 125I-fibrinogen to neutrophils was not inhibited by 10E5 (an mAb to GP IIb/IIIa complex) (Table II). RGDS at concentrations up to 1 mM showed no inhibition (Table III). A second site on fibrinogen that is important for its binding to platelet is located in the carboxy-terminal section of the \(\gamma\)-chain. Therefore, we tested the effect of the septadecapeptide CYGQQHLGGAKQAGDV on fibrinogen binding to neutrophils (Table III). Although minimal inhibition was noted at 150 \(\mu\)M, at concentrations of 250-1,000 \(\mu\)M inhibition ranged from 62 to 79\%.

An mAb to the OKM1 antigen on neutrophils (55) com-
Figure 6. Ability of fibrinogen to inhibit 125I-fibrinogen binding to neutrophils. 125I-fibrinogen (400 μg/ml) was incubated with isolated neutrophils (10^7/ml) in the presence of HBSS without Ca ++ or Mg ++ for 20 min at 4°C in the presence of the indicated concentration of fibrinogen, Zn ++ (50 μM), and Ca ++ (2 mM). The data were fit by a computer program (9) using a four parameter logistic function which calculates the values of the ordinate into relative values between 0 and 100%. The data plotted are the mean ± SEM of four experiments.

Completely (97%) prevented 125I-fibrinogen binding to neutrophils (Table II) whereas another mAb directed against a different domain of the α chain of CR3 (OKM10) (150 μM) inhibited fibrinogen binding by only 17%. mAb IMY8 of the same subtype as OKM1 did not inhibit fibrinogen binding. Consistent with the reciprocal inhibition of fibrinogen and HMWK binding to neutrophils, studies also showed that the binding of 125I-HMWK to neutrophils was partially inhibited by FNDP and not by mAb 10E5 or IMY8 (Table II). In addition, the mAb OKM1 which completely inhibited fibrinogen binding did not inhibit HMWK binding (Table II).

Competition kinetic binding experiments were performed to determine the mechanism by which fibrinogen inhibits 125I-HMWK binding to neutrophils. Binding of 125I-HMWK to neutrophils was determined in the absence or presence of increasing concentrations of fibrinogen (data not shown). When analyzed by the method of Scatchard (45), the graph of 125I-HMWK binding to neutrophils showed parallel slopes indicating no change in Kd. Increasing the fibrinogen concentration decreased the maximum number of sites for binding 125I-HMWK. This result indicated that fibrinogen is a noncompetitive inhibitor of 125I-HMWK binding to the neutrophil surface with an apparent Kd of 50 nM.

Interaction of HMWK and Fibrinogen on the Platelet Surface

Since HMWK inhibited the binding of 125I-fibrinogen to activated neutrophils, we investigated whether HMWK could also block fibrinogen binding to platelets. Since Zn ++ is a known requirement for HMWK binding to platelets (19, 20), the effect of Zn ++ on 125I-fibrinogen binding to ADP-stimulated platelets was studied. Zn ++ (50 μM) alone could not substitute for Ca ++ or Mg ++ in the fibrinogen–platelet binding studies since the number of fibrinogen binding sites per platelet in the presence of ADP and 50 μM Zn ++ alone was only 2,850 with an apparent Kd of 10^-7 M. However, in

Figure 7. Displacement of bound 125I-fibrinogen by HMWK and unlabeled fibrinogen from the neutrophil surface. Isolated neutrophils (10^7/ml) in HBBS without Ca ++ or Mg ++, pH 7.4, were incubated at 4°C in the presence of Zn ++ (50 μM), Ca ++ (2 mM), and 125I-fibrinogen (400 μg/ml). At 5 (○) and 10 (■) min a 50-fold molar excess of unlabeled HMWK was added. At 10 (△) and 28 (□) min a 50-fold molar excess of unlabeled fibrinogen (X) was added. Non-specific binding was measured in the presence of the above additives and a 50-fold molar excess of unlabeled fibrinogen was added. Nonspecific binding was measured in the presence of the above additives and a 50-fold molar excess of unlabeled fibrinogen was added. Binding was determined at the indicated time points as described in Materials and Methods. The plotted data are the mean of three experiments.
Figure 8. Concentration dependence of binding of \(^{125}\)I-fibrinogen to neutrophils. Isolated neutrophils (PMNs) (10\(^7\)/ml) in HBSS without Ca\(^{++}\) or Mg\(^{++}\), pH 7.4, were incubated with increasing concentrations of \(^{125}\)I-fibrinogen (\(^{125}\)I-FB) in the presence or absence of a 50-fold molar excess of unlabeled fibrinogen. B shows the total nonspecific and specific binding. The figure is a representative of three identically performed experiments. A represents a Scatchard plot of the data in B. C represents a plot of bound \(^{125}\)I-fibrinogen (\(\mu\)M) on the ordinate vs. log free fibrinogen (nM) on the abscissa. The line running through the points represents a manual graph of the computer-fitted data (8) from three identically performed experiments.

Table II. Effect of RGDS and mAbs on \(^{125}\)I-Fibrinogen and \(^{125}\)I-HMWK Binding to Neutrophils

<table>
<thead>
<tr>
<th>Competitor</th>
<th>(^{125})I-Fibrinogen binding inhibition*</th>
<th>(^{125})I-HMWK binding inhibition*</th>
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<tr>
<td>None</td>
<td>0</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>100</td>
<td>86 ± 2.6</td>
</tr>
<tr>
<td>HMWK</td>
<td>94 ± 5.0</td>
<td>100</td>
</tr>
<tr>
<td>FNDPs</td>
<td>26 ± 1.0</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>10E5</td>
<td>7.0 ± 1.2</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>OKM1</td>
<td>97 ± 1</td>
<td>9.8 ± 5</td>
</tr>
<tr>
<td>OKM10</td>
<td>17.0</td>
<td>ND</td>
</tr>
<tr>
<td>IMY8</td>
<td>0</td>
<td>0 ± 4</td>
</tr>
</tbody>
</table>

Human neutrophils (10\(^7\)/ml) in HBSS without Ca\(^{++}\) or Mg\(^{++}\), pH 7.4, were preincubated with each competitor in a 50-fold molar excess in the presence of 50 \(\mu\)M Zn\(^{++}\) and 2 mM Ca\(^{++}\) at 4°C for 60 min. \(^{125}\)I-Fibrinogen (1.18 \(\mu\)M) was then added, and binding was measured at 4°C after 30 min.

* Values present are mean ± SEM of three experiments.

The presence of Ca\(^{++}\) and Mg\(^{++}\), Zn\(^{++}\) appeared to significantly increase the number of fibrinogen binding sites on activated platelets almost twofold without any significant effect on the \(K_s\) (Fig. 9). Only one class of binding sites was detected under the experimental conditions. It is noteworthy that the presence of Zn\(^{++}\) did not increase nonspecific fibrinogen binding to platelets (data not shown).

Further studies were performed to determine the influence of HMWK on fibrinogen binding to activated platelets. The effect of HMWK on \(^{125}\)I-fibrinogen binding to ADP-stimulated platelets was studied in an incubation mixture containing platelets, ADP, HMWK, and various concentrations of \(^{125}\)I-fibrinogen (Table IV). HMWK at a concentration of 50 \(\mu\)g/ml (plasma concentration, 80 \(\mu\)g/ml) appeared to be a strong inhibitor of \(^{125}\)I-fibrinogen binding to activated platelets, decreasing the number of sites sevenfold without significantly altering the \(K_s\). Analysis of the data by Lineweaver-Burk plot showed that the inhibition of fibrinogen binding by HMWK was noncompetitive (data not illustrated).

**Discussion**

This study extends observations of the interaction of fibrinogen and HMWK on artificial surfaces to biological surfaces and demonstrates that fibrinogen binds to neutrophils in a specific, reversible, and saturable manner. In addition, f-
enzymes responsible for this proteolysis. Previous studies of the azurophilic granules, the presence of HNE on the neutrophil surface is probably the result of release during the migration of neutrophils. This proteolysis is probably responsible for the drop in neutrophil-bound fibrinogen observed after 4 min at 23 and 37°C, since the presence of eglion in the incubation mixture prevents the decrease at both temperatures. Since proteolysis affects primarily the α chain, it is presumably important for the binding of 125I-fibrinogen to neutrophil surface. Further studies are needed to ascertain the effects of fibrinogen binding to neutrophils on their cellular metabolism. Since HNE is liberated during blood coagulation (39) and digests fibrinogen to release a fragment containing fibrinopeptide A (63), proteolysis of fibrinogen in vivo could occur to possibly regulate the extent of thrombus formation on or about the neutrophil surface. Weitz et al. (64) recently proposed that neutrophils migrating on a fibrinogen-coated surface form zones of close contact with fibrinogen, thus preventing the access of plasma protease inhibitors to HNE released at or near the surface interface.

The binding of fibrinogen to platelets requires the presence of Ca++ since this divalent cation is necessary for the association of GP IIb and IIIa, which when in complex, function as the fibrinogen receptor on the platelet surface (31). The amount of 125I-fibrinogen bound to the activated platelet surface is increased if physiologic concentrations of Zn++ are present in addition to the Ca++ (Table III). An increase of fibrinogen binding sites on the surface of neutrophils or platelets by Zn++ may result from an effect on fibrinogen (29) or from the direct action of this cation on the cell surface receptors. In support of the former explanation, it is known that fibrinogen will bind to a zinc affinity column (52). However, our data demonstrate that Zn++ at the concentrations used in this study did not cause fibrinogen aggregates (Fig. 5). Therefore, we consider the latter explanation. The mechanism of the Zn++ action on the cell surface is unknown; it has been recently reported that Zn++ stabilizes platelet cytoskeleton by preventing proteolysis of structural elements (68) and that it enhances protein tyrosine kinase activity of human platelet membranes (15). The relevance of these observations for the unmasking of spare fibrinogen receptors might explain the observation that the number of GP IIb/IIIa complex antigenic sites detected by certain mAbs to GP IIb/IIIa is higher than the number of fibrinogen binding sites exposed by ADP (35).

The divalent cation requirements for the binding of 125I-fibrinogen to the neutrophil surface were different from those of the platelet surface. Both Zn++ and Ca++ were required for optimal binding (Figs. 4 and 10) for both cells. However, in contrast to platelets, if Ca++ alone is used, the level of fibrinogen binding to neutrophils is not greater than nonspecific binding. Binding to neutrophils occurs in the presence of HNWK are able to act as reciprocal inhibitors on the surface of neutrophils and platelets.

When studied by SDS-polyacrylamide gel electrophoresis, 125I-fibrinogen bound to the neutrophil surface undergoes proteolysis (Fig. 3), and, at 37°C, this causes a decrease in the surface-associated fibrinogen (Fig. 2). This proteolysis affects primarily the Aα chain and to a lesser extent the Bβ chain, resulting in complete disappearance of the former band. The proteolysis is prevented by a monospecific, polyclonal antibody directed towards HNE and by eglion but not by leupeptin, soybean trypsin inhibitor, or Foy, an inhibitor of cathepsin G. These results indicate that HNE is the major enzyme responsible for this proteolysis. Previous studies have shown that cathepsin G and HNE in vitro can digest fibrinogen (36, 38). Since HNE is known to be a constituent of the azurophilic granules, the presence of HNE on the neutrophil surface is probably the result of release during the

| Table IV. Effect of HNWK on 125I-Fibrinogen Binding to Platelets |
|-------------------------|-------------------------|
| Control platelet suspension | Platelet suspension with HNWK |
| Kd (M) | 6.65 ± 1.8 | 6.38 ± 1.4 (10⁻⁷ M) |
| n | 52,580 ± 3,040 | 7,200 ± 3,600 |

400 µl of platelet suspension was incubated for 5 min at 22°C with 10 µl of 125I-fibrinogen (16-400 µg), 10 µl ADP (60 µM), Ca++ (2 mM final concentration), and Zn++ (50 µM) without or with HNWK (50 µg/ml). Scatchard analysis was used to determine Kd and number of binding sites (n). The values represent the mean ± SEM of five experiments.
of Zn²⁺ alone, but the level is only approximately half that of the optimal binding in presence of both cations.

The binding of fibrinogen to the platelet GP Ib/IIIa complex has been demonstrated to be inhibited by mAbs, such as 10E5, against the heterodimer complex (11) as well as by the adhesive tetrapeptide RGDS (41). The sequence RGD, which is a recognition site for certain integrins (42), is present in the fibrinogen molecule at two separate sites (62) and appears to be important in cell binding (17). The dodecapeptide for the carboxy terminal of the γ chain is also important (27). 125I-fibrinogen binding to neutrophils, however, is not inhibited by the mAb 10E5 or by the adhesive peptide RGDS. Moreover, RGD is also present in fibronectin (37) but FNDPs, which are known to stimulate neutrophils (59) and recognize macrophages (62, 63), only weakly inhibit fibrinogen binding to neutrophils (Table II). In contrast, the sepatadecapeptide derived from the fibrinogen γ chain did inhibit fibrinogen binding to neutrophils at concentrations ≥250 μM. This finding suggests that the γ chain plays a role in the interaction on the neutrophil surface similar to its interaction with the integrin GP Ib on the platelet surface.

We tested two mAbs (OKM1 and OKM10) directed toward different epitopes (13) on the α chain of CR3 for their effect on 125I-fibrinogen binding to neutrophils. The heterodimer complex recognized by OKM1 and OKM10 is one of a family of human leukocyte differentiation antigens with distinct α subunits and a common β subunit which include the lymphocyte function-associated antigen (LFA-1), CR3, or Mac-1, and the P150,95 molecule (44). Recent epitope mapping studies have shown that CR3 is a multivalent receptor (65, 66) with at least two independent adhesion-related functions; one is identified by OKM1 and the other by OKM10 (13). OKM1 has a functional domain which is involved with such neutrophil functions as aggregation (13), spreading on plastic surfaces (4), and chemotaxis (13). Most recently Wright et al. (67) studied the adhesion of neutrophil to fibrinogen-coated surfaces. In agreement with our data, this reaction was not affected by mAbs directed against GP Ib/IIIa complex or by RGD-derived peptides and was inhibited by the carboxy-terminal peptide of the fibrinogen γ chain. However, neutrophil adherence to surface-bound fibrinogen was inhibited by OKM10 and was not inhibited by OKM1. In our studies OKM1 completely inhibited the binding of 125I-fibrinogen to neutrophils while OKM10 resulted in only 17% inhibition. Our results differ from those of Weitz et al. (64), but any difference in results could be explained by the fact that their system used surface-adsorbed fibrinogen and no direct binding studies, while our experiments were direct binding studies done in a fluid phase. The difference between surface-bound fibrinogen and fluid-phase fibrinogen has been studied with respect to platelet adhesion. Lindon et al. (29) have demonstrated that the reaction of platelets with surface-bound fibrinogen correlated with the quantity of antigenic fibrinogen but not with total fibrinogen adsorbed, implying a conformational change upon binding.

Our results do agree with those of Altieri et al. (3) who demonstrated that neither OKM10 nor RGD–containing synthetic peptide inhibited binding of fibrinogen to monocytes. In addition Altieri et al. have previously shown that binding of fibrinogen to monocytes is inhibited by OKM1 (2). These leukocyte differentiation antigens are part of the integrin receptor super family (23, 40, 44). We found that there are 140,000 sites for fibrinogen on the neutrophil, in agreement with Springer et al. (56) who found 140,000 binding sites on Mac-1, consistent with our hypothesis that CR3 (CD11b/CD18) of human neutrophils functions as a fibrinogen receptor through an RGD-independent mechanism. The data by Altieri et al. (3) suggest that the same mechanism operates during the interaction of monocytes with fibrinogen. At the present stage of knowledge, it is difficult to extrapolate our experimental data to an in vivo situation. Since the plasma fibrinogen exceeds the affinity for fibrinogen to bind to neutrophils, it is possible that all fibrinogen receptors on the neutrophils are occupied under physiological conditions in the flowing blood.

Fibrinogen and HMWK can displace each other from the surface of the neutrophil (Figs. 1 and 7). Furthermore, HMWK functions as an inhibitor of fibrinogen binding on the surface of the activated platelet (Table III). Previous studies showed that fibrinogen did not inhibit 125I-HMWK binding to unstimulated platelets (23). Activation of platelets may initiate membrane changes that allow HMWK to compete with fibrinogen. Our own studies may have been performed with neutrophils that were partially activated in the course of isolation. To begin to address this question, neutrophils were stimulated with phorbol myristate acetate (1 μM); these neutrophils released significant levels of elastase compared to resting cells and bound three- to fivefold more fibrinogen than the resting cells. Further studies are needed to characterize the mechanism of this additional receptor exposure.

The finding that upon binding to neutrophils fibrinogen is proteolized by elastase suggested that this granule enzyme may have adsorbed to the neutrophil before binding of fibrinogen to the cell surface. Since the inhibition of 125I-HMWK binding to neutrophils by fibrinogen (Table II) and 125I-fibrinogen binding to platelets by HMWK (Table IV) are noncompetitive, fibrinogen and HMWK probably do not share the same receptor(s) on either the neutrophil or platelet surface. This interpretation is reinforced by the results obtained from the experiments with various mAbs. An mAb, 10E5, to the GP Ib/IIIa complex inhibits binding of fibrinogen to the platelet surface but does not inhibit the binding of HMWK to platelets (our unpublished observation), while the mAb, OKM1 inhibits binding of fibrinogen to the neutrophil surface but does not inhibit binding of HMWK (Table II). The inhibitory effect of HMWK on the binding of fibrinogen to platelets and neutrophils may result form steric hindrance since both HMWK and fibrinogen are large asymmetric proteins. It is likely that the fibrinogen and HMWK binding sites, while distinct, are closely located on the platelet and neutrophil membrane.

The functional significance of 125I-HMWK binding to neutrophils is not fully understood. In other studies (21) we have demonstrated that neutrophil activation induced by kalikrein required the presence of HMWK, since a patient deficient in HMWK in both neutrophils (21) and plasma (12) exhibited no HNE release after contact activation. This patient's neutrophils function normally in normal plasma. Although the functional significance of binding of 125I-fibrinogen to neutrophils is not completely elucidated, our demonstration that binding of fibrinogen is inhibited by an mAb to CR3 suggests that fibrinogen may play a role in such neutrophil functions such as aggregation (2), spreading on sur-
faces, adhesion, and chemotaxis. The fact that a combined Mac-1, LFA-1, and Leu M5 leukocyte-deficiency syndrome (4) is characterized by recurrent bacterial and fungal infections, delayed umbilical cord separations, poor wound healing, and an impaired inflammatory response, suggests possible pathological implications for fibrinogen binding to neutrophils. Human fibrinopeptide B, a thrombin-derived proteolytic cleavage product of the fibrinogen β chain, has been demonstrated to cause neutrophil chemotaxis (25). Further studies have shown that this chemotactic effect occurs in the absence of degranulation, aggregation, or superoxide production, and does not involve the neutrophil receptor for C5a, N-formyl-methionyl-leucyl-phenylalanine, or LTβ (55). Thus, elastase-catalyzed fibrinogen derivatives bound to the neutrophil surface may function in the recruitment of neutrophils to the area of inflammation.

We thank Rita Stewart and Cathy Spitta for help with the manuscript and reference preparation.

This work was supported in part by the National Institutes of Health grants HL06940, an individual postdoctoral fellowship, to E. J. Gustafson; HL35553 and Research Career Development Award HL01615 to A. H. Schmaier; HL15226 to S. Niewiarowski; HL24365 to R. W. Colman; and HL19035 to R. W. Colman and Y. T. Wachtfogel. Additional support was supplied by a grant from the Ben Franklin Partnership of the State of Pennsylvania to Y. T. Wachtfogel, and a grant from the Cystic Fibrosis Foundation to Y. T. Wachtfogel. A murine monoclonal antibody that completely blocks the binding of human fibrinogen to activated platelet and fibrinogen binding to activated platelet. J. Biol. Chem. 260:11891-11896.


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