The Fibrinolytic Pathway of Human Plasma

II. THE GENERATION OF CHEMOTACTIC ACTIVITY BY ACTIVATION OF PLASMINOGEN PROACTIVATOR

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ABSTRACT The conversion of plasminogen proactivator to plasminogen activator by Hageman factor fragments results in the generation of chemotactic activity for human neutrophils. This chemotactic activity can be distinguished from that generated by Hageman factor activation of prekallikrein and is demonstrable in plasma that is genetically deficient in prekallikrein (Fletcher factor deficiency). Both the plasminogen-activating activity and chemotactic activity produced by the interaction of Hageman factor fragments and plasminogen proactivator to yield plasminogen activator were inhibited by diisopropyl fluorophosphate (DFP) indicating an essential role for the enzymatic site in both these activities. The finding that the plasminogen proactivator tolerated a dose of DFP, which completely inactivated the plasminogen activator, reveals that the active site is protected in the precursor protein.

INTRODUCTION

The Hageman factor-activatable fibrinolytic pathway of human plasma has been shown to proceed by the action of activated Hageman factor or its fragments upon plasminogen proactivator to yield plasminogen activator, which in turn converts plasminogen to plasmin (1). Activated Hageman factor and Hageman factor fragments, their substrates prekallikrein and precursor plasma thromboplastin antecedent (pre-PTA),[1] and activated PTA are all devoid of human leukocyte chemotactic activity. However, conversion of prekallikrein to kallikrein by Hageman factor fragments generated this activity for human neutrophils, which was dependent upon the active enzymatic site of kallikrein (2). The identification of plasminogen proactivator as a third Hageman factor-activatable proenzyme of human plasma prompted an examination of its possible contribution to the generation of chemotactic activity in human sera. Conversion of highly purified plasminogen proactivator to plasminogen activator was shown to result in the generation of chemotactic activity; both the plasminogen-activating activity and the chemotactic activity are dependent upon the active enzymatic site of plasminogen activator.

METHODS

Bradykinin (Sandoz Pharmaceuticals, Basel, Switzerland, or New England Nuclear, Boston, Mass.) was used as the standard for the bradykinin bioassay. Hexadimethrine bromide (Polybrene, Aldrich Chemical Co., Inc., Milwaukee, Wis.), enzodiffusion fibrin plates (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.), PTA-deficient and plasma thromboplastin component-deficient plasma (Sera-Tec Biologicals, New Brunswick, N. J.) were obtained as indicated. Fletcher factor-deficient plasma was a gift from Dr. Charles Abildgaard (University of California, Davis, Calif.).

Serum was processed for the isolation of activated Hageman factor, the Hageman factor fragments, and the active enzymes kallikrein, PTA, and plasminogen activator (3) while plasma (2) was utilized as a source of the proenzymes prekallikrein, pre-PTA, and plasminogen proactivator. Plasminogen was purified from material obtained by means of the affinity chromatographic procedure of Deutsch and Mertz (4). Plasminogen proactivator and pre-

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[1] *Abbreviations used in this paper:* DFP, diisopropyl fluorophosphate; PTA, plasma thromboplastin antecedent; PTT, partial thromboplastin time; QAE, quaternary ammonium ethyl; SP, sulphopropyl.

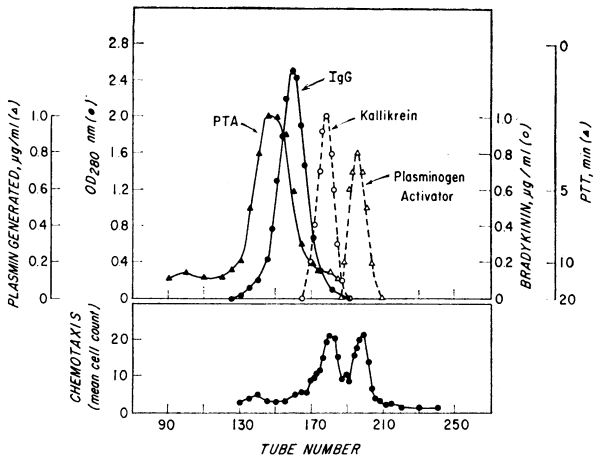

FIGURE 1 Sephadex G-150 chromatography of a DEAE-cellulose effluent of serum indicating the elution pattern of PTA, kallikrein, and plasminogen activator (above) and the assay for chemotactic activity (below).

kallikrein were isolated by sequential chromatography of human plasma on quaternary ammonium ethyl (QAE) Sephadex, sulphopropyl (SP) Sephadex, and twice on Sephadex G-150. Each precursor molecule was a unique component and contained IgG as the only contaminant on disc-gel electrophoresis or isoelectric focusing (1). The Hageman factor prealbumin fragments were purified by rechromatography of a plasma fraction obtained from QAE Sephadex on QAE Sephadex, followed by Sephadex G-100 gel filtration, SP Sephadex chromatography, and alkaline disc-gel electrophoresis (3, 5). After dialysis against 0.003 M phosphate buffer containing 0.15 M NaCl, the fragments were concentrated by ultrafiltration to 25 μg/ml and contained no contaminants upon reelectrophoresis on alkaline disc gels. Intact activated Hageman factor was purified by sequential chromatography of plasma on QAE Sephadex and carboxymethyl (CM)-cellulose, and then gel filtration on Sephadex G-100; it contained transferrin as the sole

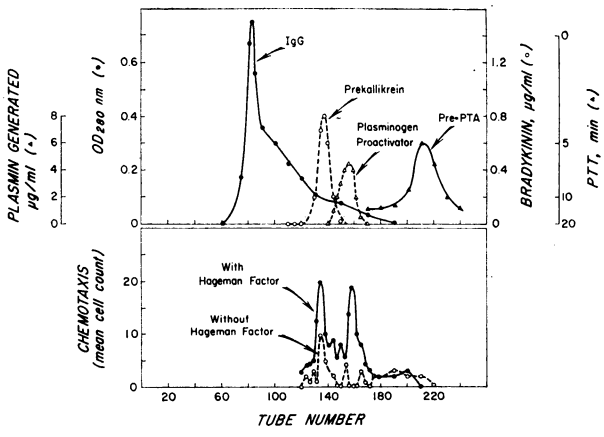

FIGURE 2 SP Sephadex chromatography of a QAE Sephadex effluent of plasma indicating the elution pattern of the proenzymes prekallikrein, plasminogen proactivator, and pre-PTA (above) and the chemotactic assay before and after the addition of Hageman factor fragments (below).

identifiable contaminant (5, 6). The initial effluent from DEAE-cellulose chromatography of serum dialyzed against 0.003 M PO_4 buffer, pH 8.0, was used as a source for the active enzymes kallikrein, PTA, and plasminogen activator (1). The assays utilized to determine prekallikrein, pre-PTA, plasminogen proactivator, and plasminogen, as well as their respective active enzymes, have been reported (1–3).

Chemotaxis of human neutrophils was quantitatively assayed by a previously described modification (7, 8) of the Boyden micropore filter technique (9) utilizing 3 μm pore size micropore filters (Millipore Corp., Bedford, Mass.) in modified Boyden chambers. Leukocytes from normal subjects were processed as in reference 8 and were suspended in Medium-199, 0.5% ovalbumin, pH 7.4, at an initial cell count of $1.3–1.6 \times 10^6$ leukocytes/ml; chemotactic agents were diluted in the same buffer. Each chemotactic reaction was studied in duplicate chambers, and the leukotactic response was expressed as the mean of the number of neutrophils per high power field, after counting five high power field from each of the duplicate filters.

RESULTS

Chemotactic activity associated with plasminogen activator. The DEAE-cellulose effluent from 40 ml of serum containing PTA, kallikrein, and plasminogen activator was concentrated and further fractionated by Sephadex G-150 gel filtration. As shown in Fig. 1, two peaks of chemotactic activity were found which were superimposable upon the peaks of plasminogen activator and kallikrein activities, whereas no chemotactic activity was observed corresponding to the elution position of either PTA or IgG. When the PTA peak was concentrated so that the partial thromboplastin time (PTT) of PTA-deficient plasma was further corrected to 1.5 min, thus approximating normal plasma levels, no chemotactic activity was demonstrable.

Generation of chemotactic activity by activation of plasminogen proactivator. The chemotactic activity, obtained upon activation of the proenzymes plasminogen proactivator and prekallikrein, was evaluated during each step in their purification procedure. The QAE Sephadex effluent from 100 ml of human plasma containing IgG and the proenzymes pre-PTA, prekallikrein, and plasminogen proactivator was then fractionated by SP Sephadex chromatography. The chromatographic fractions were assayed for chemotactic activity before and after activation with Hageman factor fragments. Upon incubation with the Hageman factor fragments, plasminogen proactivator and prekallikrein were activated and peaks of chemotactic activity were obtained in the elution position of each of these proenzymes (Fig. 2). Before activation, there was some chemotactic activity associated with the prekallikrein peak alone, but no plasminogen activation or bradykinin generation were observed. Activation of pre-PTA by Hageman factor fragments or by intact, activated Hageman factor in an

additional experiment was not associated with the generation of chemotactic activity.

The two peaks of chemotactic activity corresponding to plasminogen proactivator and prekallikrein (Fig. 2) were further assessed by combining these peaks and refractionating the mixture on Sephadex G-150. As shown in Fig. 3, two peaks of chemotactic activity were again obtained upon activation with Hageman factor fragments corresponding to the elution positions of plasminogen proactivator and prekallikrein.

When the plasminogen proactivator peak obtained from SP Sephadex chromatography (Fig. 2) was kept separate from the prekallikrein peak and was subjected to gel filtration Sephadex G-150, chemotactic activity activatable by Hageman factor corresponded predominantly to plasminogen proactivator although there was a small chemotactic peak attributable to activation of prekallikrein. This plasminogen proactivator was then pooled, concentrated, and rechromatographed on Sephadex G-150. A single peak of chemotactic activity, activatable by Hageman factor, was superimposable on the plasminogen proactivator peak.

Inhibition of chemotaxis by DFP. The chemotactic activity associated with plasminogen activator was studied for susceptibility to inactivation by diisopropyl fluorophosphate (DFP), because the chemotactic activity associated with kallikrein was destroyed by DFP (2). Highly purified plasminogen proactivator, free of detectable prekallikrein, was prepared from 100 ml plasma by the usual four-step sequential chromatographic procedure and concentrated to 3.0 ml. $\frac{6}{10}$ ml was incubated with either 60 μl of purified Hageman factor prealbumin fragments (25 μg/ml) or 60 μl phosphate-buffered saline at 37°C for 3 h. The two samples were then divided in half; one portion of each was made 10^{-3} M in DFP and the other half treated with buffer. The samples were incubated for 90 min at 37°C and dialyzed overnight against 0.003 M phosphate buffer pH 7.8 containing 0.15 M NaCl. Each preparation was then assayed for plasminogen activator and chemotactic activity. The unactivated sample possessed minimal plasminogen-activator activity and chemotactic activity (Fig. 4A). The sample, which was activated with Hageman factor fragments, generated 50 μg/ml plasmin and a mean leukocyte count of 92 (Fig. 4B). When the activated sample was treated with DFP its plasminogen-activating activity was diminished by 80% and its chemotactic activity by 98% (Fig. 4C). When the unactivated DFP-treated sample (Fig. 4D) was exposed to 30 μl of Hageman factor fragments for 3 h at 37°C, the plasminogen-activator activity and chemotactic activity were approximately two-thirds those of the activated sample never exposed to DFP.

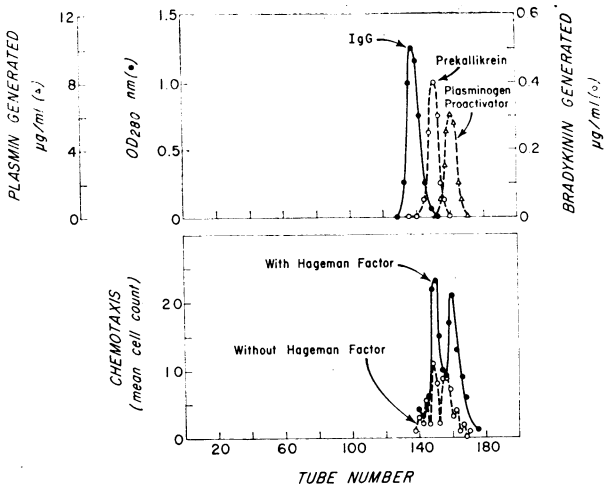

FIGURE 3 Sephadex G-150 chromatography of a mixture of prekallikrein and plasminogen proactivator obtained from SP Sephadex (Fig. 2). Beneath is shown the chemotactic assay before and after incubation with Hageman factor fragments.

Chemotactic activity of plasminogen activator isolated from prekallikrein-deficient plasma. Fletcher factor-deficient plasma (10, 11) has been shown to possess no prekallikrein (12–14) and is therefore useful in assessing the contribution of plasminogen proactivator to the generation of Hageman factor-activatable chemotactic activity. $2\frac{1}{2}$ ml of normal plasma and of Fletcher factor-deficient plasma were each dialyzed against 0.003 M PO_4 buffer, pH 8.0, and passed over a 2 × 10 cm column of QAE Sephadex equilibrated with the same buffer. 25 ml of effluent was collected, concentrated to 2.0 ml, and examined for prekallikrein, plasminogen proactivator, and chemotactic activity. The unactivated QAE Sephadex

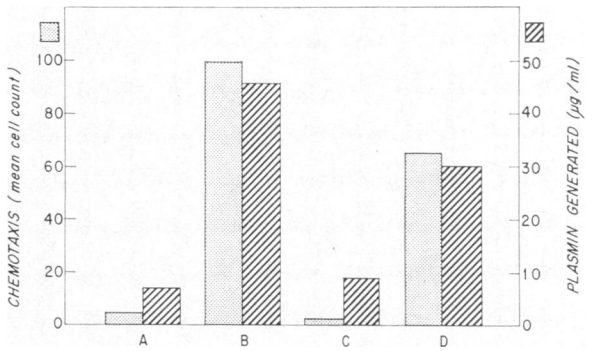

FIGURE 4 Comparison of the effect of DFP upon plasminogen activator and plasminogen proactivator. (*A*) Plasminogen proactivator, (B) plasminogen proactivator activated with the Hageman factor fragments, (C) plasminogen proactivator activated with the Hageman factor fragments, made 10^{-3} in DFP, and then dialyzed, and (D) plasminogen proactivator made 10^{-3} in DFP, dialyzed, and then activated with the Hageman factor fragments.

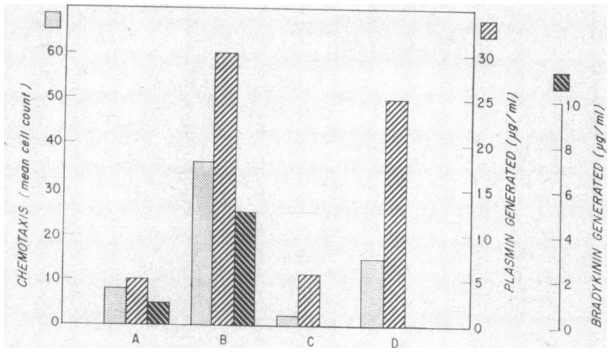

FIGURE 5 Comparison of plasminogen proactivator, (fibrinolytic activity), prekallikrein (kinin-generating activity), and chemotactic activity of a QAE Sephadex effluent obtained from either normal plasma or prekallikrein-deficient plasma. (A) Normal plasma effluent, (B) Normal plasma effluent activated with Hageman factor fragments, (C) Prekallikrein-deficient plasma effluent, and (D) Prekallikrein-deficient plasma effluent activated with Hageman factor fragments.

effluent obtained from normal plasma contained a small quantity of kallikrein, plasminogen activator, and chemotactic activity (Fig. 5A), and when activated with the Hageman fragments, exhibited a five to sixfold increase in each activity (Fig. 5B). The unactivated effluent obtained from Fletcher factor-deficient plasma possessed no kallikrein and had little plasminogen activator or chemotactic activity (Fig. 5C). When activated (Fig. 5D), this preparation exhibited a fivefold increase in plasminogen activator and in chemotactic activity in the absence of any detectable kallikrein. The recovery of plasminogen proactivator in the Fletcher factor-deficient plasma was 80% and the observed chemotactic activity was 40% of that of the normal plasma.

DISCUSSION

The plasminogen activator has been shown not only to convert plasminogen to plasmin but to exhibit chemotactic activity for human neutrophils. The chemotactic activity of plasminogen activator was distinguished from the chemotactic activity of kallikrein as follows: the chemotactic activity present in a QAE Sephadex effluent of human serum containing both kallikrein and plasminogen activator was resolved by Sephadex G-150 into two peaks of chemotactic activity, one associated with kallikrein and the other with plasminogen activator (Fig. 1); the chromatographic procedures utilized to isolate the proenzymes plasminogen proactivator and prekallikrein from plasma consistently yielded two peaks of Hageman factor-activatable chemotactic activity that were associated with plasminogen activator and kallikrein activity, respectively (Figs. 2, 3); plasminogen proactivator, purified free of prekallikrein, yielded a single Hageman factor-activatable chemotactic peak; and the QAE Sephadex effluent of normal plasma contained approximately twice the Hageman factor-activatable chemotactic activity of an equivalent effluent of prekallikrein-deficient (Fletcher factor-deficient) plasma attributable solely to the conversion of a normal content of plasminogen proactivator to activator (Fig. 5). Thus, the capacity of plasminogen proactivator to yield chemotactic activity upon conversion to plasminogen activator has been maintained throughout its purification from normal plasma. The chemotactic activity of plasminogen activator is further documented by the plasminogen-activating activity and chemotactic activity obtained upon activation of a plasma fraction from a patient genetically deficient in prekallikrein.

The chemotactic activity of kallikrein was previously shown to be dependent upon the integrity of its active site (2) and not due to a fragment released during activation. Similarly, the chemotactic activity observed upon conversion of plasminogen proactivator to plasminogen activator is not separable from the chemotactic activity of plasminogen activator by gel filtration (Fig. 1) and it is inhibited by DFP (Fig. 4). Whereas neither highly purified plasminogen proactivator nor the prealbumin Hageman factor fragments were chemotactic alone, their interaction, to convert plasminogen proactivator to activator, yielded both plasminogen-activating activity and chemotactic activity (Figs. 2, 3, 4). Both these activities were completely inhibited by a concentration of DFP which had no effect on the capacity of the plasminogen proactivator to reveal its active site after dialysis and exposure to Hageman factor fragments (Fig. 4D). Thus the plasminogen-activating and chemotactic-active site(s) is protected in the precursor molecule as is the case for prekallikrein and the precursor form of other serine esterases (15, 16).

Hageman factor activation of pre-PTA to yield PTA (another DFP-sensitive human plasma enzyme) is not associated with the appearance of chemotactic activity for human neutrophils (Figs. 1, 2) (2). Thus there is some unique specificity to the neutrophil recognition mechanism for kallikrein and plasminogen activator. Further, these two chemotactic principles appear to be equal in their contribution to chemotaxis as assessed by analysis of the γ-globulin region of normal serum (Fig. 1) and plasma (Figs. 2, 3), and by the demonstration that the residual Hageman factor-activatable chemotactic activity of prekallikrein-deficient plasma was approximately half-normal.

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