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Research Article

High molecular weight kininogen (HMW)-kininogen, the cofactor of contact-activated blood coagulation, accelerates the activation of Factor XII, prekallikrein, and Factor XI on a negatively charged surface. Although prekallikrein and Factor XI circulate as a complex with HMW-kininogen, no physical association has been demonstrated between Factor XII and HMW-kininogen, nor has the order of adsorption to surfaces of these proteins been fully clarified. In this report we explore the requirements for adsorption of HMW-kininogen to a clot-promoting surface (kaolin), in purified systems, as well as in normal plasma and plasma genetically deficient in each of the proteins of the contact system. The fraction of each coagulant protein associated with the kaolin pellet was determined by measuring the difference in coagulant activity between the initial sample and supernatants after incubation with kaolin, or by directly quantifying the amount of 125I-HMW-kininogen that was associated with the kaolin pellet. In normal plasma, the adsorption of HMW-kininogen to kaolin increased as the quantity of kaolin was increased in the incubation mixture. However, the HMW-kininogen in Factor XII-deficient plasma did not absorb appreciably to kaolin. Furthermore, the quantity of HMW-kininogen from prekallikrein-deficient plasma that adsorbed to kaolin was decreased as compared with normal plasma. These observations suggested that HMW-kininogen in plasma must be altered by a reaction involving both Factor XII and prekallikrein in order for […]

Cleavage of Human High Molecular Weight Kininogen Markedly Enhances Its Coagulant Activity

Evidence That This Molecule Exists as a Procofactor

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Abstract. High molecular weight kininogen (HMW)-kininogen, the cofactor of contact-activated blood coagulation, accelerates the activation of Factor XII, prekallikrein, and Factor XI on a negatively charged surface. Although prekallikrein and Factor XI circulate as a complex with HMW-kininogen, no physical association has been demonstrated between Factor XII and HMW-kininogen, nor has the order of adsorption to surfaces of these proteins been fully clarified. In this report, we explore the requirements for adsorption of HMW-kininogen to a clot-promoting surface (kaolin), in purified systems, as well as in normal plasma and plasma genetically deficient in each of the proteins of the contact system. The fraction of each coagulant protein associated with the kaolin pellet was determined by measuring the difference in coagulant activity between the initial sample and supernatants after incubation with kaolin, or by directly quantifying the amount of ^{125}I-HMW-kininogen that was associated with the kaolin pellet.

In normal plasma, the adsorption of HMW-kininogen to kaolin increased as the quantity of kaolin was increased in the incubation mixture. However, the HMW-kininogen in Factor XII-deficient plasma did not absorb appreciably to kaolin. Furthermore, the quantity of HMW-kininogen from prekallikrein-deficient plasma that adsorbed to kaolin was decreased as compared with normal plasma. These observations suggested that HMW-kininogen in plasma must be altered by a reaction involving both Factor XII and prekallikrein in order for HMW-kininogen to adsorb to kaolin, and to express its coagulant activity. Subsequently, the consequence of the inability of HMW-kininogen to associate with a negatively charged surface results in decreased surface activation. This assessment was derived from the further observation of the lack of prekallikrein adsorption and the diminished Factor XI adsorption in both Factor XII-deficient and HMW-kininogen-deficient plasmas, since these two zymogens (prekallikrein and Factor XI) are transported to a negatively charged surface in complex with HMW-kininogen. The percentage of HMW-kininogen coagulant activity that adsorbed to kaolin closely correlated ($r = 0.98$, slope = 0.97) with the amount of ^{125}I-HMW-kininogen adsorbed, suggesting that adsorption of HMW-kininogen results in the expression of its coagulant activity.

Since kallikrein, which is known to cleave HMW-kininogen, is generated when kaolin is added to plasma, we tested the hypothesis that proteolysis by kallikrein was responsible for the enhanced adsorption of HMW-kininogen to kaolin. When purified HMW-kininogen was incubated with purified kallikrein, its ability to adsorb to kaolin increased with time of digestion until a maximum was reached. Moreover, ^{125}I-HMW-kininogen, after cleavage by kallikrein, had markedly increased affinity for kaolin than the uncleaved starting material. Furthermore, fibrinogen, at plasma concentration (3 mg/ml), markedly curtailed the adsorption of a mixture of cleaved and uncleaved HMW-kininogen to kaolin, but was unable to prevent fully cleaved HMW-kininogen from adsorbing to the kaolin. Addition of purified kallikrein to Factor

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XII-deficient plasma, which bypasses Factor XII-dependent contact-activation, amplified the ability of its HMW-kininogen to adsorb to kaolin.

These observations indicate that HMW-kininogen is a procofactor that is activated by kallikrein, a product of a reaction which it accelerates. This cleavage, which enhances its association with a clot-promoting surface in a plasma environment, is an event that is necessary for expression of its cofactor activity. These interactions would allow coordination of HMW-kininogen adsorption with the adsorption of Factor XII, which adsorbs independently of cleavage, to the same negatively charged surface.

Introduction

High molecular weight kininogen (HMW)[1]-kininogen functions as a cofactor in the initiation of surface-activated intrinsic blood coagulation by circulating in complexes with either Factor XI (1) or prekallikrein (2), thereby facilitating their optimal orientation on a negatively charged surface (3–7). Here, the zymogens are converted to their corresponding active enzymes (Factor XIa and kallikrein) by activated Factor XII (8–10). The kallikrein that is generated converts additional Factor XII to Factor XIIa, thereby amplifying the contact-activated system of blood coagulation (8). Both intact HMW-kininogen or its light chain (M_r = 45,000) form complexes with Factor XIa and kallikrein (11), curtailing their rate of inactivation by plasma protease inhibitors (12–14). Prekallikrein can also be converted to kallikrein in the absence of a negatively charged surface, by Factor XII fragments (15), a reaction not requiring HMW-kininogen (16). The influence of solution and surface activation of the intrinsic pathway of blood coagulation, as well as the exact sequence of events in these reactions, however, is still not completely understood.

Physical studies of the interaction of plasma with hydrophilic (clot-promoting) surfaces such as glass, revealed that when normal plasma is applied to that surface, fibrinogen is antigenically detectable within 10 s (17), but after 10 min its antigenicity is lost. Concomitantly, HMW-kininogen is not initially antigenically detectable; but at 10 min it is detectable (18). Furthermore, in HMW-kininogen-deficient plasma, fibrinogen remains detectable after 10 min and in Factor XII-deficient plasma, where contact-activation cannot occur, much of the fibrinogen remains detectable on the clot-promoting surface after 10 min (19). Since no differences could be determined by physical measurements of the degree of protein thickness on the surface before or after the interaction of fibrinogen and HMW-kininogen, these data suggest that HMW kininogen binds to, replaces, or competes with fibrinogen on the clot-promoting surface (18).

This investigation was designed to assess the ability of various proteolytic digestion products of HMW-kininogen to interact with the clot-promoting surface, kaolin. To evaluate the behavior of this cofactor in the plasma environment, studies were performed in normal and contact factor-deficient plasma, as well as in purified systems containing plasma proteins. Evidence will be presented that proteolysis of HMW-kininogen amplifies its adsorption to kaolin and that plasma concentrations of fibrinogen, but not albumin, can drastically curtail the association of uncleaved HMW-kininogen with kaolin, while still allowing adsorption of cleaved HMW-kininogen.

1. *Abbreviations used in this paper:* HMW, high molecular weight; HSA, human serum albumin; PAGE, polyacrylamide gel electrophoresis.

Methods

Sodium dodecyl sulfate (SDS), methylene *bis*-acrylamide, acrylamide, high and low molecular weight standards (220,000, 130,000, 94,000, 68,000, 43,000, 30,000, 21,000), and Dowex AG-1B were purchased from Bio-Rad Laboratories, Richmond, CA. Prestained molecular weight standards (200,000, 92,000, 68,000, 43,000, 25,700, 18,400) were obtained from BRL, Bethesda, MD. Soybean trypsin inhibitor, lysozyme, human serum albumin (HSA), bovine gamma globulin, and trypsin were from Sigma Chemical Co., St. Louis, MO. Kodak XAR x-ray film was purchased from Reliance X-ray, Glenside, PA; Kaolin (acid washed), glass tubes (6 × 50 mm), and polyethylene glycol 8000 were from Fisher Scientific Co., King of Prussia, PA; inosithin (mixed soybean phospholipids) was from Associated Concentrates, New York. Human fibrinogen (Kabi 90% clottable) was obtained from Helena Laboratories, Beaumont, TX; microcentrifuge tubes 699 and 690, from Sarstedt, Inc., Princeton, NJ. [^{14}C]inulin and Na^{125}I were obtained from New England Nuclear, Boston, MA; antiserum to high molecular weight kininogen light chain, from Miles Biochemicals, Elkhart, IN; antiserum to bradykinin was a gift of Dr. David Proud. Tyrosine-8-bradykinin was purchased from Peninsula Laboratories, Inc., Belmont, CA; slab gel electrophoretic apparatus model SE 600, from Hoefer Scientific Instruments, San Francisco, CA. Iodogen was obtained from Pierce Chemical Co., Rockford, IL; Coomassie Brilliant Blue R-250 and LKB rack gamma counter, from LKB Instruments, Rockville, MD; Beckman liquid scintillation counter LS 8000, from Beckman Instruments, Inc., Fullerton, CA; Kodak RP X-Omat automated processor, from Eastman Kodak Co., Rochester, NY (courtesy of Dept. of Neuroradiology, Temple University Hospital).

Plasma. Normal pooled plasma, used as a reference standard, was purchased from George King Biomedicals, Inc., Overland Park, KS. Plasmas deficient either in Factor XI or total kininogen (20) were donated directly to us. Factor XII-deficient plasma was kindly supplied by Dr. Margaret Johnson, Wilmington, DE. Prekallikrein-deficient plasma was graciously supplied by Dr. Charles Abildgaard, Davis, CA. Aliquots of several other Factor XII-deficient plasma were a gift from Mr. George King (George King Biomedicals, Inc.).

Assay of HMW-kininogen coagulant activity (kaolin-activated partial thromboplasin time). 100 μl of total kininogen-deficient plasma, 100 μl of 20 mM Tris-Cl, pH 7.4, containing 0.15 M NaCl, 100 μl of kaolin (5 mg/ml in saline), and 100 μl of 0.2% inosithin in buffer were mixed. Normal plasma (10, 5, 2, or 1 μl) was added and incubated at 37°C for 8.5 min. 100 μl of 30 mM $CaCl_2$ was added and coagulation time was measured. This procedure was used to generate a standard curve (log-log relationship). Samples for analysis were assayed, under the same conditions, using 5 or 10 μl of sample and the data were expressed as percentage of normal pooled plasma. One unit (U) is the amount of activity in 1 ml of normal pooled plasma.

Purified proteins. Kallikrein was prepared by activation of purified

prekallikrein by Factor XII fragments (21). The activation was stopped with purified corn trypsin inhibitor (50 μg/ml) (22) which was a kind gift of Dr. Edward Kirby and Patrick McDevitt of this institution. The preparation contained no measurable Factor XI, XIa, XII, XIIa, plasminogen, or plasmin as determined by coagulant and amidolytic assays. Two different kallikrein preparations were used. The first preparation was free of IgG and the second contained 10–20 mg IgG/mg kallikrein. Both preparations yielded similar results in the experiments described.

HMW-kininogen was prepared by the method of Kerbiriou and Griffin (23) and was primarily a single band of M_r = 120,000 on nonreduced SDS-polyacrylamide gel electrophoresis (PAGE). Upon reduction, 75–90% remained M_r = 120,000, as assessed by scanning densitometry of the gels.

Fibrinogen was purified from fresh human citrated plasma, as previously described (24). Thrombin-coagulable protein was 95% of the total. The preparation of purified fibrinogen was freed of plasminogen by passing it through a lysine-sepharose column. SDS-PAGE in 3.5% gels demonstrated the presence of one homogenous fibrinogen band and the absence of von Willebrand's factor and fibronectin. This fibrinogen was used in all experiments that were performed with purified proteins.

Kaolin adsorption experiments. Kaolin (10 mg) was suspended in 1 ml of 20 mM Tris-Cl, pH 7.4, containing 0.15 M NaCl, and 3 mg fibrinogen, at 23°C. Various volumes of the suspension were transferred to 0.4-ml microcentrifuge tubes, centrifuged in an Eppendorf microcentrifuge for 1 min at 12,000 *g* at 23°C, and the supernatants were discarded. The prepared kaolin pellets were retained for use as a negatively charged surface. In some experiments, kaolin (10 mg/ml) was added directly to the above-mentioned buffer containing fibrinogen, before the addition of the other reactants. Buffers containing fibrinogen (3 mg/ml), HSA (30 mg/ml), and HMW-kininogen, were prepared. In the experiments where plasma was adsorbed to kaolin, it was directly added to a prepared kaolin pellet. One-half of each solution or plasma sample was transferred to the tube containing the kaolin pellet, mixed, and incubated at 23°C for the times indicated. The presence of fibrinogen prevented adsorptive losses during transfer (16). After centrifugation, the supernatants were transferred to clean tubes. The original sample and supernatants from the kaolin pellets were then assayed for coagulant activity. The percent coagulant activity adsorbed to the kaolin was calculated as (initial activity − residual supernatant activity)/(initial activity) × 100.

Using [^{14}C]inulin, we found that 10–25% of the fluid was trapped by the kaolin, when the kaolin pellets were prepared with buffer. Furthermore, it was difficult to efficiently remove the buffer without removing some kaolin. However, when fibrinogen was added to the kaolin, the trapped fluid decreased to 3% and kaolin could be easily separated. An assessment of the amount of plasma trapped by the kaolin pellet after incubation was determined by incubating 99 μl plasma and 1 μl [^{14}C]inulin with kaolin pellets ranging in weight from 125 to 500 μg. After incubation for 15 min at 23°C, the pellets were centrifuged. The starting material, supernatants, and pellets were counted for radioactivity. Trapped fluid averaged 3% for each amount of kaolin used and therefore no corrections were necessary.

To obtain an independent determination of the amount of coagulant proteins associated with the kaolin pellets, normal plasma was incubated with kaolin for various times. The pellets were washed six times with buffer, resuspended in the initial sample volume, and assayed for prekallikrein, Factor XI, or high molecular weight kininogen coagulant activity. The activity in the pellet was always less than the difference between starting material and supernatant in agreement with a previous study (25) showing inhibition of the enzymes in the coagulant assay by kaolin. Therefore, in all subsequent experiments, we measured the difference between starting material and supernatant in order to determine the percent coagulant protein associated with the kaolin pellet.

Determination of HMW-kininogen antigen in plasma. Radial immunodiffusion (26) was performed by using an antibody to HMW-kininogen light chain.

Preparation and analysis of cleaved HMW-kininogen. HMW-kininogen, in 0.15 M Tris-acetate, pH 8.0, was incubated with kallikrein at 37°C. At specified times, aliquots were transferred to tubes containing soybean trypsin inhibitor (6.5-fold molar excess compared with kallikrein) in order to stop the cleavage process. To monitor the distribution of the cleaved product, 5-μl aliquots were removed from each tube and added to glass tubes 6 × 50 mm, containing 70 μl H_2O and 25 μl buffer containing 0.4 M Tris-Cl, pH 6.8, and 8% SDS. The samples were reduced with 2-mercaptoethanol (0.8 M) and either boiled for 5 min or incubated overnight at 37°C. Polyacrylamide gel electrophoresis was carried out in the presence of SDS on 10% slab gels (16 × 18 mm plates) according to the method of Laemmli (27). The gels were run at 36 mA/gel for 4½ h or overnight at 7.5 mA/gel and stained in 0.5% Coomassie Brilliant Blue R-250 in 5:5:1 ($CH_3OH/H_2O/CH_3COOH$).

Stained gels and autoradiograms for selected experiments were scanned using a SD 3000 Spectrodensitometer (Schoeffel Instrument Div., Kratos, Inc., Westwood, NJ). The relative peak areas were quantified by weighing tracings on standard paper.

Radiolabeling of HMW-kininogen. 125 μg of purified HMW-kininogen was radiolabeled by a minor modification of the Iodogen method (28) at pH 8.0 in the presence of 0.75 M NaCl. After stopping the reaction with sodium metabisulfite (50 μg/ml), the labeled material was gel filtered on a (0.5 × 10 cm) G-50 column, equilibrated with 50 mM sodium acetate buffer, pH 5.2, 0.15 M NaCl, and 1% polyethylene glycol 8000, in order to remove the free ^{125}I. The peak contained 14% of the total radioactivity. The specific radioactivity of the peak tube was 1.4 × 10^6 cpm/μg. The HMW-kininogen coagulant activity was monitored during the radiolabeling procedure and was not altered.

Radiolabeling of tyrosine-8-bradykinin. Tyrosine-8-bradykinin was iodinated by the chloramine T method (29). The labeled peptide was separated from the free iodine by elution from Dowex AG-1B with distilled water. The peak material was diluted 1:4 in 0.2 M Tris acetate:0.01 M Na_2 EDTA, pH 6.4, containing 1 mg/ml lysozyme (Tris-lysozyme buffer) and stored at −70°C.

Measurement of bradykinin release. Bradykinin was measured using a radioimmunoassay according to the method of Proud et al. (29). To prepare individual samples for assay, 3 μl of the sample was added to 97 μl of Tris-lysozyme buffer. The sample was mixed and then 400 μl 95% ethanol was added. The sample was placed at 4°C for at least 10 min before centrifugation at 12,000 *g* for 5 min. Subsequently, the supernatant was removed and the pellet reextracted with ethanol, spun at 12,000 *g*, and the supernatants combined. This sample was applied to a 3 ml QAE A-50 Sephadex column (4°C, 0.0075 M Tris buffer, pH 8.0) and the bradykinin eluted with the same buffer. Percent recovery for the procedure was 82%. Radioimmunoassay (RIA) was performed as outlined previously (29) using a high affinity bradykinin antisera at a concentration of 1:100,000. Duplicate samples were incubated at 4°C for 18 h. Polyethylene glycol 8000 precipitation was used to separate free from bound bradykinin. Results were calculated from a log-logit plot of bradykinin, ranging from (0 to 500 pg/ml), vs. relative binding.

Results

Effect of kaolin concentration on the adsorption of contact factors in normal and contact factor-deficient plasma. When normal

plasma was incubated with kaolin, and the supernatant assayed for HMW-kininogen coagulant activity, HMW-kininogen adsorption to kaolin directly increased with increasing kaolin concentration (Fig. 1 *A*). The adsorption of this cofactor in Factor XI-deficient plasma was similar to what was observed with normal plasma. In contrast, adsorption of HMW-kininogen was decreased in prekallikrein-deficient plasma. In Factor XII-deficient plasma, little or no adsorption of HMW-kininogen was observed. We found, however, that during prolonged storage or freezing and thawing of the Factor XII-deficient plasma, an increased amount of HMW-kininogen adsorbed to kaolin. Therefore, for subsequent experiments, we used a batch of Factor XII-deficient plasma which was freshly collected and snap-frozen (George King Biomedicals, Inc.).

Factor XII was found to adsorb to the same extent in all plasma samples tested (Fig. 1 *B*), in agreement with the suggestion (3) that its adsorption is not dependent upon its cleavage or the cleavage of any other plasma proteins.

Prekallikrein (Fig. 2 *A*) from either normal or Factor XI-deficient plasma was similar in its adsorption characteristics with maximal adsorption occurring at a kaolin concentration of 1.25 mg/ml. Prekallikrein adsorption to kaolin from HMW-kininogen-deficient or Factor XII-deficient plasma, was <1%. Thus, prekallikrein adsorption to kaolin in plasma not only requires HMW-kininogen but also Factor XII.

At the lowest concentration of kaolin studied, 81% of the Factor XI (Fig. 2 *B*) from normal plasma was adsorbed, and at the highest kaolin concentration, 93% was adsorbed. In contrast, the Factor XI which was adsorbed onto kaolin from HMW-kininogen-deficient plasma ranged from 14 to 24% and from Factor XII-deficient plasma, ranged from 9 to 37% of normal.

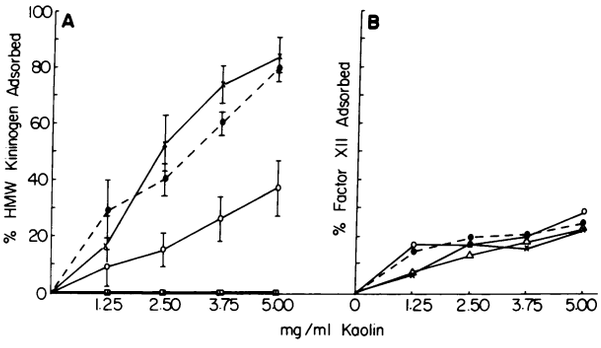

Figure 1. Effect of kaolin concentration on adsorption of HMW-kininogen (*A*) and Factor XII (*B*) from deficient plasmas. Kaolin pellets, 0.125–0.5 mg, were prepared as in Methods. 100 μl of normal (●), Factor XII-deficient (□), Factor XI deficient (×), prekallikrein-deficient (○), or HMW-kininogen-deficient (△) plasma was then suspended and incubated with the kaolin for 15 min at 23°C. The starting material and supernatants were assayed for HMW-kininogen and Factor XII coagulant activity and the percentage adsorbed was calculated by difference. The data represent the mean±SD of three to six experiments.

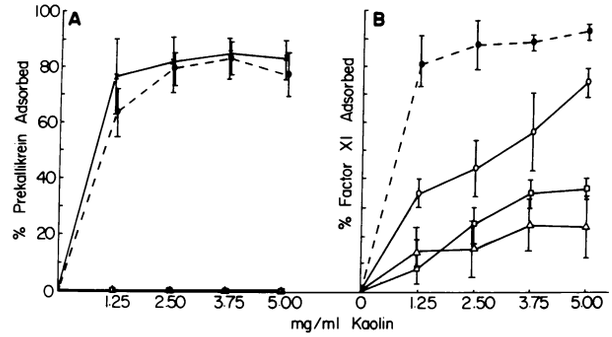

Figure 2. Effect of kaolin concentration on adsorption of prekallikrein (*A*) and Factor XI (*B*) from deficient plasmas. Kaolin pellets, 0.125–0.5 mg, were prepared as in Methods. 100 μl of normal (●), Factor XII-deficient (□), Factor XI-deficient (×), prekallikrein-deficient (○), or HMW-kininogen deficient (△) plasma was then suspended and incubated with the kaolin for 15 min at 23°C. The starting material and supernatants were assayed for prekallikrein and Factor XI coagulant activity and the percentage adsorbed was calculated by difference. The data represent the mean±SD of three to six experiments.

The amount of Factor XI that adsorbed to kaolin in prekallikrein-deficient plasma was also diminished, as compared with normal, especially at the lowest concentration, where only 35% was adsorbed and reached a maximum of 74%. Thus, prekallikrein and Factor XI adsorption depended not only on HMW-kininogen but also on Factor XII.

These findings indicate that both prekallikrein and Factor XII are involved in an alteration of HMW-kininogen that increases its adsorption to kaolin in plasma and ultimately the adsorption of Factor XI.

Relationship between adsorption of HMW-kininogen coagulant activity and adsorption of ^{125}I-HMW-kininogen to kaolin. To confirm that the decrease in plasma HMW-kininogen coagulant activity, after exposure to kaolin, was actually due to physical removal of the protein by kaolin, we performed two experiments to analyze the behavior of HMW-kininogen. In the first study, the residual HMW-kininogen in the supernatant, after incubation with kaolin, was determined by its coagulant activity (Fig. 3, *x* axis). We also measured the adsorption of HMW-kininogen to kaolin by determining the percent ^{125}I-HMW-kininogen directly associated with the kaolin pellet (Fig. 3, *y* axis). Each group of samples was generated by incubating plasma (normal or prekallikrein-deficient) with kaolin, for various times. We observed an excellent correlation (r = 0.97) between the two sets of data, and a slope of 0.98, indicating that the decrease in coagulant activity was a direct result of physical removal of the HMW-kininogen by the kaolin. Moreover, using ^{125}I-HMW-kininogen, there was close agreement between the sum of the supernatant and pellet radioactivity as compared with the radioactivity of the starting material.

These observations were reproduced in a second study by incubating normal plasma with kaolin and assaying the super-

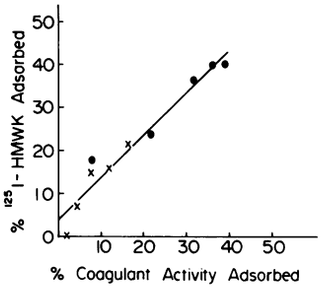

Figure 3. Relationship between HMW-kininogen coagulant activity and ^{125}I-HMW-kininogen adsorbed to kaolin. 320 μl of normal plasma (•) or prekallikrein-deficient plasma (×) plus 80 μl saline was mixed. 250 μl of the mixture was transferred to a precoated kaolin pellet (625 μg). 50 μl was removed and centrifuged at 2, 5, 10, 15, and 20 min. Each starting sample and supernatant was assayed for HMW-kininogen coagulant activity (as in Fig. 2). Prekallikrein-deficient plasma was used for the lower values, and normal plasma was used for the higher values. In a parallel experiment, ^{125}I-HMW-kininogen (5 μl), 75 μl saline, and 320 μl of normal plasma (•) or prekallikrein-deficient plasma (×) was mixed. 250 μl of each mixture was incubated with a precoated kaolin pellet (625 μg) and 50 μl was removed and centrifuged at 2, 5, 10, 15, and 20 min. The starting material, supernatants, and pellets were counted for gamma radioactivity. After calculating the percentage of radioactivity adsorbed, linear regression analysis was performed by correlating the results with that obtained for the functional (coagulant) data. (r = 0.97; slope = 0.98; intercept = 4.3%).

natant for coagulant activity as well as antigen (using antibody to light chain of HMW-kininogen). A correlation coefficient of 0.98 and a slope of 0.97 were observed when the data were analyzed in that experiment (data not shown).

From the data of Fig. 1 and Fig. 3, it is evident HMW-kininogen was physically adsorbed to kaolin in normal plasma, but not from Factor XII-deficient plasma. It thus appears that an alteration in the HMW-kininogen molecule enabled HMW-kininogen to adsorb to kaolin in a plasma environment. Factor XII is known to be activated on exposure to kaolin and then to activate prekallikrein to kallikrein, which in turn cleaves HMW-kininogen. Therefore, we investigated the possibility that cleavage of HMW-kininogen promoted its adsorption to kaolin.

Cleavage of purified HMW-kininogen by kallikrein. To assess the distribution of cleavage products of HMW-kininogen as a function of time, we incubated HMW-kininogen, that was >90% intact, with kallikrein for various times, and stopped the reaction with soybean trypsin inhibitor (Fig. 4). The disappearance of the 120,000-M_r species was followed by the appearance of a heavy chain (M_r = 65,000) reaching a maximum at 20 min, and a light chain (M_r = 56,000) reaching a maximum at 15 min. A small amount of degraded light chain (M_r = 45,000), as described by Mori and Nagasawa (30), was observed between 0 and 60 min. After 60 min, when the 56,000-M_r species began to decrease, the 45,000-M_r species rapidly increased. The reciprocal relationship of the 56,000-M_r light chain and 45,000-M_r degraded light chain suggested that the 56,000-M_r species was a short-lived intermediate and the 45,000-M_r species was an end-product of the HMW-kininogen proteolysis.

Effect of purified fibrinogen on the adsorption of uncleaved and cleaved HMW-kininogen as determined by coagulant activity. Previous studies (17–19) have suggested that fibrinogen may be a plasma component that affects HMW-kininogen adsorption to activating surfaces. This observation allowed us to assess the adsorption to kaolin in a purified system of virtually intact HMW-kininogen (90% of protein 120,000-M_r), HMW-kininogen that was partially cleaved (65% of protein 120,000-M_r), and HMW-kininogen that was fully cleaved by kallikrein (no 120,000-M_r component). Parallel experiments were performed in the absence (Fig. 5 *A*) and presence (Fig. 5 *B*) of fibrinogen at plasma concentrations (3 mg/ml). The 90% intact HMW-kininogen adsorbed to kaolin in the absence of fibrinogen to a limited extent. With cleavage, the extent of adsorption at a given kaolin concentration increased (Fig. 5 *A*). The presence of fibrinogen decreased the adsorption of the 90% intact and 65% intact HMW-kininogen but had no effect on fully cleaved HMW-kininogen (Fig. 5 *B*). Thus, cleavage of HMW-kininogen amplified its adsorption to kaolin in the presence of a concentration of fibrinogen similar to that in plasma.

The actual amount of fibrinogen (3.9 mg/ml) adsorbed to 12.5 mg of kaolin was determined by measuring the fibrinogen ($E^{1\%}_{280\ nm}$ = 13.6) before and after centrifugation of the kaolin pellet. The adsorbed fibrinogen was 44 μg or 17.6 μg/5 mg kaolin. From Fig. 5, 4.8 μg of uncleaved HMW-kininogen was

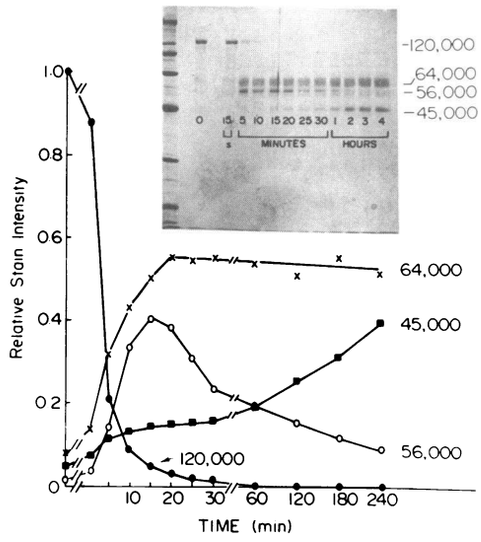

Figure 4. Cleavage of HMW-kininogen by kallikrein. HMW-kininogen (65 μg) was incubated with kallikrein (1.4 μg) in a final volume of 30 μl (weight ratio of kallikrein to HMW-kininogen of 1:46). At the times indicated, 2 μl was transferred to a tube containing 5 μl 2-mercaptoethanol, sample buffer, and water in a final volume of 100 μl. The samples were each incubated in the 2-mercaptoethanol mixture for 15 min at 37°C in order to destroy the kallikrein activity. Subsequently, all samples were incubated at 37°C overnight to complete the reduction process. 50 μl of each reduced sample was then subjected to SDS-PAGE, as described in Methods. The standards utilized were a mixture of high and low molecular weight proteins obtained from Bio-Rad Laboratories (see Methods).

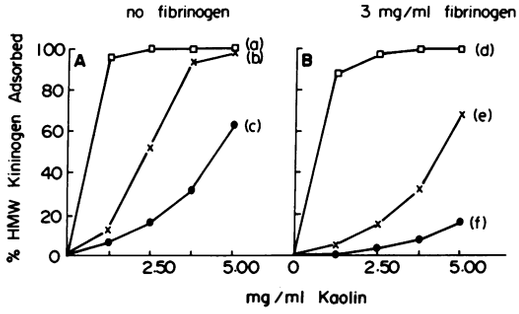

Figure 5. Effect of fibrinogen on adsorption of intact and cleaved HMW-kininogen. Three preparations of HMW kininogen were used. The first preparation contained 90% of the M_r = 120,000 band (*c* and *f*). A second preparation contained 65% of the M_r = 120,000 band (*b* and *e*) and the third preparation was fully cleaved by kallikrein (*a* and *d*). 100 μl of each preparation, at 1 U/ml, was incubated in the absence (*A*) or presence (*B*) of 3 mg/ml purified plasminogen-free fibrinogen, with kaolin (0–5 mg/ml final concentration) for 10 min at 23°C. The starting material and supernatants from the kaolin pellets were assayed for HMW-kininogen coagulant activity. Each point is the average of duplicate determinations and the figure is representive of two to three similar experiments.

adsorbed in the absence of fibrinogen, whereas in the presence of fibrinogen, only 1.8 μg was adsorbed, which is a difference of 3.0 μg. Substitution of 30 mg/ml HSA for fibrinogen resulted in similar results to those with buffer alone (data not shown). Therefore, fibrinogen displayed specificity in its ability to prevent uncleaved HMW-kininogen from adsorbing to kaolin.

Effect of cleavage on adsorption of ^{125}I-HMW-kininogen to kaolin. To ascertain which cleavage events were critical for the adsorption of HMW-kininogen to kaolin, radiolabeled HMW kininogen was incubated for various times with plasma kallikrein (Fig. 6). The cleavage profile (data not shown) was similar to Fig. 4 except that the rate of cleavage was slower, owing to a lower ratio of kallikrein to HMW-kininogen in the incubation mixture. As cleavage progressed, increasing amounts of HMW kininogen were found to adsorb to kaolin, until maximal adsorption was observed between 3 and 4 h of incubation. The maximal adsorption followed the maximal formation of the 56,000-M_r species and also paralleled bradykinin release. Thus, the first two cleavages of HMW-kininogen by kallikrein greatly enhance its ability to adsorb to kaolin at plasma concentrations of fibrinogen.

Determination of ^{125}I-HMW-kininogen that adsorbed to kaolin in the presence of fibrinogen. To identify and quantify the actual species of HMW-kininogen that adsorbed to kaolin, various amounts of ^{125}I-HMW-kininogen starting material (Fig. 7 *a*) or ^{125}I-HMW-kininogen that was partially cleaved by kallikrein (Fig. 7 *b*), were mixed with fibrinogen and HSA and incubated with kaolin, in the presence and absence of a fivefold excess of unlabeled HMW-kininogen. A maximum of 2,600 cpm (0.265 μg) from the starting material was associated with 100 μg kaolin, and a similar amount of radioactivity was present in the pellet in the presence of additional fivefold excess unlabeled HMW kininogen (Fig. 7 *a*). The radioactivity that was adsorbed to the kaolin could not be eluted by SDS. Moreover, the labeled HMW-kininogen in the supernatant was uncleaved (Fig. 7 *a*, inset).

In contrast, 18,000 cpm (1.84 μg) from the cleaved HMW-kininogen were associated with kaolin, and all but 2,500 cpm (14%) were displaced by the excess unlabeled, cleaved HMW-kininogen (Fig. 7 *b*). The kaolin-associated material was then eluted with SDS and subjected to electrophoresis. The SDS-extracted kaolin pellet Fig. 7 *b* (inset), contained 15% uncleaved HMW-kininogen (M_r = 120,000), equivalent to the adsorption observed before the chase with unlabeled HMW kininogen and 85% cleavage products (heavy and light chains). The unadsorbed material, however, contained 85% uncleaved HMW-kininogen and 15% cleavage products. This experiment, in agreement with Fig. 5, suggests that cleavage of HMW-kininogen markedly amplifies its ability to adsorb to kaolin in the presence of fibrinogen, and that uncleaved HMW-kininogen has little to no ability to adsorb to a negatively charged surface, under such conditions.

Effect of kallikrein on the adsorption of HMW-kininogen from Factor XII-deficient plasma. To directly test the hypothesis

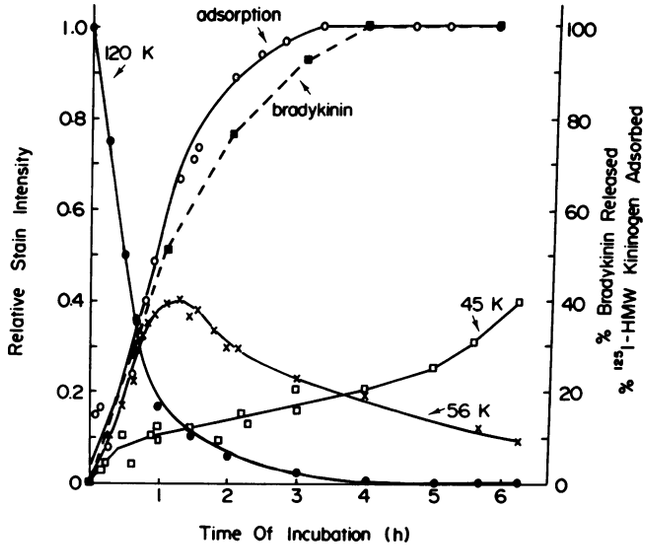

Figure 6. Kaolin adsorption of cleaved ^{125}I-HMW-kininogen in a purified system containing fibrinogen. Aliquots of cleaved HMW-kininogen (3 μl) from a timed digest (25 μg kallikrein and 2,500 μg HMW-kininogen in a final volume of 535 μl) were removed for bradykinin determination (■) after stopping the reaction with soybean trypsin inhibitor (40 μg). Additional 3-μl aliquots were incubated with 97 μl of fibrinogen (3 mg/ml) in buffer, and then 50 μl of each mixture was added to 0.125 mg prepared kaolin pellets for 6 min at 23°C. The starting material and supernatants were counted for gamma radioactivity and the percentage of ^{125}I-HMW-kininogen adsorbed to kaolin was calculated (○). After SDS-PAGE, the percentage of each M_r form 120,000 (●), 56,000 (×), 45,000 (□) was calculated after densitometric scanning of the stained gel (see Methods).

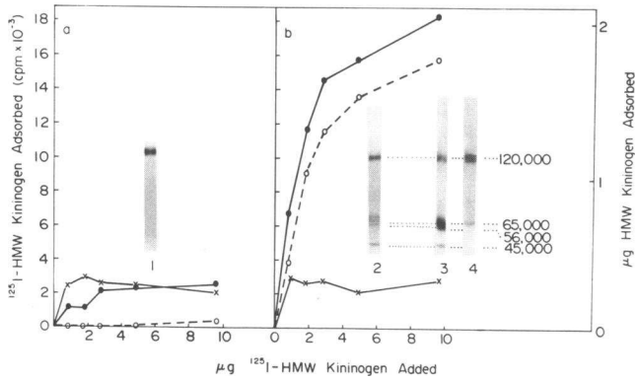

Figure 7. Effect of cleavage by kallikrein on the adsorption of ^{125}I-HMW-kininogen to kaolin. Various amounts of ^{125}I-HMW-kininogen starting material (*a*) were diluted with unlabeled HMW-kininogen 1:120 in order to reduce the specific radioactivity to 9,800 cpm/μg. ^{125}I-HMW-kininogen, which was incubated with kallikrein (1:48 wt/wt) for 2 h at 37°C (*b*), was also incubated with 218 μg fibrinogen, 2,180 μg human serum albumin, and 100 μg of kaolin at 23°C for 6 min in the presence and absence of an additional fivefold excess unlabeled HMW-kininogen. The samples were centrifuged at 12,000 *g* at 23°C for 1 min and the supernatants and pellets counted for radioactivity. The kaolin-associated radioactivity and amount of HMW-kininogen are plotted vs. the amount of HMW kininogen incubated. ●, ^{125}I-HMW-kininogen alone; ×, ^{125}I-HMW-kininogen plus unlabeled HMW-kininogen; ○, specific adsorption (the difference between the above). (*Inset*) The pellets, obtained by incubating cleaved ^{125}I-HMW-kininogen with kaolin, were washed with 100 μl saline three times and this wash, containing <6% of the total radioactivity, was discarded. The pellets were then incubated for 10 min at 23°C with 1% SDS in Tris-Cl, pH 6.8. The soluble extracts as well as the unadsorbed material, were run reduced on a 10% SDS-PAGE, and an autoradiogram was performed at −70°C and percentage of total radioactivity was calculated as in Methods. This photograph represents the 5 μg ^{125}I-HMW-kininogen sample. *1*, uncleaved starting material; *2*, cleaved (partially) starting material; *3*, SDS-extracted pellet; *4*, supernatant.

that kallikrein can render HMW-kininogen capable of adsorption to kaolin in a plasma environment, purified kallikrein was incubated with Factor XII-deficient plasma (Fig. 8) before the mixture was exposed to kaolin (curves a and c) and compared with normal plasma (curve b). At various times, a portion was removed and the supernatants were assayed for residual HMW-kininogen coagulant activity. The amount of HMW-kininogen that adsorbed to kaolin increased with time of incubation to kaolin, unlike what occurred in the sample without kallikrein (curve d). Therefore, kallikrein can directly promote kaolin adsorption of HMW-kininogen in Factor XII-deficient plasma.

Discussion

The discovery that HMW-kininogen is an essential determinant of the rate of initiation of blood coagulation on in vitro surfaces opened up a new line of inquiry into the mechanism of contact-activated coagulation. Many of the reactions previously known to occur on negatively charged surfaces, including the reciprocal activation of Factor XII and prekallikrein, and the transformation of Factor XI into an active enzyme, are dramatically accelerated in the presence of HMW-kininogen (3–6). The observations that this cofactor forms complexes with prekallikrein (2) and Factor XI (1) led to the hypothesis that the binding to HMW-kininogen optimally positions these zymogens on clot-promoting surfaces for subsequent activation by Factor XIIa. However, since Factor XII adsorbs to negatively charges surfaces independently of HMW-kininogen (3) (Fig. 1 *B*), current concepts have not provided for coordination of the binding of this initiator of contact-activated coagulation with the cofactor, HMW-kininogen. We now present evidence that this cofactor exists as a procofactor, and that proteolysis by plasma kallikrein greatly augments its adsorption to a kaolin surface, in the presence of fibrinogen concentrations occurring in plasma (Figs. 6 and 7). Fibrinolysis cannot explain the ability of cleaved HMW-kininogen to adsorb to kaolin, since in a purified system (Fig. 6), kallikrein (the only enzyme in the experiment) was inactivated before incubation with plasminogen-free fibrinogen and kaolin. We therefore conclude that not only is HMW-kininogen necessary for optimal positioning of the zymogens, prekallikrein, and Factor XI on a negatively charged surface but that in its cleaved form, is actually responsible for transporting the zymogens or enzymes to the surface.

Purified uncleaved HMW-kininogen adsorbed to kaolin even in the presence of high concentrations of human albumin (30 mg/ml), but this adsorption was prevented by plasma concentrations of purified fibrinogen (Fig. 5). However, fibrinogen failed to prevent cleaved HMW-kininogen from adsorption to kaolin

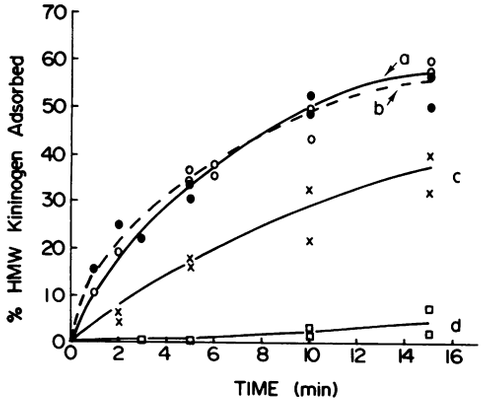

Figure 8. Effect of kallikrein on HMW-kininogen adsorption to kaolin in Factor XII-deficient plasma. Purified kallikrein (0.92 U/ml) (*a*), 0.46 U/ml (*c*), or buffer (*d*) was incubated with Factor XII-deficient plasma for 3 min before incubation with kaolin (2.5 mg/ml). At various times, 50-μl aliquots were centrifuged. The starting material and supernatants were then assayed for HMW-kininogen coagulant activity. Normal plasma was included for comparison (*b*).

(Fig. 7). Since HMW-kininogen in plasma must interact with a negatively charged surface in the presence of ~3 mg/ml fibrinogen in order to become a cofactor for the activation of Factor XI and prekallikrein, a mechanism must be operative that permits HMW-kininogen to associate with the activating surface. Cleavage of human HMW-kininogen in solution would serve this purpose. Sugo et al. (31) showed that when bovine HMW-kininogen was "activated" by limited proteolysis by bovine plasma kallikrein, it became a much more efficient cofactor of Factor XII activation than the intact form. A logical explanation of this experiment is that active HMW-kininogen adsorbs more avidly to the kaolin employed, facilitating the kallikrein-mediated cleavage of Factor XII on a negatively charged surface.

The requirement for Factor XII in the proteolysis of HMW-kininogen, therefore, appears to be indirect, requiring first, the conversion of Factor XII to XIIa and Factor XII fragments on a negatively charged surface, which in turn, cleaves prekallikrein to yield kallikrein. Support for this conclusion derives from the observation of greatly diminished adsorption of HMW-kininogen to kaolin from prekallikrein-deficient plasma (Fig. 1 *A*) as well as the promotion of HMW-kininogen adsorption in Factor XII-deficient plasma incubation of the plasma with kallikrein (Fig. 8). Moreover, when we quantified the adsorption of prekallikrein and Factor XI (zymogens requiring HMW-kininogen for optimal adsorption to negatively charged surfaces), we observed that the amount of prekallikrein (Fig. 2 *A*) and Factor XI (Fig. 2 *B*) in Factor XII-deficient and HMW-kininogen-deficient plasma that was adsorbed was greatly diminished, as compared with normal. Therefore, the amount of Factor XI from Factor XII-deficient plasma that adsorbed to kaolin represents the suboptimal adsorption of this zymogen, without the assistance of HMW-kininogen, since the adsorption was similar in HMW-kininogen-deficient plasma. This adsorption, in the absence of HMW-kininogen, however, renders the molecule markedly less active in a coagulant assay (S. Schiffman, personal communication), and less susceptible to cleavage (7). Therefore, HMW-kininogen, whether alone or complexed with either prekallikrein or Factor XI, must be cleaved before it can substantially associate with a negatively charged surface, in the presence of plasma.

It is of interest that Factor XII adsorption to kaolin from all contact-factor-deficient plasmas was similar to normal. This suggests that adsorption of Factor XII is independent of HMW-kininogen as well as independent of cleavage. Furthermore, at a time when only 15% of the Factor XII from normal plasma was adsorbed, 29% of the HMW-kininogen, 65% of the prekallikrein, and 81% of the Factor XI were adsorbed, consistent with the catalytic role of Factor XII in the alteration of HMW-kininogen.

It is also evident from our data, however, that there must be a second mechanism for cleavage of HMW-kininogen in plasma, since HMW-kininogen did adsorb, albeit slowly, to kaolin in prekallikrein-deficient plasma. We recently observed that in a purified system, Factor XIa can cleave HMW-kininogen (32). Furthermore, the slightly diminished HMW-kininogen-adsorption from Factor XI-deficient plasma (Fig. 1 *A*), at the lowest kaolin concentration, suggests an involvement of Factor XIa in the cleavage of HMW-kininogen. It is possible that the slow activation of Factor XII, which is known to occur in prekallikrein-deficient plasma (4), eventually activates enough Factor XI to cleave HMW-kininogen. Further investigations are now underway in our laboratory to determine the role of Factor XIa in HMW-kininogen cleavage and surface-adsorption in plasma. Finally, Wiggins (33) has recently shown that Factor XIIa, at stoichiometric concentrations, can cleave HMW-kininogen, but only in the presence of kaolin.

The present report demonstrates that intact HMW-kininogen is a procofactor that is activated by kallikrein. The requirement of proteolytic cleavage of a cofactor by its product to provide maximum acceleration is not unique to contact-activated coagulation. Factor V functions as a procofactor for the Factor Xa-catalyzed conversion of prothrombin to thrombin. Cleavage of Factor V by thrombin markedly enhances its coagulant activity (34), since Factor Va has increased affinity for the platelet surfaces, as compared with unaltered Factor V (35). Factor VIII:C also exists as a procofactor, which when activated by thrombin, greatly enhances the activation of Factor X by Factor IXa (36).

Certain investigators have postulated, although none has demonstrated (37), a physical complex existing between HMW-kininogen and Factor XIIa on the surface in an attempt to explain the acceleration of contact-activated coagulation by HMW-kininogen. The present study, however, provides evidence for a functional, rather than physical, relationship between these two proteins. The requirement for cleavage of HMW-kininogen by Factor XII-dependent proteases, before surface adsorption, allows for the temporally ordered proximity of HMW-kininogen, with its complexed prekallikrein and Factor XI, to Factor XIIa. Viewed in this way, diffusion of kallikrein into solution (38) would allow propagation of proteolysis, not only by activation of additional amounts of Factor XII (6), but also by cleavage of HMW-kininogen, in order to maximize its cofactor activity.

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