Hageman-Factor-Dependent Fibrinolysis: Generation of Fibrinolytic Activity by the Interaction of Human Activated Factor XI and Plasminogen

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Human coagulation factor XI has been purified, and upon activation with Hageman factor fragments, was found to convert the fibrinolytic proenzyme plasminogen to plasmin. This proactivator activity was shown to be functionally and antigenically distinct from prekallikrein. When the gamma-globulin fractions of plasma deficient in Hageman factor, prekallikrein and factor XI were isolated, factor-XI-deficient plasma possessed two-thirds of the plasminogen proactivator activity of the Hageman-factor-deficient plasma, while prekallikrein deficient plasma had only one-third of the plasminogen proactivator activity. Thus, the Hageman-factor-dependent plasminogen proactivator previously reported to be present in the gamma-globulin fraction of normal human plasma is a function of prekallikrein and factor XI, while the activity observed in prekallikrein-deficient plasma is attributable to factor XI. When compared utilizing digestion of iodinated fibrin, prekallikrein and factor XIa had similar potency per active site; they were, however, far less active than urokinase.

Despite the potential importance of fibrinolysis in both inflammation and homeostasis, little is known about intrinsic fibrinolytic pathways in human plasma. In 1959, Niewiarowski and Prow-Wartelle' reported that kaolin activation of Hageman factor increased the euglobulin fibrinolytic activity of plasma. Iatridis and Ferguson2 extended these studies and demonstrated a requirement for Hageman factor (factor XII) and suggested that Hageman factor was an indirect activator of plasminogen. Colman3 first reported that plasma kallikrein could activate plasminogen, and other investigators have shown that activated factor XII acts upon a proenzyme that in turn directly activates plasminogen; this proenzyme was subsequently identified as prekallikrein.4,5 In a recent publication, we demonstrated that two factors in the gamma-globulin fraction of normal plasma contribute to Hageman-factor-dependent fibrinolysis. These factors were functionally defined as plasminogen proactivators,4 since they could be isolated as proenzymes and were activatable by activated Hageman factor. One of these factors was identical to prekallikrein.6 The second factor, which was present in prekallikrein-deficient plasma, copurified with a second Hageman factor substrate, factor XI. This article represents further studies on the factor-XI-associated plasminogen proactivator activity and demonstrates that both prekallikrein and factor XI account for the Hageman-factor-dependent plasminogen proactivator activity observed in the gamma-globulin fraction of normal plasma.
MATERIALS AND METHODS

Hexadimethrine bromide and diisopropylfluorophosphate (DFP; Aldrich Chemical Co., Inc., Milwaukee, Wisc.); Enzodiffusion fibrin plates (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, Calif.); quaternary aminioethyl (QAE) Sephadex A-50, carboxymethyl (CM) sepharose, diethyaminoethyl (DEAE) Sephadex A-50, Sepharose 4B, and concanavalin A (Con-A) Sepharose (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.); hemostatic phosphatide (cephalin) and bovine serum albumin (BSA) 4x recrystalized; (INC Nutritional Biochemicals Division, Cleveland, Ohio); kaolin and octanoic acid (Fisher Chemical Co., Fair Lawn, N.J.); polyacrylamide gel reagents (Bio-Rad Laboratories, Richmond, Calif.); Bz-Pro-Phe-Arg-pNA HCl (Pentapharm LTD. Basel, Switzerland); Hageman-factor-deficient plasma (Sera Tec Biologicals, New Brunswick, N.J.); and prekallikrein-deficient and factor-XI-deficient plasma (George King Biomedica, Inc., Salem, N.H.) contained 0.38% citrate and were obtained as indicated. The concentrations of prekallikrein, as assessed by radial immunodiffusion, and factor XI, as assessed by functional analysis compared to our standard factor XI preparations, were within normal limits when determined in the Hageman-factor-deficient plasma (35 µg/ml and 6 µg/ml, respectively), as were the concentrations of factor XI in prekallikrein-deficient plasma (7 µg/ml) and the concentration of prekallikrein in factor-XI-deficient plasma (30 µg/ml). Purified human fibrinogen (plasminogen free) and human thrombin were gifts from Dr. John Finlayson, N.I.H., Bethesda, Md. Hirudin was obtained from Dr. Harvey Gralnick, N.I.H., and Dr. Robert Rosenberg, Sidney Farber Cancer Research Center, Boston, Mass. Urokinase was obtained from Abbott Labs, Inc. (Chicago, Ill.) and was 40% pure with a molecular weight of 54,000.

Preparation of Plasma Proteins

Prekallikrein, kallikrein, Hageman factor fragments,4,6 and plasminogen7 were isolated as previously described. Factor XI was purified from 4 liters of human plasma by a modification of previously published procedures. Plasma was desalted on Sephadex G-25 and fractionated by sequential QAE Sephadex A-50 and CM Sepharose chromatography; the conditions and buffers were the same as those described in references 4 and 6. The factor XI pool was dialyzed and fractionated on SP Sephadex, pH 8.1, and concanavalin A Sepharose, as previously described.8 The final preparation contained 160 U coagulant activity/mg if 1 U is assumed to equal the activity present in pooled normal human plasma.

Activation of Prekallikrein and Factor XI With HFF

The functional activity of Hageman factor fragments (HFF) was assessed by bioassay on a guinea pig ileum9 and adjusted such that 5 µl of HFF generated 10 ng of bradykinin following a 2-min incubation at 37°C with 0.2 ml of fresh EDTA-treated plasma. The final concentration was 20 µg/ml. Prekallikrein was incubated with an equal volume of HFF (1:20 molar ratio) for 15 min at 37°C to achieve maximal activation. Factor XI required overnight incubation at room temperature at a molar ratio for HFF/factor XI of 1:10 in order to achieve maximal activation.10

Assays

Coagulation Assays

The partial thromboplastin time (PTT) was measured by the method of Proctor and Rapaport.11 Prekallikrein and factor XI were determined by a modification of the PTT using congenitally deficient plasma.12

Fibrinolytic Assays

Plasmin was assayed with fibrin plates prepared with purified reagents according to the method of Deogny et al.13 Seven milliliters of 2% agarose in water was mixed with 7 ml of 0.25% fibrinogen in barbital buffer (0.1 M barbital, 1.7 mM CaCl2, 0.7 mM MgCl2, 9.4 mM NaCl) pH 7.4 plus 75 µl of human thrombin (20 NIH U/ml). The mixture was allowed to solidify at room temperature in 100 x 15 mm plastic dishes (Falcon). Routinely, 10 µl of test sample and 10 µl of plasminogen (1 mg/ml) were incubated at 30°C for 90 min in polypropylene microfuge (Beckman) tubes. A 7.5-µl quantity was applied to wells of the fibrin plate, which were incubated at 30°C. After the appearance of lytic zone

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(usually 6–12 hr), the plates were photographed at intervals and the diameter of lysis measured from the photograph. Addition of 50 μg of urokinase directly to each plate yielded no detectable lysis.

In some experiments, plasmin was assayed by the procedure of Moroz and Gilmore. Briefly, purified human fibrinogen was labeled with 125I by the chloramine-T method in 0.05 M phosphate buffer, pH 8.0, and had a specific activity of 1.5–1.7 μCi/μg. The radioactive fibrinogen was over 95% clottable upon addition of thrombin. It was adjusted to an activity of 40,000–75,000 cpm/μg with unlabeled fibrinogen and 10 μg fibrinogen were rotated for 3 hr at room temperature in polystyrene tubes (4.5 ml volume). Unbound 125I-fibrinogen was aspirated, and 0.5 ml of a 10 mg/ml solution of bovine serum albumin was added to bind unoccupied polystyrene sites. After standing for 20 min at room temperature, the tubes were aspirated and washed four times with tris-buffered saline. Tris-buffered saline (0.2 ml) containing 2 U purified human thrombin were then pipetted into each tube, and the tubes were incubated for 5 min at 37°C. The thrombin was aspirated, the tubes were washed twice with tris-buffered saline, and aspirated to near dryness before introduction of enzyme reaction mixtures. Fibrinolytic assays were performed by addition of 0.1 ml of plasmin (or other enzyme) source. After incubation for varying intervals, the reactions were terminated by rapid addition of 2 1.0-mI aliquots of tris-buffered saline. After each addition, tube contents were pooled, quantitatively transferred to new uncoated tubes, and counted in a gamma scintillation spectrometer. Appropriate control tubes were incubated in all experiments and these blank values were substrated from test values. This assay has been estimated to be 10 times more sensitive than the fibrin plate method and can detect as little as 0.2 mg plasmin/ml.

Amidolytic Activity of Kallikrein

Buffer or sample (0–100 μl) was diluted with 1 ml of phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA and incubated at 37°C for 2 min. Next, 150 μl of 1 mM Bz-Pro-Phe-Arg-pNA was added and the absorbance at 405 nm monitored with a Gilford spectrophotometer equipped with a thermostated cuvette holder and X-Y recorder. Milliunits of kallikrein were calculated from the change in optical density 405 nm/mm and divided by the molar extinction coefficient for paranitroaniline as described previously.

Preparation of Antisera Affinity Columns and Ouchterlony Double Diffusion

Rabbit anti-human prekallikrein and rabbit anti-human factor XI were prepared by injecting 20 μg of purified protein emulsified in complete Freund’s adjuvant into the popliteal lymph nodes of New Zealand rabbits. Intramuscular booster injections were given at 2 and 8 wk, with 100 μg of protein emulsified in incomplete Freund’s adjuvant. The immunoglobulin fraction was obtained by octanoic acid precipitation of plasma. The precipitate was discarded and the supernatant dialyzed against PBS. These fractions were used without further purification. Antiserum were absorbed with 25% v/v of either prekallikrein- or factor-XI-deficient plasma or with 0.5 mg purified human IgG/ml antisera before octanoic acid treatment. Antiprekallikrein serum gave a single precipitin line when tested against normal plasma or purified prekallikrein (100 μg/ml) and gave no precipitin with prekallikrein-deficient plasma or purified factor XI (50–500 μg/ml). Anti-factor-XI serum gave no precipitin with normal plasma, since the factor XI concentration is so low, but gave a strong precipitin with purified factor XI (100 μg/ml) and none with purified prekallikrein (100 μg/ml). The immunoglobulin fractions were coupled to CI-Sepharose 4B by the cyanogen bromide method. Ouchterlony double diffusion was performed in 1% agarose in PBS with 1 mM EDTA.

Polyacrylamide Gel Electrophoresis (PAGE)

Alkaline disc gel electrophoresis was performed according to Ornstein and Davis. SDS-PAGE was run using the same buffer system, as modified by King and Laemmli, with the exception that 4 M urea was added to the sample buffer. Electrophoresis was carried out in 6 × 100 mm tubes or in a 1.5-mm vertical slab (Bio-Rad Laboratories, N.Y.). Molecular weights (mol wt) of prekallikrein and kallikrein were estimated in reduced samples using reduced carbonic anhydrase (mol wt 29,000), ovalbumin (mol wt 43,000), transferrin (mol wt 90,000), and ß-galactosidase (mol wt 130,000) as standards.
**RESULTS**

Comparisons of Gamma-Globulin Plasminogen Activators in Plasma Deficient in Prekallikrein, Factor XI, or Hageman Factor

Fifty milliliters of plasma deficient in either prekallikrein, factor XI, or Hageman factor were dialyzed against 20 mM Tris-HCl buffer, pH 8.1. Each deficient plasma was applied to one of three identical 3.8 x 20 cm columns of QAE Sephadex, equilibrated in the same buffer. The fraction that did not bind was concentrated to 10 ml by positive pressure filtration through a UM-10 membrane (Amicon). The concentrates were designated as prekallikrein peak I, factor XI peak I, or Hageman factor peak I. The relative amount of IgG in each of the plasma and peak-I concentrates was determined by radial-immunodiffusion plates (Hyland Division, Travenol Labs, Costa Mesa, Calif.) as a check on recovery. The ratios of IgG in the peak I to IgG in the plasma were: 5.7 (prekallikrein-deficient), 5.4 (factor-XI-deficient), and 6.7 (Hageman-factor-deficient). The three peak-I samples were activated overnight with Hageman factor fragments (HFF) and assayed for plasminogen activator utilizing Hyland fibrin plates. The data (Fig. 1) indicate that prekallikrein is responsible for approximately two-thirds of the plasminogen-activating activity in factor-XII-deficient peak I, while factor XI contributes approximately one-third of the activity.

Immunologic Nonidentity of the Factor-XII-Dependent Plasminogen Proactivators

Monospecific rabbit antisera to prekallikrein and factor XI were prepared by absorption with purified IgG or with each respective deficient plasma. The nonidentity of prekallikrein and factor XI was demonstrated by ouchterlony double diffusion (Fig. 2). In addition, the antiserum to prekallikrein yielded no precipitin with factor XI, and the antiserum to factor XI yielded no precipitin with prekallikrein. Together, these data indicate that these two Hageman factor substrates do not share any antigenic determinants. The immunoglobulin fraction of these antisera was then coupled to Sepharose CL-4B. Two 1-ml columns of each
immobilized antibody were equilibrated with 10 mM Tris-HCl, 0.15 M NaCl buffer. Each column was loaded with 300 µl of a kallikrein (50 µg/ml) or factor XIa (15 µg/ml) preparation. The protocol was as follows: Column A—anti-factor XI, sample-kallikrein; column B—antiprekallikrein, sample-kallikrein; column C—antiprekallikrein, sample-factor XIa; column D—anti-factor-XI, sample-factor XIa. Two column volumes were collected and concentrated to 300 µl by dialysis against 10% Ficol in PBS. The concentrates and original samples were then assayed for plasminogen activator activity (Fig. 3). Significant reduction of fibrinolytic activity occurred when kallikrein was applied to antiprekallikrein columns but not to anti-factor-XI columns. Conversely, factor XIa fibrinolytic activity was absorbed by anti-factor-XI columns but not by antiprekallikrein columns. In each case, the diminution of fibrinolytic activity paralleled the loss of either kallikrein or factor XIa activity when each protein was passed over the homologous immunoadsorbent. When each protein was passed over the heterologous antibody and concentrated back to the original volume, over 90% of the protein applied was recovered. Although a representative experiment is shown, this study was repeated three times, each of which yielded the same result.
Functional Independence of the Factor-XII-Dependent Plasminogen Proactivators

Additional evidence that the activity in factor XI preparations was not due to contaminating prekallikrein is shown in Fig. 4. Factor XI was activated with HFF and assayed with a specific kallikrein substrate, Bz-Pro-Phe-Arg-pNA. The amount of amidolytic activity was compared with dilutions of a second kallikrein preparation. If it is assumed that all the amidolytic activity in the factor XI preparation was due to kallikrein, then the amount of contaminating kallikrein was equal to 1:640 dilution of the kallikrein sample (Fig. 4A). Figure 4B compares the plasminogen activator activity of the same two preparations. The plasminogen activator activity in the factor XI preparation corresponds to a 1:3–1:4 dilution of the kallikrein preparation. Thus, the amount of kallikrein in the factor XI preparation is insufficient by two orders of magnitude to account for the fibrinolytic data.

Functional Comparison of Purified Factor XI and Prekallikrein

Figure 5 shows an 8% SDS-PAGE of purified factor XI. Human XI is a single species of apparent mol wt 158,000 in nonreduced samples. Upon reduction, factor XI dissociates into two identical subunits of 80,000 daltons. Efforts to
Fig. 4. (A) Amidolytic activity of serial dilutions of a kallikrein preparation compared to that of a factor Xla sample. (B) Plasminogen-activating activity of serial dilutions of the same kallikrein preparation compared to the undiluted factor Xla sample.

demonstrate the Hageman factor dependence of the factor-XI-associated plasminogen activator were complicated by the relative inability to activate factor XI in the fluid phase with the same facility as prekallikrein. It required greater than 8 hr at 23°C to activate factor XI with HFF (Fig. 6) while prekallikrein was activated within 15 min under similar conditions.

Purified factor XI and purified prekallikrein were then adjusted to 30× plasma
concentration and assayed for plasminogen activator and plasminogen proactivator activity. Serial dilutions of Hageman-factor-deficient plasma were compared with factor XI in a PTT assay utilizing factor-XI-deficient plasma as a substrate. Prekallikrein was similarly quantitated using prekallikrein-deficient plasma. Activation of prekallikrein and factor XI were performed overnight to insure factor XI activation, and samples were assayed utilizing fibrin plates prepared with purified reagents. As shown in Fig. 7, at comparable plasma concentration, factor XIa had approximately one-half the activity of the kallikrein preparation, and in each case, the activity was Hageman-factor-dependent.
Comparison of the Plasminogen Activating Ability of Kallikrein and Factor Xla With Urokinase

In order to compare the plasminogen-activating ability of kallikrein, factor Xla, and urokinase on an active site basis, the digestion of ¹²⁵I-fibrin bound to polystyrene tubes was used as an assay for plasmin. The concentration of urokinase used was $7.4 \times 10^{-6} M$, kallikrein was set at $3 \times 10^{-6} M$, and the concentration of factor Xla active sites was $3.6 \times 10^{-6} M$, (molarity $\times 2$ since it has 2 active sites/mole). Ten-microliter samples of each enzyme were incubated with 10 µl plasminogen (2 mg/ml) for 30 min at 37°C. The plasmin generated was then assayed by addition to ¹²⁵I-fibrin-coated tubes for 10 min at 23°C; the reaction was stopped by transfer to noncoated tubes and the release of fibrin peptides determined. The fibrinolytic activity was determined in controls consisting of the plasminogen solution incubated with an equal volume of buffer as well as each enzyme incubated with buffer and were subtracted from the data shown. Incubation of plasminogen with HfF at the concentration used to activate prekallikrein was not different from buffer. As seen in Fig. 8, a dose response for urokinase was obtained between a 12,000- and 100,000-fold dilution. The values obtained for the undiluted kallikrein and factor Xla preparations are shown and indicate an activity similar to a 50,000-fold dilution of the urokinase with the kallikrein being slightly more potent than the factor Xla.

DISCUSSION

The two Hageman factor substrates, prekallikrein and factor XI, are gammaglobulin glycoproteins with respective molecular weights of 88,000$^6$ and 156,000$^{18,22}$ daltons. Both proenzymes are activated by Hageman factor through a mechanism...
involving limited proteolysis.\textsuperscript{6,8,21} That kallikrein can activate plasminogen has now been reported from several laboratories.\textsuperscript{3,5,6} We now suggest that both Hageman factor substrates participate in Hageman-factor-dependent fibrinolysis. The identification of factor XI as a plasminogen proactivator is based on the following observations: (1) the plasminogen-activating activity associated with factor XI is Hageman-factor-dependent; (2) the activity associated with factor XI is functionally and immunochemically distinct from prekallikrein; (3) factor XI procoagulant and profibrinolytic activity copurify to homogeneity (as assessed by reduced and nonreduced SDS-PAGE); and (4) the ratio of plasminogen-activating activity between purified prekallikrein and factor XI correlates well with the fibrinolytic defect in the gamma-globulin fractions of prekallikrein- and factor-XI-deficient plasma.

This conclusion is at variance with observations previously reported by other workers. Vennerod and Laake\textsuperscript{23} reported that plasma deficient in prekallikrein was devoid of gamma-globulin-associated plasminogen activator or plasminogen proactivator activity, however, the use of HFf as the activator may restrict one's ability to observe this reaction, since it is a weak factor XI activator but retains full activity upon prekallikrein.\textsuperscript{9} Bouma and Griffin reported that purified factor Xla did not activate plasminogen.\textsuperscript{24} The latter authors did observe plasminogen activator activity in the gamma-globulin fraction of normal or prekallikrein-deficient plasma,\textsuperscript{25} but this activity was not Hageman-factor-dependent. Whether this is an additional fibrinolytic factor is not clear. Although it has recently been reported that HFf has plasminogen-activating activity,\textsuperscript{26} at the concentrations used in this study to activate the Hageman factor substrates, the contribution of HFf was negligible. When compared to kallikrein and factor Xla on an active site basis, HFf was 18-fold less potent using the digestion of \textsuperscript{125}I-fibrin.

Ogston et al.\textsuperscript{27} have reported a Hageman-factor-dependent plasminogen activator that could be adsorbed from plasma by crushed glass. The depleted plasma could then be reconstituted by addition of a gamma-globulin fraction from normal plasma or factor-XI-deficient plasma. Wuepper\textsuperscript{28} demonstrated that adsorption of plasma with crushed glass rendered it prekallikrein-deficient plasma, thus, reconstitution with any prekallikrein would correct the abnormality in Hageman factor activation as well as any direct effect of kallikrein upon plasminogen. However, the profile of inhibition by plasma protease inhibitors as well as the apparent molecular size of this factor resembled those of factor XI.\textsuperscript{29} It is therefore possible that this activity (called Hageman factor cofactor) was a function of factor XI as well as prekallikrein.

An estimate can be made regarding the relative potency of the two Hageman-factor-dependent plasminogen activators. The current estimates for plasma levels of prekallikrein and factor XI indicate that prekallikrein is at least three times more abundant. Prekallikrein contributes 60% of the plasminogen activating activity in the gamma-globulin fraction of normal plasma, while factor XI contributes approximately 30%. Alternatively, the ratio of activator activity between purified kallikrein and factor Xla, when both are at relative plasma concentration, was 2:1 (Fig. 7). Thus, kallikrein and factor Xla appear to have similar plasminogen-activating capability when assessed by the fibrin plate method. The result is confirmed by the assay utilizing the digestion of \textsuperscript{125}I-fibrin. Factor Xla
is actually more potent in a molar basis; however, if one accounts for its content of two active sites/mole, kallikrein is slightly more potent/active site (Fig. 8).

Earlier data by Colman suggested that the kinetics of interaction of kallikrein with plasminogen was stoichiometric and that catalytic conversion of plasminogen to plasmin did not occur. Yecies and Kaplan have shown that prekallikrein and kallikrein do not form a stable complex with plasminogen, thus, the reason for the apparent stoichiometry is not clear. At a high molar ratio of kallikrein to plasminogen, one can demonstrate cleavage of plasminogen to yield the two-chain structure characteristics of plasmin as well as incorporation of $^{32}$P-DFP into the plasmin light chain. There is, in addition, sufficient prekallikrein in plasma to potentially activate 10%–20% of the plasminogen present. Regardless of mechanism, kallikrein and factor XIa are exceedingly weak activators of plasminogen when compared to a plasminogen activator such as urokinase. Nevertheless, kallikrein and factor XIa have relevance to the phenomenon of kaolin-activatable fibrinolysis, since this pathway is not activatable in plasma deficient in Hageman factor or HMW-kininogen, and the rate of plasmin generation is markedly diminished in prekallikrein-deficient plasma. However, the abnormality seen in each of these deficient plasmas is a function of the contribution made by each of these proteins to Hageman-factor activation as well as their ability to directly activate plasminogen. Since kaolin-activatable fibrinolysis is normal in factor-XI-deficient plasma, the effect of kallikrein appears more important than that of factor XIa; however, factor XIa, as well as activated Hageman factor, may contribute to the gradual evolution of plasmin in prekallikrein-deficient plasma.

Since kallikrein, factor XIa, and HFF are such weak plasminogen activators when interacted with purified plasminogen (compared to urokinase), one must seek other as yet unidentified plasma activators of plasminogen that are generated as a consequence of Hageman-factor activation. Thus, the abnormalities seen in congenitally deficient plasmas indicated above may relate to the need for each protein to activate Hageman factor, and their contribution to the direct conversion of plasminogen to plasmin may be small. On the other hand, it appears appropriate to seek nonenzymatic cofactors that may function in plasma to augment plasminogen activation; it is also possible that the kinetics of activation of plasminogen bound to kaolin or fibrin by any of these enzymes may be considerably different from that seen with purified components in solution.

REFERENCES


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RJ Jr Mandle and AP Kaplan