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

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Abstract. Fibrinogen inhibited ^{125}I-high molecular weight kininogen (HMWK) binding and displaced bound ^{125}I-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 ^{125}I-fibrinogen to neutrophils reached a plateau by 30 min and did not decrease. At 23 and 37°C, the amount of ^{125}I-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 ^{125}I-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 ^{125}I-fibrinogen. Fibronectin degradation products (FNDP) partially inhibited binding, but prekallikrein and factor XII did not. The binding of ^{125}I-fibrinogen at 4°C was reversible with a 50-fold molar excess of fibrinogen or HMWK. Binding of ^{125}I-fibrinogen, at a concentration range of 5–200 μg/ml of added radioligand, was saturable with an apparent K_d of 0.17 μM and 140,000 sites/cell. The binding of ^{125}I-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 IIb/IIIa 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 ^{125}I-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 γ-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).[1] 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 XI 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 ^{125}I-fibrinogen

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1. *Abbreviations used in this paper*: CR3, complement receptor type 3; FNDP, fibronectin degradation product; GP, glycoprotein; HBSS, Hanks' balanced salt solution; HMWK, high molecular weight kininogen; HNE, human neutrophil elastase; LMWK, low molecular weight kininogen.

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 ^{125}I-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 (chloramide, 1,3,4,6-tetrachloro-3 α, 6 α-diphenyl-glycoluril) was obtained from Pierce Chemical Co. (Rockford, IL). ^{125}I-Na (50 mCi/mmol) was obtained from ICN Pharmaceuticals (Irvine, CA). *N*-Butyl-phthalate was obtained from Fisher Scientific Co. (Pittsburgh, PA). Apiezion oil (a mixture of silicon oils) was obtained from Apiezion 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). Methoxysuccinyl-Ala-Ala-Pro-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 ditrizoate 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 NH_4Cl, 2.7 mM $KHCO_3$, and 3.7 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 (10^7–10^9 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 $\sim 8 \times 10^8$ 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 10^7 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 $NaHCO_3$, 0.3 mM $Na_2H_2PO_4$, 2.65 mM KCl, 137 mM NaCl, 12.5 mM glucose, and 3.5 mg/ml BSA, pH 7.35, containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$).

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 ^{125}I-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 Pixley (Temple University, Philadelphia, PA). Foy, an inhibitor of cathepsin G but not HNE (18), was kindly provided by Dr. Frederick Kueppers (Temple University, Philadelphia, PA). Eglin, an inhibitor of both cathepsin G and HNE, was generously provided by Dr. Hans-Peter Schnebli, (Ciba-Geigy AG, Basel, Switzerland) (53). HNE 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 ^{125}I-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. Fibronectin degradation products (FNDPs) were obtained from Dr. Andrei Budzynski (Temple University, Philadelphia, PA) and have been characterized previously (61). A peptide, CYGQQHHLGGAKQAGDV, modeled from a portion of the γ chain of fibrinogen, was purchased from Peninsula Laboratories, Inc. (Belmont, CA).

Antibodies

A monospecific 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 OKM1 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 Rao) 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 Z^{++}-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 $ZnSO_4$. 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 activity in 1 ml of pooled normal plasma. HNE activity was measured by a chromogenic assay using the substrate, methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitro-anilide (61).

Binding Experiments

In all binding experiments, neutrophils were at a final concentration of 10^7/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 polypropylene centrifuge tube (Sarstedt, Inc., Princeton, NJ). 400 μg ^{125}I-fibrinogen or 1 μg ^{125}I-HMWK as well as additions were added to yield a total volume of 350–450 μl. After variable 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 Apiezion/*N*-butyl-phthalate) in polypropylene 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/μg) 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, and the results were expressed as micrograms of fibrinogen bound per 10^7 neutrophils. In a typical experiment total binding was the amount of ^{125}I-fibrinogen bound in the absence of unlabeled 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 IIe 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).

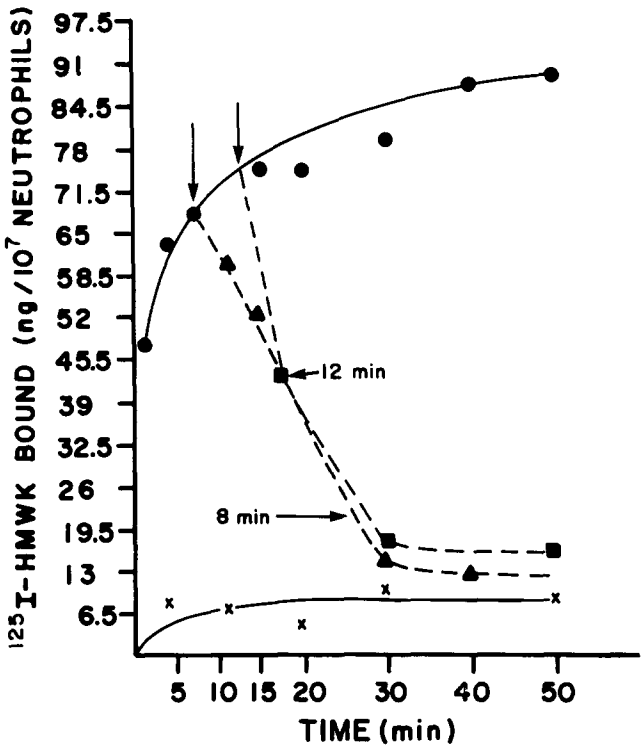

Figure 1. Displacement of bound ^{125}I-HMWK by fibrinogen from the neutrophil surface. Isolated neutrophils (10^7/ml) in HBSS without Ca^{++} or Mg^{++}, pH 7.4, were incubated with Zn^{++} (50 μM), Ca^{++} (2 mM), and ^{125}I-HMWK (1 μg/ml) at 23°C (●). At 8 (▲) and 12 (■) min, a 50-fold molar excess of unlabeled fibrinogen was added. Nonspecific binding (X) was measured by ^{125}I-HMWK binding in the presence of the above divalent cations and a 50-fold molar excess of unlabeled HMWK. The binding was determined at the indicated time points as described in Materials and Methods. The plotted data are the mean of four experiments.

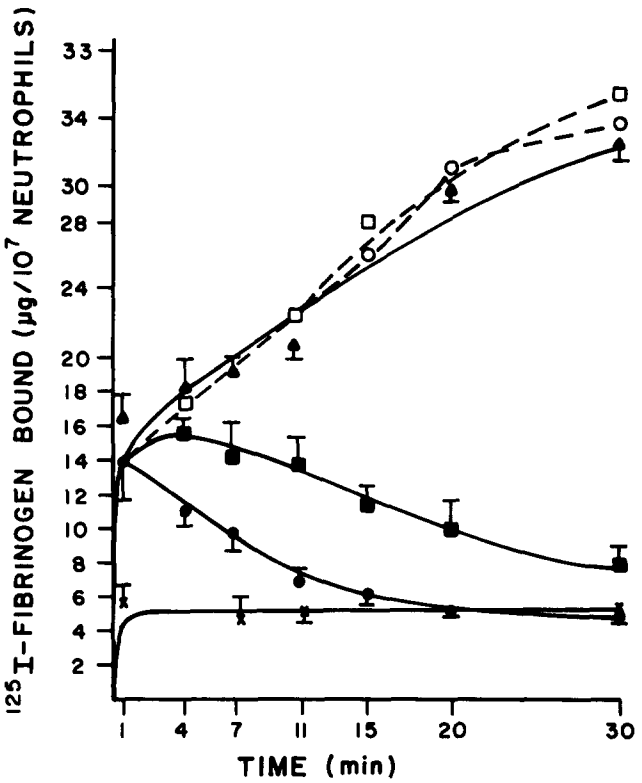

Figure 2. Binding of ^{125}I-fibrinogen to neutrophils at various temperatures. Isolated neutrophils (10^7/ml) in HBSS without Ca^{++} or Mg^{++}, pH 7.4, were incubated with ^{125}I-fibrinogen (400 μg/ml) in the presence of Zn^{++} (50 μM) and Ca^{++} (2 mM) at 4 (▲), 23 (■), or 37°C (●). Simultaneously, eglin (50 μm) was added to incubations at 23 (○) and 37°C (□) in addition to the cations and fibrinogen. Nonspecific binding was determined with all the above additions plus a 50-fold molar excess of unlabeled fibrinogen. At the designated time points, samples were removed and the amount of ^{125}I-fibrinogen bound to neutrophils was determined as indicated in Materials and Methods. The plotted results are the mean ± SEM of three experiments where indicated.

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 proteolyzed. 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 Aα chain, as well as minimal cleavage of the Bβ 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.

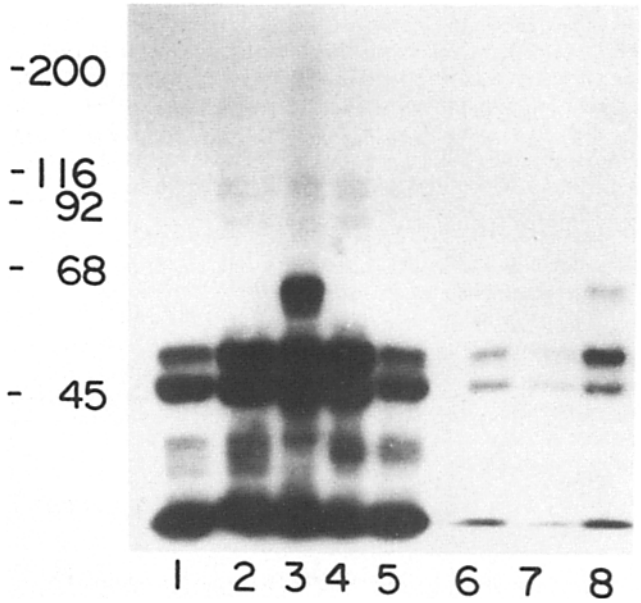

Figure 3. Proteolysis of ^{125}I-fibrinogen bound to neutrophils. ^{125}I-Fibrinogen was incubated with neutrophils (10^7/ml) in the presence of 50 μM Zn^{++}, 2 mM Ca^{++}, and various inhibitors for 20 min at 4°C, and then centrifuged through an oil mixture for 2 min. The pellet was solubilized in 4% SDS. The pellet and supernatant were run in a 7.5% polyacrylamide gel containing SDS after the sample was reduced by boiling for 10 min in the presence of 2% β-mercaptoethanol. Lanes *1–5* contained ~10-fold the amount of radioactivity as lanes *6* and *7* and 5-fold that in lane *8*. Inhibitors present: none (lane *1*); 0.5 mM leupeptin (lane *2*); 0.5 μM monospecific polyclonal antibody to HNE (lane *3*); 0.5 μM soy bean trypsin inhibitor (lane *4*); 1 mM Foy (lane *5*); none (lane *6*); 0.5 mM leupeptin (lane *7*); and 0.5 μM eglin (lane *8*). The three bands in lane *8* are exactly the position of the Aα, Bβ, and γ chains in the control fibrinogen (not shown). The numbers to the left of the gel represent molecular mass markers in kilodaltons.

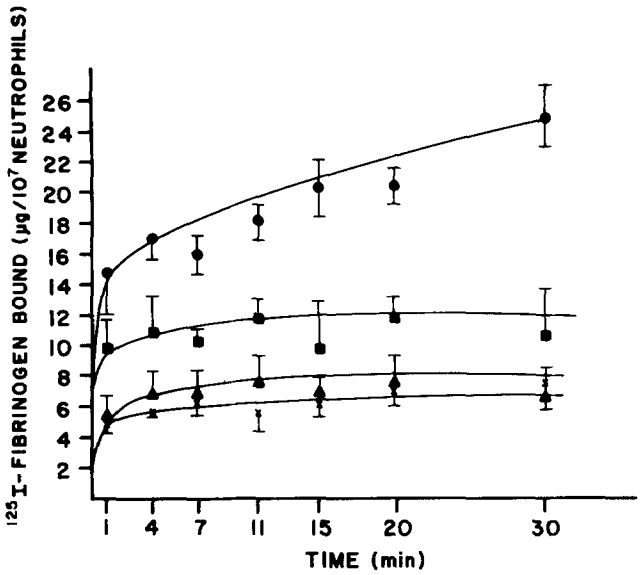

Figure 4. Effect of divalent cations on binding of ^{125}I-fibrinogen to neutrophils. Isolated neutrophils (10^7/ml) in HBSS without Ca^{++} or Mg^{++}, pH 7.4, were incubated at 4°C with ^{125}I-fibrinogen (400 μg/ml) in buffer containing various cations. In each experiment where an addition was made, the concentrations of Zn^{++} and Ca^{++} were 50 μM and 2 mM, respectively. At the designated time points, binding of ^{125}I-fibrinogen was determined as indicated in Materials and Methods. Binding was determined in the presence of Zn^{++} alone (■), Zn^{++} and Ca^{++} (●), and Ca^{++} alone (▲). Nonspecific binding was measured in the presence of all divalent cations, ^{125}I-fibrinogen, and a 50-fold molar excess of unlabeled fibrinogen (X). The plotted data are the mean ± SEM of four experiments.

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 μM). 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. Nonspecific 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^{++}-free Tyrode's solution, reapplied to the column with Zn^{++} buffer, eluted identically (not shown).

Specificity of Binding of ^{125}I-Fibrinogen to Neutrophils

To ascertain whether the binding of ^{125}I-fibrinogen to neutrophils was specific, we first tested the capacity of other proteins besides unlabeled fibrinogen to inhibit binding of ^{125}I-fibrinogen to neutrophils (Table I). The binding of ^{125}I-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 ^{125}I-fibrinogen to neutrophils was concentration dependent (Fig. 6). Using the mean ± SEM for each point from four experiments, unlabeled fibrinogen inhibited the binding of ^{125}I-fibrinogen to neutrophils 50% at a concentration of 2.8 ± 1.3 μM, which gave a calculated apparent K_i of 0.49 ± 0.30 μM. 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 ^{125}I-Fibrinogen to Neutrophils

Binding of ^{125}I-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 ^{125}I-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 ^{125}I-fibrinogen was displaced by HMWK.

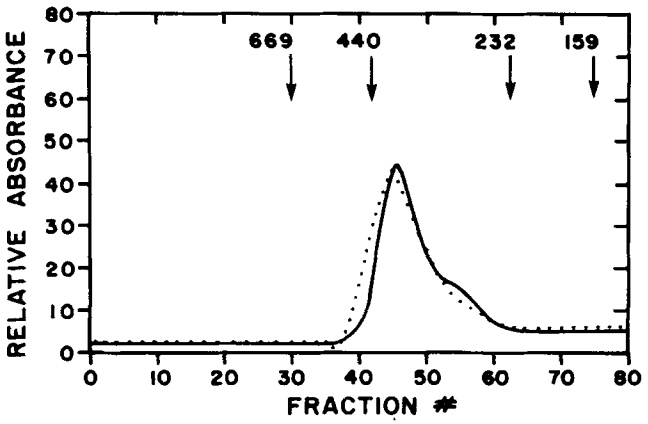

Figure 5. Effect of Zn^{++} on the elution of fibrinogen from HPLC column. Elution of fibrinogen (800 μg) from TSK-4000 column equilibrated with Tyrode-albumin buffer free of Zn^{++} ions (——). Elution of fibrinogen from TSK-4000 column equilibrated with Tyrode's albumin solution containing 50 μM Zn^{++} (- - -). Molecular mass markers are indicated with the arrows. See Material and Methods for more explanation.

Table I. Specificity of Binding of ^{125}I-Fibrinogen to Neutrophils

Protein competitor*	^{125}I-Fibrinogen binding inhibition‡
	%
None	<1
Fibrinogen	100
FNDPs	26 ± 1
HMWK	94 ± 5
Factor XII	<1
Prekallikrein	10 ± 4

^{125}I-fibrinogen (1 mg/ml, 3 μM) was incubated for 20 min at 4°C with human neutrophils (10^7/ml) in HBSS in the presence of 50 μM Zn^{++}, 2 mM Ca^{++}, and various proteins.
* Each competitor was added in a 50-fold molar excess of fibrinogen.
‡ Values present are the mean ± SEM of three experiments.

Determination of the Number of Binding Sites and Dissociation Constant of Binding of ^{125}I-Fibrinogen to Neutrophils

Since at low concentrations of added fibrinogen the binding of ^{125}I-fibrinogen to neutrophils was specific and reversible, studies were performed under equilibrium conditions to determine if binding was saturable. Increasing concentrations of ^{125}I-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 ^{125}I-fibrinogen increased, the level of specific binding increased until it leveled off at ~120 μg/ml of added ^{125}I-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 μM 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 μM added fibrinogen. This result characterizes one saturable binding site with an apparent K_d of 0.15 μM. This K_d is not significantly different from the K_i of 0.49 μM 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 γ chain of fibrinogen (27), the effect of these agents on fibrinogen binding to neutrophils was investigated. The binding of ^{125}I-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 γ-chain. Therefore, we tested the effect of the septadecapeptide CYGQQHHLGGAKQAGDV on fibrinogen binding to neutrophils (Table III). Although minimal inhibition was noted at 150 μM, at concentrations of 250–1,000 μM inhibition ranged from 62 to 79%.

An mAb to the OKM1 antigen on neutrophils (55) com-

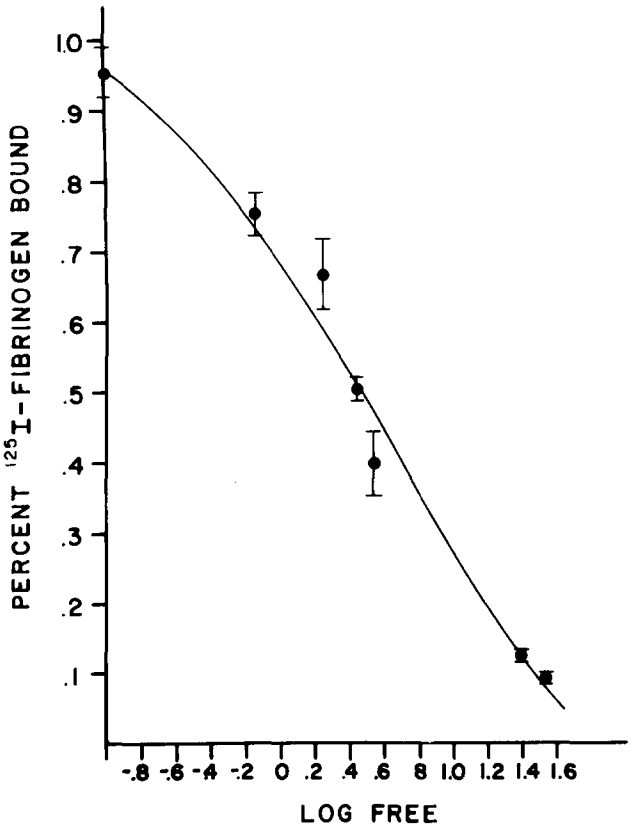

Figure 6. Ability of fibrinogen to inhibit ^{125}I-fibrinogen binding to neutrophils. ^{125}I-fibrinogen (400 μg/ml) was incubated with isolated neutrophils (10^7/ml) in the presence of HBSS without Ca^{++} or Mg^{++} for 20 min at 4°C in the presence of the indicated concentration of fibrinogen, Zn^{++} (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.

pletely (97%) prevented ^{125}I-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 ^{125}I-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 ^{125}I-HMWK binding to neutrophils. Binding of ^{125}I-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 ^{125}I-HMWK binding to neutrophils showed parallel slopes indicating no change in K_d. Increasing the fibrinogen concentration decreased the maximum number of sites for binding ^{125}I-HMWK. This result indicated that fibrinogen is a noncompetitive inhibitor of ^{125}I-HMWK binding to the neutrophil surface with an apparent K_i of 50 nM.

Interaction of HMWK and Fibrinogen on the Platelet Surface

Since HMWK inhibited the binding of ^{125}I-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 ^{125}I-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 K_d of 10^{-7} M. However, in

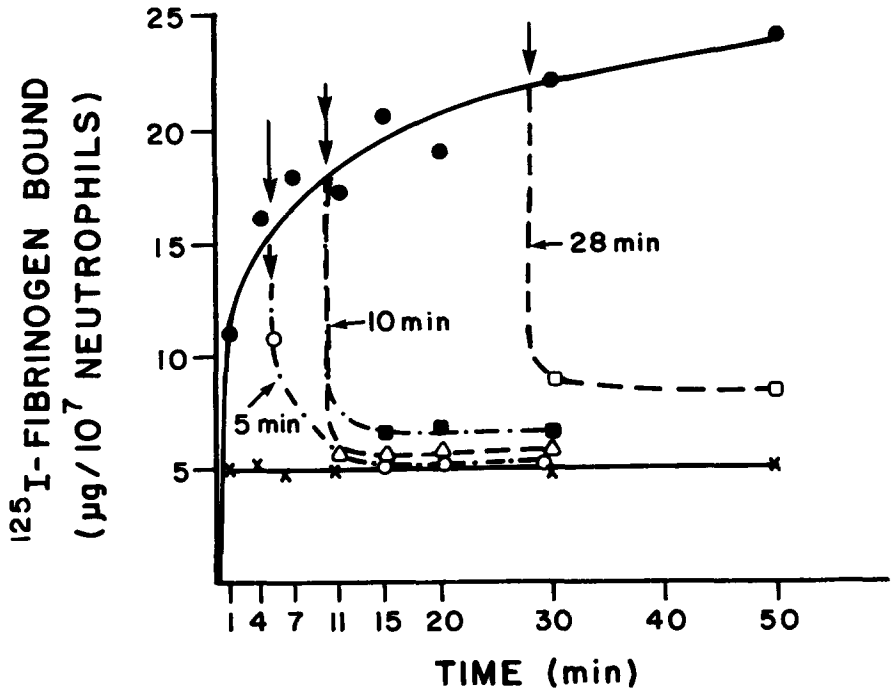

Figure 7. Displacement of bound ^{125}I-fibrinogen by HMWK and unlabeled fibrinogen from the neutrophil surface. Isolated neutrophils (10^7/ml) in HBBS without Ca^{++} or Mg^{++}, pH 7.4, were incubated at 4°C in the presence of Zn^{++} (50 μM), Ca^{++} (2 mM), and ^{125}I-fibrinogen (400 μg/ml). At 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 was added. Nonspecific binding was measured in the presence of the above additives and a 50-fold molar excess of unlabeled fibrinogen (X). Binding was determined at the indicated time points as described in Materials and Methods. The plotted data are the mean of three experiments.

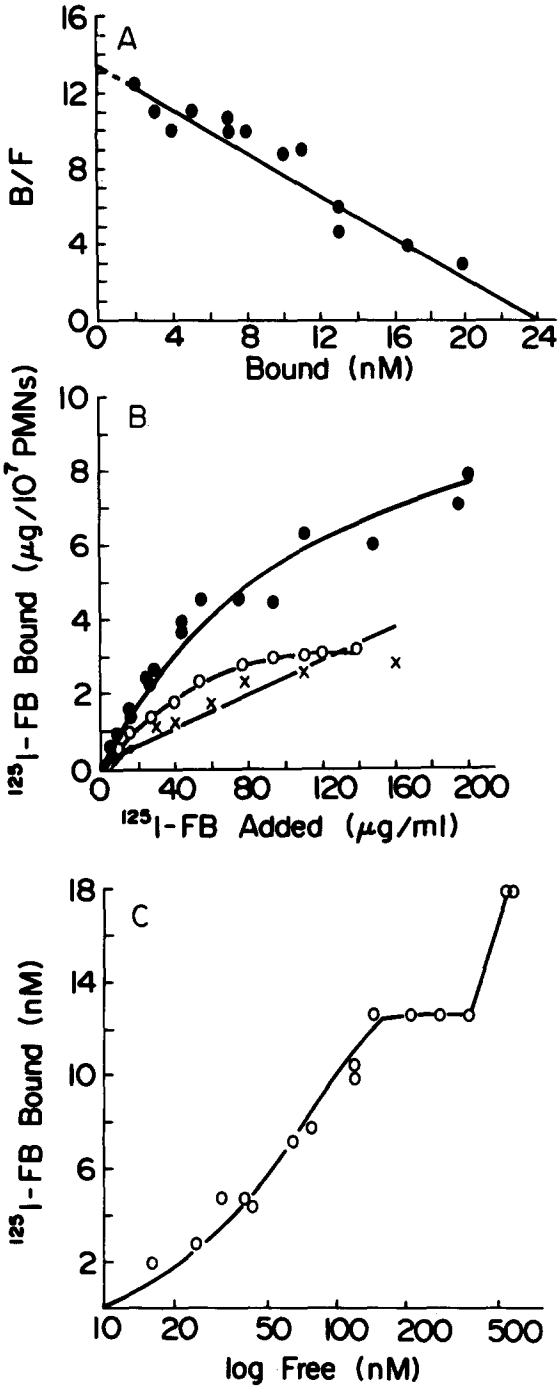

Figure 8. Concentration dependence of binding of ^{125}I-fibrinogen to neutrophils. Isolated neutrophils (*PMNs*) (10^7/ml) in HBSS without Ca^{++} or Mg^{++}, pH 7.4, were incubated with increasing concentrations of ^{125}I-fibrinogen (*^{125}I-FB*) in the presence or absence of a 50-fold molar excess 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 (μM) 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

Competitor	^{125}I-Fibrinogen binding inhibition*	^{125}I-HMWK binding inhibition*
	%	%
None	0	0 ± 3
Fibrinogen	100	86 ± 2.6
HMWK	94 ± 5.0	100
FNDPs	26 ± 1.0	22 ± 4
10E5	7.0 ± 1.2	0 ± 3
OKM1	97 ± 1	9.8 ± 5
OKM10	17.0	ND
IMY8	0	0 ± 4

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 μM Zn^{++} and 2 mM Ca^{++} at 4°C for 60 min. ^{125}I-Fibrinogen (1.18 μM) 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_d (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 μg/ml (plasma concentration, 80 μg/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_d. 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, fi-

Table III. Effect of the Peptides Derived from Fibrinogen on ^{125}I-Fibrinogen Binding to Neutrophils

Concentration	Inhibition	
	CYGQQHHLGGAKQGDV	RGDS
μM	%	%
50	ND	1.0
150	17.0	ND
250	62.0	ND
500	71.4	4.5
1,000	79.5	0.0

The protocol was identical to that of Table II. The concentration of the peptides is indicated. The inhibition is expressed as the mean of two separate experiments.

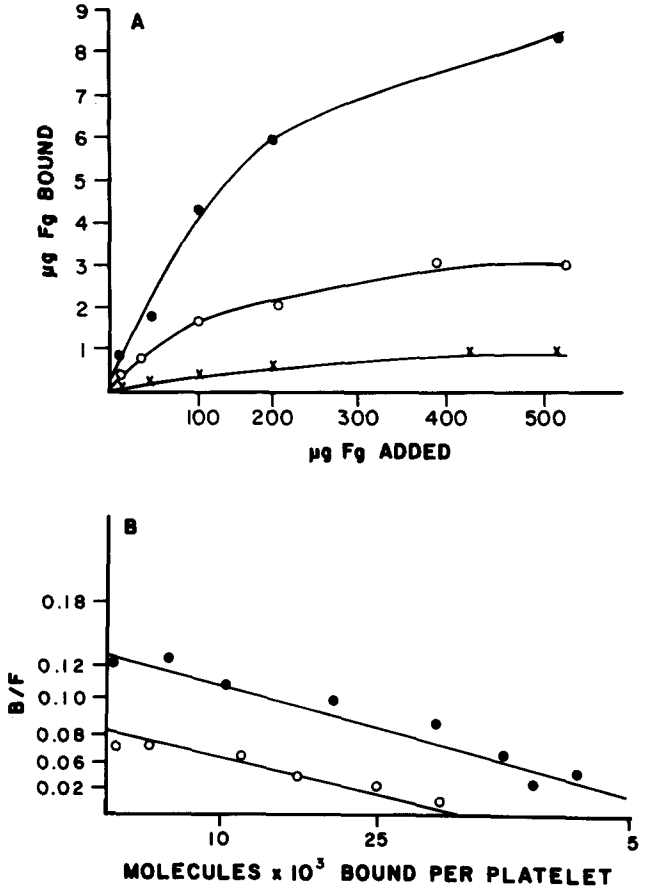

Figure 9. Effect of zinc and calcium on the binding isotherm of ^{125}I-fibrinogen ADP-stimulated platelets. Washed ADP-stimulated platelets (3×10^8/ml) in Tyrodes buffer containing Ca^{++} (2 mM) and Mg^{++} (1 mM), pH 7.35, were incubated with increasing concentrations of ^{125}I-fibrinogen. The total binding was measured in the presence (●) and in the absence (○) of 50 μM $ZnCl_2$. (X) Nonspecific binding of fibrinogen to nonactivated platelets in the presence or absence of $ZnCl_2$. *A* is a binding isotherm from a single experiment, and *B* represents a Scatchard plot of the specific binding calculated from the data in *A*. The correlation coefficient was 0.95. In five experiments the number of fibrinogen binding sites per platelet in the presence of Zn^{++} were 52,580 ± 3,040 (K_d = 6.6×10^{-7} M ± 1.8×10^{-7} M) and in the absence of Zn^{++} were 31,200 ± 4,300 (K_d = 5.2×10^{-7} M ± 0.7×10^{-7} M).

brinogen and HMWK are able to act as reciprocal inhibitors on the surface of neutrophils and platelets.

When studied by SDS-polyacrylamide gel electrophoresis, ^{125}I-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 eglin 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 preparation of the 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 eglin 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 ^{125}I-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 ^{125}I-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 ^{125}I-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

Table IV. Effect of HMWK on ^{125}I-Fibrinogen Binding to Platelets

	Control platelet suspension	Platelet suspension with HMWK
K_d	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 ^{125}I-fibrinogen (16–400 μg), 10 μl ADP (60 μM), Ca^{++} (2 mM final concentration), and Zn^{++} (50 μM) without or with HMWK (50 μg/ml). Scatchard analysis was used to determine K_d and number of binding sites (n). The values represent the mean ± SEM of five experiments.

of Zn^{++} 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 IIb/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). ^{125}I-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 septadecapeptide derived from the fibrinogen γ chain did inhibit fibrinogen binding to neutrophils at concentrations $\geqslant$250 μM. This finding suggests that the γ chain plays a role in the interaction with CR3 on the neutrophil surface similar to its interaction with the integrin GP IIb 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 ^{125}I-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 IIb/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 ^{125}I-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 (C11b/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 ^{125}I-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 elastate 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 proteolyzed 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 ^{125}I-HMWK binding to neutrophils by fibrinogen (Table II) and ^{125}I-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 IIb/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 ^{125}I-HMWK binding to neutrophils is not fully understood. In other studies (21) we have demonstrated that neutrophil activation induced by kallikrein 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 ^{125}I-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 LTB_4 (55). Thus, elastase-catalyzed fibrinogen derivatives bound to the neutrophil surface may function in the recruitment of neutrophils to the area of inflammation.

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