Heparin Cofactor Activities in a Family With Hereditary Antithrombin III Deficiency: Evidence for a Second Heparin Cofactor in Human Plasma

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Plasma levels of antithrombin-heparin cofactor, determined by heparin-dependent antithrombin assay, and antithrombin III antigen were measured in 22 members of a large kindred predisposed to venous thrombosis. While 11 members had reduced plasma levels of both antithrombin-heparin cofactor and antithrombin III antigen, the levels of antithrombin-heparin cofactor were always greater than the levels of antithrombin III antigen: 66% (±7%) and 49% (±5%) of normal plasma, respectively. Pooled normal plasma and plasma from one of the affected family members (60% antithrombin-heparin cofactor and 47% antithrombin III antigen) were fractionated by heparin-agarose affinity chromatography. Antithrombin-heparin cofactor, which eluted from heparin-agarose with buffer containing 0.4 M NaCl and did not cross-react with antibody specific for antithrombin III and did not inhibit factor Xa at an appreciable rate in the presence of heparin, was designated heparin cofactor A. Antithrombin-heparin cofactor, which eluted from heparin-agarose with buffer containing 2.0 M NaCl, was functionally and antigenically identified as antithrombin III. The concentrations of heparin cofactor A in normal and patient plasma were similar (4.5 x 10^{-7} M), while the concentration of antithrombin III in patient plasma (8.0 x 10^{-7} M) was only 50% of normal (1.6 x 10^{-6} M). The functional properties of both heparin cofactor A and antithrombin III obtained from patient plasma were normal. From the results of the present study it would appear that the antithrombin-heparin cofactor concentration measured in patient plasma reflects the combined concentrations of heparin cofactor A and antithrombin III. Since heparin cofactor A does not cross-react with antibody to antithrombin III, the concentration of antithrombin III antigen in patient plasma is thus lower than the concentration of antithrombin-heparin cofactor.

In 1939, Brinkhous and coworkers demonstrated that the anticoagulant activity of heparin was dependent on a plasma component that was termed heparin cofactor.1 In subsequent years, the term antithrombin II was used to describe this heparin-dependent thrombin inhibitor in plasma, while the term antithrombin III was used to describe the heparin-independent thrombin inhibitor in plasma.2 In 1968, Abildgaard isolated antithrombin III and demonstrated that antithrombin III also had heparin cofactor activity,3 suggesting that antithrombin II and antithrombin III were one and the same protein. Briginshaw and Shanberge4,5 and Tollefsen and Blank,6 however, have provided evidence that a second heparin cofactor, different from antithrombin III, is also present in plasma. Thus, the heparin cofactor activity in plasma may reflect more than one heparin-dependent thrombin inhibitor.

In 1965, Egeberg reported that an inherited deficiency of antithrombin III (heparin cofactor) was related to a thrombotic tendency.7 Numerous studies have since confirmed this observation.8-23 In the present article, we describe a family predisposed to venous thrombosis. Members of the family were found to be deficient with respect to plasma levels of antithrombin III antigen and antithrombin-heparin cofactor, but demonstrated a characteristic and reproducible disparity between the two levels. The presence of a second antithrombin-heparin cofactor, heparin cofactor A,*

*We have chosen to use the nomenclature of Briginshaw and Shanberge,4,5 i.e., heparin cofactor A, at the present time, to describe the “second antithrombin-heparin cofactor” we have obtained by heparin-agarose affinity fractionation of plasma.
nium sulfate precipitation essentially as described previously.\textsuperscript{29} The preparation was judged to be homogeneous by sodium dodecyl sulfate polyacrylamide gel electrophoresis.\textsuperscript{30} Antithrombin III concentrations were determined spectrophotometrically using an extinction coefficient value of 0.61 ml - mg\textsuperscript{-1} - cm\textsuperscript{-1} at 280 nm.\textsuperscript{31} Using a molecular weight of 65,000,\textsuperscript{29} 1 mole of antithrombin III was required to neutralize 1 mole of thrombin in the presence or absence of heparin. This preparation of antithrombin III was used as a standard in the heparin cofactor assays described below. Human factor X was purified to homogeneity, as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis, using a procedure incorporating aspects of published procedures.\textsuperscript{32} The concentration of factor X was determined spectrophotometrically using an extinction coefficient value of 1.16 ml - mg\textsuperscript{-1} - cm\textsuperscript{-1} at 280 nm.\textsuperscript{33} Factor Xa was prepared by incubation of factor X with partially purified Russell's viper venom (RVV, Sigma),\textsuperscript{34} which was covalently bound to agarose (Sepharose 4B, Pharmacia). Complete conversion of factor X to factor Xa was performed in the presence of 10 mM calcium. RVV-agarose was removed by centrifugation.

**Routine Assays for Antithrombin-Heparin Cofactor and Antithrombin III Antigen**

Patient plasma was screened for antithrombin-heparin cofactor and antithrombin III antigen by the Clinical Coagulation Laboratory, North Carolina Memorial Hospital. Plasma levels of antithrombin-heparin cofactor were determined using an antithrombin III assay kit (Abbott quantichrome, Abbott Laboratories) as described by the manufacturer. Plasma levels of antithrombin III antigen were determined by rocket immunoelectrophoresis essentially as described previously.\textsuperscript{29} Briefly, 30-\textmu l samples were electrophoresed in 0.9% agarose gel containing 1% goat anti-human antithrombin III antibody (Atlantic Antibodies). After staining with Coomassie blue R-250, rocket heights were determined. Patient plasma levels of antithrombin III antigen were determined from the rocket height using a standard curve. The standard curve was prepared by plotting rocket heights on log-log paper against the concentration of pooled normal plasma (20 donor): undiluted, 1:2, 1:4, and 1:8. The range of plasma antithrombin III antigen levels in an independent population of 30 normal individuals was 88%-130%. The range of antithrombin-heparin cofactor, determined as described above, was also 88%-130%.

**Plasma Fractionation**

Plasma was obtained from normal healthy volunteers and from one member of the C kindred (V-4) who had no symptoms of thrombosis and was not taking Coumadin. Plasma was obtained by double plasmapheresis using acid-citrate-dextrose as anticoagulant. PEG was added to plasma (normal and patient) to a final concentration of 3.3% (w/v). After stirring for 15 min at 4\textdegree C, the precipitate that formed was removed by centrifugation at 10,000 g for 15 min. The supernatant was decanted and one-tenth volume of 1.0 M BaCl\textsubscript{2} added. After stirring for 30 min at 4\textdegree C, the precipitate was removed by centrifugation as above. The supernatant was decanted and the concentration of heparin cofactor determined by heparin-dependent antithrombin assay. Five milliliters of the supernatant were applied to a heparin-agarose column (2.5 \times 30 cm, ~40 mg heparin/ml packed gel) equilibrated with 0.05 M Tris - HCl (pH 7.4), 0.4% citrate (starting buffer). The column was washed with starting buffer until the absorbance (A\textsubscript{280}) of the effluent was 0.050. The column was then washed with the starting buffer containing 0.4 M NaCl. After the absorbance of the effluent again reached 0.050, the NaCl concentration in the buffer was increased to 2.0 M and the column washed until all 280 nm absorbing material had been eluted. Fractions were assayed for antithrombin-heparin cofactor as described below. Samples were diluted for assay such that the final NaCl concentration was <0.2 M. The recovery of antithrombin-heparin cofactor was essentially 100% (99.9% \pm 2.3%, 3 runs). Fractions containing heparin cofactor A (0.4 M NaCl wash) were pooled and concentrated to a final concentration, determined by thrombin inhibition, of 10\textsuperscript{-4} M (A\textsubscript{280} \approx 8.0). Fractions containing antithrombin III (2.0 M wash) were also pooled and concentrated to a final concentration of 10\textsuperscript{-4} M (A\textsubscript{280} \approx 0.050). The pool of heparin cofactor A and antithrombin III obtained from both normal and patient plasma was used for functional characterization (described below) without further purification. The amount of antithrombin III antigen in the pools was determined as described above.

**Heparin Cofactor Concentration Determination**

The concentration of heparin cofactor in plasma was determined by heparin-dependent antithrombin or heparin-dependent antifactor Xa assay. Plasma was added to a solution containing thrombin or factor Xa, 0.05 M Tris - HCl (pH 7.4), 0.15 M NaCl, 0.1% PEG, and 5 \mu g/ml heparin (0.825 USP U/ml; ~3.5 \times 10\textsuperscript{-3} M). The enzyme concentration was 4.0 \times 10\textsuperscript{-4} M in the final total volume of 1.0 ml. Under these conditions, approximately 2 al of plasma completely neutralized thrombin and factor Xa in less than 10 min. In the absence of heparin there was no measurable enzyme inhibition in 10 min. The heparin cofactor concentration in a given sample (plasma or column fraction) was determined by adding increasing amounts of sample to the enzyme-heparin solution and incubating at room temperature for 10 min. The amount of residual enzyme was determined by removing samples (0.1 ml) and adding to a solution 0.8 ml containing 1.2 \times 10\textsuperscript{-4} M TosGlyProArgNa\textsubscript{N}, 0.1 M TEA (pH 8.0), 0.1 M NaCl, and 0.1% PEG. TosGlyProArgNa\textsubscript{N} hydrolysis was terminated by the addition of acetic acid (0.1 ml). The amount of substrate hydrolyzed was determined spectrophotometrically at 400 nm (E\textsubscript{400} = 1.16 \times 10\textsuperscript{-4} M\textsuperscript{-1} - cm\textsuperscript{-1}, p-nitroanilide) using a Beckman Acta CIII spectrophotometer. The amount of substrate hydrolyzed was proportional to the concentration of enzyme as long as the A\textsubscript{400} was less than 0.200 for thrombin and 0.150 for factor Xa. The percentage of residual enzyme was plotted as a function of sample volume added to the enzyme solution. The x-intercept corresponds to the equivalence point (V\textsubscript{eq}), i.e., volume of sample containing an amount of heparin cofactor equivalent to the amount of enzyme in the assay solution. The concentration of heparin cofactor was calculated using the following equation,

\[
\frac{V}{V_{eq}} \cdot [E] = [\text{heparin cofactor}]
\]

where V is the assay solution volume and [E] is the enzyme concentration.

**Characterization of Antithrombin III and Heparin Cofactor A**

The functional properties of antithrombin III and heparin cofactor A, obtained from normal and patient plasma as described above, were determined by measuring the rate of thrombin or factor Xa inhibition in the presence and absence of heparin. In the absence of heparin, antithrombin III or heparin cofactor A was added to a solution containing 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG, and 5.0 \times 10\textsuperscript{-4} M enzyme (thrombin or factor Xa). The final concentration of antithrombin III or heparin cofactor A was 10\textsuperscript{-7} M. Samples were removed at timed intervals and the amount of residual enzyme determined by synthetic substrate assay as described above. Under these conditions the reaction follows pseudo-first-order kinetics. The apparent first-order rate constant, k\textsuperscript{app} was determined from
the following equation,
\[ \ln \frac{[E]}{[E_0]} = -k_{app} \cdot t \quad (2) \]
where \([E_0]\) is the initial enzyme concentration and \([E]\) is the concentration of enzyme at time \(t\). The specific activity of the inhibitor, \(I\) (antithrombin III or heparin cofactor A), can be expressed in terms of the apparent second-order rate constant value, \(k_{app}'\), which is described by the following.
\[ k_{app}' = k_{app}/[I] \quad (3) \]

In the presence of heparin, the rate of enzyme inhibition is greatly accelerated, which required that the procedure described above be modified. Specifically, a solution containing Polybrene (0.25 mg/ml final concentration) and TosGlyProArgNaN \((1.2 \times 10^{-4} M)\) was added at a fixed time after the addition of inhibitor to the enzyme solution containing heparin. The amount of Polybrene added was sufficient to neutralize the heparin present in all experiments. Substrate hydrolysis was terminated by the addition of acetic acid and the amount of residual enzyme determined as described above. The \(k_{app}'\) value was calculated using equation 2, with \(t\) being the time of addition of Polybrene/substrate to the assay solution. The \(k_{app}''\) value was calculated using equation 3. The rate of thrombin inhibition by antithrombin III was also determined in the presence of synthetic substrate as described previously.23

CASE REPORT

The propositus is a 48-yr-old American physician now residing in Vellore, India. He was well until the age of 24, when, as a medical student, he developed the sudden onset of pleuritic chest pain and hemoptysis after a long drive in an automobile. A deep venous thrombosis and pulmonary embolism were diagnosed clinically based on physical findings in the leg and an abnormal chest film. He was given anticoagulant for 3 mo. A year later, he again developed chest pain and hemoptysis and was diagnosed as having a pulmonary embolism. A ligation of the inferior vena cava was performed and he was treated with Coumadin. Over the next several years, he remained on Coumadin and did well except for symptoms of lower extremity edema and venous stasis ulcers. A venogram during this time showed marked alterations of the deep venous system, modest collateral flow, and an intact ligation of the inferior vena cava. At the age of 33, it was recommended that he stop Coumadin. Six years later, at the age of 39, he developed a thrombosis in the right arm following venipuncture and 2 wk later he again developed chest pain and hemoptysis. Recurrent pulmonary embolism was again diagnosed and Coumadin was restarted. He was first seen at North Carolina Memorial Hospital as a Visting Scientist in the Clinical Coagulation Laboratory.

Laboratory data at the time of initial evaluation of Coumadin revealed a prothrombin time 28.9 sec (control 12.1), partial thromboplastin time 200 sec (57.3), antithrombin-heparin cofactor 68% (88%-130%), and antithrombin III antigen 40% (88%-130%). Plasma levels of alpha-1-antitrypsin, alpha-2-macroglobulin, and C-esterase inhibitor C were all normal.

Family History

The family was originally from the Minneapolis–St. Paul area of Minnesota. There is no known history of consanguinity. History was available on five generations and members of two generations were studied (Fig. 1). Plasma levels of antithrombin-heparin cofactor and antithrombin III antigen for subjects investigated are presented in Table 1.

Subject I-1 had no history of thrombosis.

Table 1. Antithrombin-Heparin Cofactor and Antithrombin III Antigen Levels in Family C

<table>
<thead>
<tr>
<th>Subject</th>
<th>Antithrombin-Heparin Cofactor %</th>
<th>Antithrombin III Antigen %</th>
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*Antithrombin-heparin cofactor levels were determined by routine assay as described in Materials and Methods.
‡Antithrombin III antigen levels were determined as described in Materials and Methods.
¶On Coumadin at time of testing.
death at age 76 of nonthrombotic causes. An aunt (III-1) is alive with no history of thrombosis. An uncle (III-2) died at age 72 with no history of thrombosis.

Subject IV-3, the oldest brother of the propositus, developed venous thrombosis and pulmonary embolism at age 48. He is presently on Coumadin. Another brother (IV-8) has no history of thrombosis. A sister (IV-5) died at 6 wk of age.

Subject V-9, a nephew of the propositus, developed venous thrombosis at the age of 18 and has postphlebitis syndrome.

Eight subjects (IV-6, IV-7, V-3, V-4, V-5, V-7, V-14, and V-16) have abnormal levels of antithrombin-heparin cofactor and antithrombin III antigen but have had no symptoms of thrombosis.

RESULTS

Plasma obtained from five normal males was assayed for antithrombin-heparin cofactor. The concentration of antithrombin-heparin cofactor varied from $1.85 \times 10^{-6}$ M to $2.15 \times 10^{-6}$ M, with the pool, $2.05 \times 10^{-6}$ M. The antithrombin-heparin cofactor concentration in patient plasma was $1.25 \times 10^{-6}$ M or 61% of normal pool. The antithrombin III antigen level in patient plasma was 47% of normal. PEG precipitation and barium citrate adsorption of normal and patient plasma did not decrease the amount of antithrombin-heparin cofactor. However, because of the slight increase in volume, the concentrations decreased to $1.85 \times 10^{-6}$ M and $1.13 \times 10^{-6}$ M, respectively.

Heparin-Agarose Affinity Fractionation of Normal and Patient Plasma

Normal and patient plasma were fractionated by heparin-agarose affinity chromatography. As shown in Fig. 2, two peaks of antithrombin-heparin cofactor activity were resolved by stepwise elution from the column. The first peak of activity, eluting with buffer containing 0.4 M NaCl, was designated heparin cofactor A. Heparin cofactor A did not cross-react with antibody specific for antithrombin III and did not inhibit factor Xa at an appreciable rate in the presence of heparin. The second peak of heparin cofactor activity, eluting with buffer containing 2.0 M NaCl, was immunologically identical to antithrombin III and inhibited thrombin and factor Xa in the presence and absence of heparin at rates consistent with previously reported values for purified antithrombin III. The pooled heparin cofactor A, rechromatographed as described in Fig. 2, again eluted with buffer containing 0.4 M NaCl. Heparin cofactor activity was not found in the 2.0 M NaCl wash. Likewise, antithrombin III was not eluted with buffer containing 0.4 M NaCl when rechromatographed.

The concentrations of heparin cofactor A and antithrombin III in normal and patient plasma, determined from the results of heparin-agarose chromatography, are summarized in Table 2. The concentration of heparin cofactor A was approximately $4.5 \times 10^{-7}$ M in both normal and patient plasma. The concentration of antithrombin III in patient plasma was approximately 50% of normal, in good agreement with the 47% plasma level of antithrombin III antigen.

Characterization of Antithrombin III

To examine the functional properties of the antithrombin III obtained from patient plasma, the rate of thrombin inhibition by antithrombin III was determined as a function of heparin concentration. The apparent second-order rate constant, $k_{app}^{app}$ for the antithrombin III/thrombin reaction increased as the heparin concentration was increased to approximately 4.0 $\mu$g/ml. The effectiveness of heparin in enhancing the antithrombin III/thrombin reaction decreased, however, as the heparin concentration was increased above...
obtained from normal and patient plasma, respectively. In the absence of heparin, the $k'_{\text{app}}$ values for the antithrombin III/thrombin and antithrombin III/factor Xa reactions were $7.7 \times 10^5 \text{ M}^{-1} \cdot \text{min}^{-1}$ and $4.5 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$, respectively, with antithrombin III obtained from either plasma.

**Characterization of Heparin Cofactor A**

The rates of thrombin and factor Xa inhibition by heparin cofactor A were also determined as a function of heparin concentration. As shown in Fig. 4, the rate of thrombin inhibition by heparin cofactor A increased as the heparin concentration was increased to approximately $10 \mu\text{g/ml}$. The maximum $k'_{\text{app}}$ value was $2.8 \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$, which is tenfold slower than the antithrombin III/thrombin reaction rate in the presence of an optimal heparin concentration. The rate of factor Xa inhibition by heparin cofactor A was less than $5 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$ with heparin concentration up to $40 \mu\text{g/ml}$. In the absence of heparin, the $k'_{\text{app}}$ value for the heparin cofactor A/thrombin reaction was $3.3 \times 10^9 \text{ M}^{-1} \cdot \text{min}^{-1}$ and for the heparin cofactor A/factor Xa reaction $<10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$.

**Heparin-Dependent Antifactor Xa Activity of Plasma**

The observation that heparin cofactor A did not inhibit factor Xa at an appreciable rate in the presence of heparin suggested that the concentration of antithrombin III in plasma could be specifically measured by heparin-dependent antifactor Xa assay. A comparison between thrombin neutralization and factor Xa neutralization by patient plasma is shown in Fig. 5. Since the initial enzyme concentrations were equiva-

![Fig. 3. Antithrombin III/thrombin and antithrombin III/factor Xa reaction rates as a function of heparin concentration. The rates of thrombin (A) and factor Xa (B) inhibition by antithrombin III obtained from normal plasma (•) or patient plasma (○) were determined at varying heparin concentration as described in Materials and Methods.](image-url)
Fig. 4. Heparin cofactor A activity versus heparin concentration. Heparin cofactor A was added to a solution containing 5.0 × 10^{-9} M thrombin (θ) or 5.0 × 10^{-9} M factor Xa (Ⅲ). 0.1 M TEA (pH 8.0), 0.1 M NaCl, 0.1% PEG, and heparin. The heparin cofactor A concentration was 5.0 × 10^{-4} M. The apparent second-order rate constant was determined as described in Materials and Methods.

Fig. 5. Antithrombin-heparin and anti-factor-Xa-heparin cofactor in patient plasma. Patient plasma was added to a 1.0-mI solution containing either thrombin (θ) or factor Xa (Ⅲ), heparin (5.0 μg/ml), 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.1% PEG. The initial concentrations of thrombin and factor X were 4.0 × 10^{-8} M. After a 10-min incubation, samples were removed and the amount of residual enzyme determined as described in Materials and Methods.

Discussion

Since the original study by Egeberg was reported, a number of cases of familial thrombophilia have been shown to be associated with a congenital antithrombin III deficiency.8-23 The deficiency appears to be inherited as an autosomal dominant trait with plasma levels of antithrombin III, typically, around 50% of normal in affected family members. In many of the kindreds examined it would appear that the decrease in antithrombin III antigen is associated with a similar decrease in antithrombin-heparin cofactor activity.

Exceptions have been found, however, where the plasma level of antithrombin III antigen is normal, but the level of antithrombin-heparin cofactor is only 50% of normal.17 In the present report we have described a kindred, with a history of thrombosis, where the plasma level of antithrombin-heparin cofactor in affected family members is greater than the plasma level of antithrombin III antigen. The apparently high level of antithrombin-heparin cofactor could not be accounted for by enhanced levels of other plasma protease inhibitors. Nor was it due to a “superactive” antithrombin III, as the antithrombin III obtained from one member of the family was functionally normal although present in reduced amounts. The results of plasma fractionation suggested, instead, that the apparent excess of antithrombin-heparin cofactor was due to the existence of a second heparin cofactor, which we have termed heparin cofactor A. Since heparin cofactor A is antigenically different from antithrombin III, it contributed functionally, but not immunologically, to the plasma concentration of antithrombin-heparin cofactor.

It is not clear at the present time why other studies involving congenital antithrombin III deficiencies have not shown the same discrepancy between plasma levels of antithrombin III antigen and antithrombin-heparin cofactor. Our results indicate that the plasma concen-
tation of heparin cofactor A is lower (~fourfold) than antithrombin III. In addition, it would appear that the rate of thrombin inhibition by heparin cofactor A is considerably slower (~tenfold), both in the presence and absence of heparin, than the rate of thrombin inhibition by antithrombin III. We have also observed that the optimal heparin concentration for thrombin inhibition by heparin cofactor A is greater than that required by antithrombin III. Taken together, our results suggest that heparin cofactor A might not be detected in an antithrombin-heparin cofactor assay if the assay detects only the initial rate of thrombin inhibition. If the assay measures thrombin neutralization, as in the present study, sufficient time must be given to allow the heparin cofactor A present to completely react with the thrombin. It is apparent that the heparin concentration in the assay is important in determining what length of time is sufficient. Since heparin cofactor A does not have significant antifactor Xa activity, the easiest means of measuring plasma antithrombin III activity is by heparin-dependent antifactor-Xa assay.

The present study has provided evidence that in at least one case of congenital antithrombin III deficiency, there is not a concomitant decrease in heparin cofactor A. Nevertheless, the patient studied in the present report has not had clinical thrombosis. The high level of antithrombin-heparin cofactor, relative to antithrombin III, in members of the kindred who have had thrombosis reflects normal levels of plasma heparin cofactor A and suggests that normal levels of heparin cofactor A are not sufficient to prevent thrombosis in antithrombin III deficiencies. However, it remains to be determined whether deficiencies in heparin cofactor A, in the presence of normal levels of antithrombin III, predispose an individual to thrombosis. At the present time, the physiologic significance of heparin cofactor A in the regulation of hemostasis is not known.

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Heparin cofactor activities in a family with hereditary antithrombin III deficiency: evidence for a second heparin cofactor in human plasma

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