RAPID COMMUNICATION

A Frameshift Mutation Leading to Type 1 Antithrombin Deficiency and Thrombosis

By R.J. Olds, D.A. Lane, G. Finazzi, T. Barbui, and S.-L. Thein

Type 1 antithrombin III (ATIII) deficiency, which is the commonest form of inherited ATIII defect, is characterized by a quantitative reduction in both immunologically and functionally detectable protein. This condition is associated with a high incidence of thromboembolic disorder. Previous investigations have shown that the ATIII genes in the majority of cases are grossly intact, but the precise underlying molecular defects remain unknown. We have investigated the molecular basis of a type 1 ATIII deficiency in an Italian kindred by enzymatic amplification of the ATIII gene sequences in affected family members and direct sequencing of the amplified genomic DNA. A novel mutation, the deletion of a single T in the second position of codon 119, was identified in each of the affected individuals. The resulting frameshift leads to a premature termination in codon 126, effectively resulting in a null allele.© 1990 by The American Society of Hematology.

ANTITHROMBIN III (ATIII) is the most important physiologic inhibitor of thrombin,1 and has an action against a number of other serine proteases of the coagulation system. It is a protein of 432 amino acids and a member of the large serine protease inhibitor (serpin) family, whose members include α1-antitrypsin, heparin cofactor II, α2-antiplasmin, and the plasminogen activator inhibitors.2 The ATIII gene, which has been localized to 1q23-25,3 spans approximately 19 kb and consists of 7 exons that code for a signal peptide of 32 amino acids as well as the mature protein.4

The prevalence of inherited deficiency of ATIII has been estimated at 1:2,000 to 1:5,000.5,6 Deficiency is inherited in an autosomal dominant fashion and is associated with a risk of thromboembolic disease, which is related to the type of ATIII defect.5 The majority of inherited deficiencies are characterized by a decrease in both immunologic and functional activity to approximately half the normal level. These cases have been classified as type 17 and represent individuals who produce a diminished quantity of normal ATIII, probably because of a null allele. A small proportion have a low concentration of a variant ATIII detectable in the plasma (type 1b), suggesting inefficient translation or increased turnover of the variant protein. Other ATIII-deficient individuals have normal levels of immunologically determined ATIII but reduced functional ATIII activity. A variant ATIII protein can usually be isolated from the plasma, representing about 50% of the total ATIII (type 2). Several type 2 and 1b variants have now been analyzed at the molecular and genetic levels, and in each case the variant has been produced by a single base substitution leading to an amino acid change that interferes with the binding of ATIII to heparin or its interaction with thrombin.8

Although type 1 deficiencies are much more common, the molecular basis underlying these defects remains largely uncharacterized. Previous studies have shown that both copies of the ATIII gene in the majority of these patients are grossly intact,9 which suggests that the molecular defects are likely to be due to point mutations, that is single base substitutions or minor insertions or deletions, as noted in several other single gene disorders, such as the thalassemias11 and hemophilia A.12 We have investigated the genetic basis of a type 1 ATIII deficiency in an Italian kindred with three affected members by directly sequencing amplified DNA from the exons and the flanking intron regions of the ATIII gene. The mutation involved the deletion of a T from the second position of codon 119, in each of the affected family members. This novel mutation results in a frameshift leading to a premature termination of protein translation within seven codons. The mutation was not identified in affected individuals from 28 other kindreds with type 1 deficiency.

MATERIALS AND METHODS

Family history. The propositus was an Italian male who presented at the age of 19 after mild trauma with bilateral iliofemoral thromboses and pulmonary embolism. Initial management consisted of heparin infusion supplemented by ATIII concentrates to produce a therapeutic prolongation of the activated partial thromboplastin time. After 3 days warfarin was instituted and the heparin and ATIII concentrates gradually withdrawn. The patient has been maintained on warfarin for 2 years with no thrombotic recurrence. His sister developed thrombosis of the calf veins in the 10th week of her first pregnancy, and was anticoagulated with heparin intravenously, followed by maintenance with subcutaneous heparin. The pregnancy proceeded to term, with delivery of a healthy infant. Prophylaxis was not continued and she remains well 10 months later. No history of thrombotic disease was found in either parent.

ATIII assays. Plasma was obtained from the propositus, his sister, and both parents. Functional (heparin cofactor) ATIII assays, using a two-stage amidolytic method, and immunologic ATIII assays, using the Laurell technique, were performed as previously described.13 The presence of a variant ATIII protein in plasma was investigated by crossed immunoelectrophoresis14 in the presence of heparin.

Amplification and direct sequencing of genomic DNA. High molecular weight DNA was extracted from peripheral blood leuko-

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cytes by standard methods. The polymerase chain reaction (PCR) was used to amplify specific regions of the ATIII gene for analysis of known DNA polymorphisms, to produce templates for DNA sequencing, and for hybridization with allele-specific oligonucleotide probes. Sequences of the oligonucleotide primers are shown in Table 1. DNA amplification was achieved by addition of 500 ng of genomic DNA to the following: (1) pairs of oligonucleotide primers (20 pmol each); (2) 10 µL dNTP solution (containing 2 mmol/L of each dATP, dTTP, dCTP, dGTP); (3) 10 µL 10X PCR buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl pH 8.3, 25 mmol/L MgCl2); (4) 2 U AmpliTaq DNA polymerase (ILS Ltd, London, England); (5) water to 100 µL. Thermal cycling conditions were denaturation at 94°C for 1 minute, annealing at 56°C (50°C for primers P7 and P8) for 1 minute, and extension at 72°C for 2 minutes, for 30 cycles. The first cycle was modified to provide a denaturation phase of 1.5 minutes while in the last cycle the extension temperature was maintained for 10 minutes. PCR product was visualized in 1% agarose gels stained with ethidium bromide and the amplified DNA of interest was isolated using a unidirectional electroclutor (International Biotechnologies Inc, Cambridge, England). Purified PCR product was sequenced directly by the dyeoxy chain termination method (Sequenase, United States Biochemical, Cleveland, OH) using nested oligonucleotide primers complementary to the coding and noncoding strands (Table I). To analyze the site polymorphism for P1 within exon 3A,16 16 µL of PCR product was incubated with excess of the restriction enzyme according to the manufacturer’s instructions, the digested product electrophoresed in 1% agarose and visualized by staining with ethidium bromide.

**Oligoprobe hybridization.** Two 19-bp oligonucleotides were synthesized, one complementary to the identified mutation 5'-AAGAAGTGGTCATGACAG-3', the other to the normal sequence 5'-AAGAAGTGGTCATGACAG-3' and were end-labeled with 32P-dATP (Amersham, England) by a kinase reaction. Amplified DNA of exon 3A sequences from the ATIII genes of each of the affected family members and from 17 other individuals with type 1 ATIII deficiency, was blotted onto a nitrocellulose membrane. The labeled allele-specific oligoprobe were hybridized sequentially to the membrane, with stringent washes being performed for 5 minutes at 50°C for both probes.

**Allele-specific priming of PCR.** As confirmation and extension of the results obtained by allele-specific oligoprobe hybridization, the technique of allele-specific priming of the PCR was used to screen individuals from a total of 28 kindreds with type 1 ATIII deficiency for the presence of the mutation identified by direct sequencing. The basis of the method lies in the observation that oligonucleotides that are not complementary to their templates at the 3' end do not, under stringent conditions, act as primers in the PCR. Two allele-specific primers were synthesized with the single base deletion site one base 5' to the 3' end (normal 5'-ATCTGAGAAAACCTGATCTGAC-3'; mutant 5'-ATCTGAGAAAACCTGAGCTAC-3'). A further base mismatch (T) was incorporated into both the normal and mutant allele-specific oligonucleotides toward the 3'-end to promote destabilization of primer-template binding. A common downstream primer (5'-GGTGGAGAATGCATTGGACCTG-3') was used in the amplification for both alleles. As a test of efficacy of amplification a pair of primers allowing amplification of a fragment from an unrelated gene (delta globin; upstream primer 5'-GACACACTGATGAGAAGCCCAAT-3', downstream primer 5'-GAAGAGCAGGTAAGTAAAAGAACC-3') was included in each reaction as an internal control.

### Table 1. Oligonucleotide Sequences

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P20</td>
<td>5'-GGTTTTTCTTGGTGTGCGCAG</td>
</tr>
<tr>
<td></td>
<td>P21</td>
<td>5'-TTGGAGGCTATTTGATGTGAGTC</td>
</tr>
<tr>
<td>2</td>
<td>P1</td>
<td>5'-GGTGGAGAATGCATTGGACCTT</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>5'-GGTGGAGAATGCATTGGACCTT</td>
</tr>
<tr>
<td></td>
<td>S1</td>
<td>5'-GCAACAGCAAGCCTGGGAC</td>
</tr>
<tr>
<td></td>
<td>S2</td>
<td>5'-TGGAATATCTGAGGGTGGAC</td>
</tr>
<tr>
<td>3A</td>
<td>P3</td>
<td>5'-AGTCAGAGACTGACAGCAGATGTC</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>5'-AGGGTTGTAATCTTGTGTGAGTC</td>
</tr>
<tr>
<td></td>
<td>S3</td>
<td>5'-CATGTGAACTGGCGACCCGAC</td>
</tr>
<tr>
<td></td>
<td>S4</td>
<td>5'-TGCTTCAGAGCACACGGACTT</td>
</tr>
<tr>
<td>4</td>
<td>P7</td>
<td>5'-CTTAAATAATGATGATGCTG</td>
</tr>
<tr>
<td></td>
<td>P8</td>
<td>5'-CTTCCACCTTTGTTGGTACACT</td>
</tr>
</tbody>
</table>

Even-numbered primers are complementary to the noncoding strand; odd-numbered primers are complementary to the coding strand.

### Table 2. ATIII Functional and Immunologic Quantitation

<table>
<thead>
<tr>
<th>Subject</th>
<th>Activity (%)</th>
<th>Antigen (%)</th>
</tr>
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<tbody>
<tr>
<td>Propositus</td>
<td>53</td>
<td>33</td>
</tr>
<tr>
<td>Sister</td>
<td>64</td>
<td>48</td>
</tr>
<tr>
<td>Father</td>
<td>62</td>
<td>56</td>
</tr>
<tr>
<td>Mother</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Normal</td>
<td>85-115</td>
<td>85-125</td>
</tr>
</tbody>
</table>

![Fig 1. Sequencing gel autoradiograph for the exon 3A coding strand of the propositus. Each of the four reactions has been loaded in duplicate, in the order shown, to facilitate reading of the sequence. The PCR simultaneously amplifies the wild-type and the mutant allele so that both alleles are sequenced. If both alleles were normal, only a single band should be present in each ladder. The arrow indicates the position of the deleted T in the mutant allele, resulting in a single base shift in one allele beyond the mutation site; thus the sequences of the two alleles are one step apart.](from www.bloodjournal.org by guest on September 24, 2017. For personal use only.)
DNA of known normal sequence was used as a normal control and DNA from one of the family members with the mutation was used as the mutant control. The PCR was performed as described above, except that the reactions were performed in a total volume of 25 μL (with each component being added in one quarter of the quantity used in a 100 μL reaction). The annealing temperature was 65°C. Products were visualized as described above.

RESULTS

ATIII assays. Results are presented in Table 2. The functional and immunologic assays of ATIII in the propositus, his sister, and father are consistent with type 1 ATIII deficiency. The mother's results are within normal limits. No variant form of ATIII was detected in the plasma from the propositus, using crossed immunoelectrophoresis (data not shown).

ATIII gene polymorphisms. Amplification of a fragment of DNA encoding part of the 5'-untranslated region of the ATIII gene and exon 1 using primers P20 and P21 produces fragments of 708 or 632 bp because of the presence of the previously described length polymorphism. The propositus and his sister were each homozygous for the shorter-length allele, while the father was heterozygous for the short and long alleles. Digestion of the amplified DNA from the exon 4 region (primers P7 and P8) with Pst I allowed analysis of a Pst I site polymorphism located within codon 305. Amplification using primers P7 and P8 produces a fragment of 531 bp but the presence of the Pst I site results in two fragments of 325 and 206 bp after digestion with the enzyme. Each of the family members examined here was heterozygous for the presence of the Pst I polymorphism.

DNA sequence. The nucleotide sequence of exon 2, coding for the last 19 residues of the signal peptide and the first 104 amino acids of the mature protein, was normal. Similarly, the intron sequences flanking exon 2 were normal. In exon 3A, deletion of a single T was identified in the second position of codon 119 in each of the affected individuals, ie, the propositus, his father, and sister (Fig 1). The mutation was confirmed by sequencing both DNA strands, by allele specific oligonucleotide hybridization, and by allele-specific priming of PCR (Fig 2); in each case the results clearly demonstrate that the affected individuals were heterozygous for the deletion in codon 119. None of the DNA samples from the other 28 individuals with type 1 ATIII deficiency showed amplification with the mutant allele-specific primer, and they all amplified with the normal allele-specific primer. Similarly, specific oligonucleotide hybridization did not show that any of the 17 individuals examined had the mutant allele.

Fig 2. Agarose gel demonstrating allele-specific priming of the PCR for the normal allele (top) and for the mutant allele (bottom). Lane 1, marker φx174 Hae III; 2, normal control; 3 through 5, father, son, and daughter, respectively of the affected family; 6 through 11, other individuals with type 1 ATIII deficiency; 12, water blank. The upper band of 742 bp in each sample track results from the amplification of a fragment of the delta globin gene and acts as an internal control of the success of the PCR. Both the normal allele-specific and the mutant allele-specific bands of 334 bp are seen in the affected family members (top and bottom lanes), whereas the other individuals (lanes 6 through 11) show amplification with the normal allele-specific primer only. This demonstrates that the mutation is present in one of the ATIII alleles of the affected family members but absent in the other individuals.
site polymorphism within the ATIII gene showed that both copies of the gene in the affected individuals are present and grossly intact. Such a gross mapping strategy will not detect small rearrangements within an allele, or disturbance of the 3'-end of the gene, but it can at least indicate the presence or absence of both alleles.

We elected to search for the genetic basis of the deficiency by sequencing amplified DNA of the exons and the flanking intron regions that code for the mature ATIII protein. A single base deletion in exon 3A, a T in the second position of codon 119, was identified in one allele of the affected individual. The effect of the deletion is to produce a frameshift that results in a stop codon at codon 126 (Fig 3), compared with the normal ATIII protein of 432 amino acids. The truncated form of the protein was not detectable in the plasma, suggesting that either the protein is not expressed or is rapidly degraded. Having characterized the mutation, we used allele-specific primer amplification to screen 28 other kindreds with type 1 ATIII deficiency for the codon 119 single base deletion, but the mutation was not identified in any of the individuals. Results from this technique, and allele-specific oligoprobe hybridization, confirmed that the mutation was heterozygous in the affected members of the Italian family.

One other mutation within the ATIII gene has been described, in preliminary form, in association with a type 1 ATIII-deficient phenotype. This produced an amino acid substitution within the signal peptide of the ATIII preproprotein, but further details of the effect of this mutation have not been published yet. A previous survey of ATIII gene structure in type 1-deficient patients suggested gross deletion or rearrangement of the gene was an uncommon mechanism of ATIII deficiency. Using the simple expedient of screening the polymorphisms known to be closely linked to the ATIII gene, we confirmed, as an initial step, that whole gene deletion was not the mechanism of deficiency in this family, or in 17 of 28 other kindreds we have examined (Olds RJ, Thein SL: unpublished observations, May 1990). By analogy with other single gene disorders, such as the thalassemias and hemophilia A, likely genetic lesions include point mutations, which result in premature termination of translation or affect transcription of the gene or processing of the RNA. The possibility of trans-acting lesions has also been suggested in ATIII deficiency, although there is no evidence to date to support this. The present demonstration of a mutation affecting a single nucleotide suggests that point mutations may not be an uncommon cause of ATIII type 1 deficiency, and it seems likely that a variety of mutations will be found.

ACKNOWLEDGMENT

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REFERENCES

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A frameshift mutation leading to type 1 antithrombin deficiency and thrombosis

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