Antithrombin III Deficiency: Decreased Synthesis of a Biochemically Normal Molecule

By Daniel R. Ambruso, Bruce D. Leonard, Roger D. Bies, Linda Jacobson, Wm. E. Hathaway, and E. Basil Reeve

A 29-yr-old white female has suffered from recurrent venous thromboses over the last 12 yr. Plasma antithrombin III (AT-III) levels were 48% of normal by immunoelectrophoresis and 56% by chromogenic assay. Three of four siblings and the father had similar AT-III levels without associated venous thromboses. Heparin-Sepharose chromatography demonstrated normal behavior of the patient's AT-III. Her purified AT-III could not be distinguished from AT-III purified from a normal control either by SDS polyacrylamide gel electrophoresis or by crossed immunoelectrophoresis, and the heparin cofactor activity and the progressive antithrombin activity of both AT-III samples were identical. Turnover studies were made in the patient using her own purified AT-III labeled with 125I (1I). The results did not differ significantly from studies made with autologous I-AT-III in two normal control women. Her fractional breakdown rate of 0.54 total plasma AT-III per day compared with 0.45 and 0.52 in the controls. These studies indicate that the patient synthesizes a normal AT-III molecule at half normal rates.

ANTITHROMBIN III-HEPARIN cofactor (AT-III) is a major inhibitor of procoagulant serine proteases. Diminished activity of this plasma protein can occur as an inherited disorder and often is associated with decreased amounts of apparently normal protein.1, 3 Individuals affected with this hereditary deficiency are predisposed to thromboembolic disease.1, 3 We report a patient with hereditary AT-III deficiency and recurrent venous thrombosis. Kinetic studies of thrombin inactivation by purified AT-III and protein turnover studies with autologous AT-III indicate that this patient has a normally functioning AT-III molecule synthesized at a rate of only 50% of normal.

MATERIALS AND METHODS

Coagulation Studies and Antithrombin III Assays

Plasma was separated by centrifugation at 1400 g for 30 min from blood drawn by the two-syringe technique. Nine volumes of blood were mixed with one volume of citrate anticoagulant (3 vol 0.1 mole/liter trisodium citrate to 2 vol 0.1 mole/liter citric acid). Prothrombin time, partial thromboplastin time, thrombin time, fibrinogen, fibrinogen-fibrin split products, fibrin monomer, and euglobulin lysis time were measured by standard techniques.1 Progressive antithrombin activity was determined in defibrinated plasma* and expressed as the ratio between the subject's value and that of normal control plasma or of a standard pool of plasma tested simultaneously. AT-III-heparin cofactor activity was measured with a chromogenic substrate1 and AT-III antigen level was measured by rocket immunoelectrophoresis1 using a rabbit anti-human AT-III antibody purchased from Behring Diagnostics, Sommerville, N.J., or prepared by us. Results were expressed in terms of percent of the value of a standard plasma pool. Crossed immunoelectrophoresis was performed on plasma as described previously.3

Antithrombin III Preparation

Antithrombin III was purified by a modification of methods described previously.7, 4 Fresh citrate plasma (40 ml) was heat defibrinated, diluted 1/1 with Tris-citrate-NaCl buffer (0.01 mole/liter, 0.01 mole/liter, 0.145 mole/liter, pH 7.4; buffer 1) and applied at 0.5–1.0 ml/min to a 1 x 8 cm heparin-agarose column.5 After washes with the above buffer and a similar buffer (0.3 mole/liter in NaCl from which citrate was omitted), the AT-III eluted was eluted by a linear salt gradient formed from two identical beakers containing 0.01 mole/liter, pH 7.4, Tris buffers with 0.15 and 1.3 mole/liter NaCl, respectively. The fractions containing AT-III as determined by rocket immunoelectrophoresis and heparin cofactor activity were separated, diluted to -0.3 M salt by adding buffer 1 from which the citrate was omitted and concentrated on a short, heparin-agarose column. SDS-PAGE showed homogeneous AT-III preparations (Fig. 1). Specific activity (activity of purified AT-III with A280 of 1.0 expressed as percent of activity of 1 ml of a standard citrated plasma pool) was 1200%–1300%. For turnover studies, the heparin-agarose, sterilized during its preparation and not previously used, was washed with 100 vol of sterile, pyrogen-free isotonic saline before the application of the plasma. The buffers were made in sterile, pyrogen-free water, and these and all glassware were autoclaved.

131I-Antithrombin III Preparation and Turnover Studies

Labeling with radioactive iodine (1I) was performed with iodine monochloride.6 Radioactive iodine unbound to AT-III was removed by washing the radiolabeled AT-III (*)-AT-III) on a small heparin-agarose column with buffer 1.7 SDS-PAGE showed homogeneous I-AT-III preparations that ran identically with unlabeled AT-III (Fig. 1). Specific activity of AT-III preparations was unchanged by the iodination. Immediately before i.v. injection, the I-AT-III preparations were passed through Millipore filters previously treated with a few milliliters of sterile 1 mg/ml human albumin solution (Armour Pharmaceutical) to prevent surface denaturation. About 0.2 mg I-AT-III, pyrogen-free as shown by the Limulus test, containing 3–10 μCi 131I (New England Nuclear) was injected per study. For radioactive counting, plasma samples were handled as described previously1 and whole body counts were made in the...
ANTITHROMBIN III DEFICIENCY

CASE REPORT

S.H. is a 29-yr-old white female with a history of recurrent thromboses. The first episode occurred at 20 yr of age as an ileofemoral thrombosis with pulmonary embolus. She was on birth control pills at the time. Over the subsequent 5 yr she had several episodes of superficial venous thrombosis involving both legs and was treated with bed rest and short-term oral anticoagulant therapy.

We first evaluated her when she was 25 yr old. At that time she was not on anticoagulant medications and had no clinical evidence of thrombosis, and her prothrombin, partial thromboplastin and thrombin times were all within normal limits. Other studies then included: fibrinogen 240 mg/dl (normal range, 200-450 mg/dl), fibrinogen-fibrin split products <10 μg/ml, negative fibrin monomer, normal thromboelastogram, and euglobulin lysis time 135 min (normal range, 90-300 min). Progressive clotting AT-III activity was 0.17 (normal range, 0.66-1.38) and AT-III antigen by immunoelectrophoresis was 54% (normal range, 83%-117%).

During the next 4 yr, S.H. noted progressive swelling and pain in her right leg after periods of standing. At 27 yr of age she developed acute swelling of the right leg and tenderness over the right femoral vein. At this time she was 6-8 wk pregnant. A venogram documented thrombosis involving right femoral, internal, and external iliac veins. Thrombectomy and therapeutic abortion were performed, and she was started on coumadin. Subsequently, she has not developed any severe thrombotic episodes.

Although no other family members have had thromboembolic episodes, the patient’s father and three siblings demonstrated low levels of AT-III (Fig. 2). Plasma levels of cholesterol and triglycerides were normal in the propositus and D.H.Jr.

RESULTS

Coagulation and Immunochemical Studies

Measurements of plasma AT-III levels by progressive thrombin inhibition, interaction with chro-
Table 1. Plasma Antithrombin III Assays* in S.H. Obtained Over 4 yr

<table>
<thead>
<tr>
<th>Assay</th>
<th>Level</th>
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<tbody>
<tr>
<td>Progressive serum activity</td>
<td>0.26 ± 0.09 (6)†</td>
</tr>
<tr>
<td>Chromogenic (84%–118%)†</td>
<td>56% ± 4% (7)†</td>
</tr>
<tr>
<td>Antigen (83%–117%)†</td>
<td>48% ± 9% (7)†</td>
</tr>
</tbody>
</table>

*Assays were performed as described in Materials and Methods.
†Normal range.
Numbers represent mean ± SD of assays performed during the 4-yr observation period. The number of determinations appears in parentheses.

Plasma antithrombin III assays in S.H. obtained over 4 yr are summarized in Table 1. Both AT-III antigen level and heparin cofactor activity were decreased to half normal. Activity measured by the progressive clotting assay† was also markedly decreased.

Figure 3 shows the pattern of AT-III on crossed immunoelectrophoresis of plasma. In normal plasma there are two peaks representing bound and unbound AT-III.† The patient’s pattern was identical to that of normal control plasma except that the areas under the peaks were smaller. This is consistent with decreased protein and not with an abnormal protein. Measurement of the ratio of the areas under the peaks (area peak 1/area peak 2, Fig. 3) as previously described verifies this quantitatively. The ratio for S.H. was 0.26 ± 0.03 (n = 4, mean ± SD) and that of normal controls 0.24 ± 0.03 (n = 17). The course of inactivation of thrombin by purified AT-III from the patient and from a normal control is shown in Fig. 4. The extent of inactivation of thrombin at each time point was essentially the same for the patient and the control.

Turnover Studies

Figure 5 compares the results of turnover studies made in S.H. aged 29 yr, 58.2 kg, with those made in two control healthy women, R.Ho. aged 65, 57.3 kg, and M.S. aged 50, 57.0 kg. For each study, 131I-AT-III was prepared from AT-III freshly separated from the subject’s own plasma. At the time of the study, S.H. had been off all anticoagulant drugs for 10 days; she was healthy and showed no clinical or laboratory evidence of venous thrombosis. The plasma and whole body radioactivity measurements in the three women show no great differences, and Table 2 confirms this by summarizing values of several parameters derived from these measurements. Table 2 also shows that the plasma AT-III antigen levels in S.H. are about half those in R.Ho. and M.S.

DISCUSSION

Antithrombin III, an important inhibitor of coagulation proteases, plays a central role in hemostasis as

![Fig. 3. Crossed immunoelectrophoresis of normal AT-III (A) and S.H.'s AT-III (B).](image)

![Fig. 4. Progressive antithrombin activity of AT-III purified from S.H. (broken line) and a normal subject (solid line) against human thrombin (see text for experimental details).](image)
evidenced by the clinical association of thromboembolic disease with decreased levels of this plasma protein. Several varieties of inherited abnormality of AT-III have been described. Our patient demonstrates the commonest form, which is inherited as an autosomal dominant. In our patient’s plasma, AT-III levels were 40–50% normal and the AT-III exhibited no gross chemical abnormality. While some subjects with this abnormality, such as S.H., suffer recurrent severe attacks of venous thrombosis, others, such as the patient’s father and siblings, are free from clinical disturbances of coagulation. In this article we provide additional evidence that the AT-III of patients with this deficiency is chemically normal and define the abnormality responsible for the low plasma AT-III level.

Biochemical properties of our patient’s protein were characterized by normal crossed immunoelectrophoresis of S.H.’s plasma AT-III and normal heparin-agarose chromatography during purification of AT-III from S.H.’s plasma. As shown by SDS-PAGE (Fig. 3), the purified AT-III was homogeneous and ran with the same Rf as AT-III purified from a normal control. Specific activity, expressed as the percent heparin cofactor activity of a purified sample divided by the absorbance at 280 nm of 1 cm depth of the solution, was 1200% and ran at the high end of our normal AT-III range. Progressive antithrombin activity (Fig. 4) was indistinguishable from normal. Thus, any major chemical change in the AT-III molecule is unlikely.

In a steady state, synthetic rate, $S$, equals breakdown rate, $B$. Defining $V$ as plasma volume, $C$ as plasma AT-III concentration, and $k_B$ as the fraction of plasma AT-III broken down per day in a steady state:

$$S (mg/day) = B (mg/day) - k_B V \cdot C$$  

For instance, with normal plasma AT-III level of ~0.13 mg/ml, S.H.’s measured plasma volume of 2400 ml and the average normal $k_B$ based on our human studies of about 0.5/day, $S = B = 156$ mg/day. Equation 1 can be rearranged to show the relation...
must not significantly affect the \( ^{1}I\text{-AT-III} \) turnover. Our previous studies in dogs with screened \( ^{1}I\text{-AT-III} \) indicate that our methods of purifying and \( ^{1}I \)-labeling AT-III do not alter the biologic behavior. Further, the studies of the \( ^{1}I \)-AT-III injections, as would result from rapid breakdown of altered \( ^{1}I\text{-AT-III} \).

Table 2 presents the parameters calculated from the 3 turnover studies of Fig. 5 and shows that S.H.'s \( k_B \) values were essentially normal as compared with the two controls, studies of Collen and collaborators,\(^9\) and other studies of ours.\(^9\) Scully et al.\(^9\) also found about a 50% value for \( k_B \) using a KABI preparation of AT-III labeled with \( ^{1}I \). Synthetic rate, \( S \), can now be calculated from equation 1 by inserting the normal value of \( k_B \) and the half normal value for AT-III concentration, \( C \). \( S \) turns out to be 50% of the normal rate. Figure 6 reinforces this conclusion. In this figure the turnover studies on S.H. of Fig. 5 are compared with the calculated plasma and whole body radioactivity curves that would be seen if fractional breakdown rate, \( k_B \), was doubled but distribution of AT-III between plasma and interstitial fluids was unchanged. Equation 2 shows that this would also result in a 50% normal level of plasma AT-III. The parameters used in the calculation are shown in Table 2. The calculated plasma and whole body radioactivity curves, labeled T, fall much more steeply than those determined in the turnover studies.

In summary, we have described a patient with AT-III deficiency, severe thromboembolic complications, and an AT-III molecule that is biochemically normal but is synthesized at half the normal rate. This results in half the normal plasma AT-III level. Evaluation and classification of other patients by these methods will aid in understanding the pathophysiology of their diseases and their clinical management.

REFERENCES


Fig. 6. Comparison of turnover studies in S.H. from Fig. 5 with theoretical studies, S.H.. For S.H., the synthetic rate was assumed to be normal, but the fractional rate was assumed to be twice normal.

\[ C = \frac{S}{k_B V} \]  

This equation shows that if changes in \( V \) are excluded, concentration (\( C \)) of plasma AT-III could be halved by halving \( S \), doubling \( k_B \), or changes in both \( S \) and \( k_B \). Turnover studies with \( ^{1}I\text{-AT-III} \) that reflect the behavior of S.H.'s native AT-III allow measurement of \( k_B \). However, the \( ^{1}I\text{-AT-III} \) must be prepared from S.H.'s plasma, and the purification and iodination
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