Molecular Genetic Survey of 16 Kindreds With Hereditary Antithrombin III Deficiency

By Susan Clark Bock and Edward V. Prochownik

Molecular genetic techniques were utilized to examine antithrombin III (ATIII) gene status in 16 independently ascertained kindreds with hereditary ATIII deficiency. In one of these families antithrombin III deficiency is caused by hemizygosity of the ATIII locus. In the remaining 15 kindreds, two copies of the ATIII gene are present and appear to be grossly normal at the level of whole genome.

Antithrombin III (ATIII) is a plasma glycoprotein that is in large part responsible for maintaining the fluidity of blood. It inhibits thrombin and other activated proteinases of the coagulation cascade by forming stoichiometric 1:1 inhibitor–enzyme complexes with them. The rate of antithrombin III-mediated thrombin inactivation is modulated by the acidic mucopolysaccharide heparin, increasing by over three orders of magnitude in the presence of this cofactor.

Studies of families with inherited antithrombin III deficiency have provided evidence that ATIII is an important physiologic inhibitor of thrombin. Affected individuals in such families have functional anticoagulant levels that are about 50% of normal and tend to suffer recurring episodes of pulmonary embolism and deep vein thrombosis during adult life. More than 50 families with ATIII deficiency have been reported in the literature (see Thaler and Lechner for review). The human ATIII structural gene locus maps to chromosome 1, subregion 1q23-q25, and the mode of inheritance in all cases of ATIII deficiency studied so far is autosomal dominant.

Two categories of ATIII deficiency have been defined on the basis of ATIII antigen levels in the plasma of affected individuals. The majority of ATIII deficiency kindreds belong in the type I ("classical") deficiency group and have a quantitatively abnormal phenotype in which antigen and heparin cofactor activity levels are both reduced to about 50% of normal. Possible molecular genetic explanations for type I deficiencies include complete or partial deletion or rearrangement of one ATIII allele or mutations that interrupt anticoagulant biosynthesis at the RNA or protein level.

The second category of ATIII deficiency affects about 20% of families with hereditary thrombosis and has been termed type II ("functional") deficiency. Affected individuals from these kindreds produce dysfunctional ATIII molecules; they have reduced heparin cofactor activity levels (approximately 50% of normal) but levels of ATIII antigen are often normal or nearly normal. In addition, variant ATIII molecules, but not thrombotic symptoms, are inherited in several other families. Studies of dysfunctional and variant ATIIIs have shown that the abnormalities in ATIII molecules from different kindreds are heterogeneous, and the amino acid substitutions responsible for dysfunction have been identified in four cases.

cDNA clones for human ATIII have been isolated, and the structure of the gene has been determined. In addition, two restriction fragment length polymorphisms in the ATIII locus have been identified. The availability of probes, a

Southern blotting, suggesting that small deletions, insertions or limited nucleotide substitution(s) in the antithrombin III gene, or "trans-acting" defects at other loci involved in the processing, modification, and secretion of biologically active ATIII are responsible for the observed anticoagulant disorders.

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hemizygous at these markers). In these studies Southern blots were hybridized to a mixed probe containing human ATIII and CI inhibitor \(^{26}\) cDNA sequences. ATIII is located in subregion q23-q25 of chromosome 1,\(^4\) while CI inhibitor maps to chromosome 11, q12-q13.1.\(^{25}\) CI inhibitor thus serves as an internal gene dosage and blotting control in this protocol. Control DNA for the quantitative gene dosage experiment was extracted from cultured fibroblasts of the GM2025 cell line (del 1q21-1q25; Human Genetic Mutant Repository, Camden, NJ). Following autoradiography films were scanned on a densitometer.

**RESULTS**

The flow chart shown in Fig 1 depicts the experimental scheme used to study ATIII gene status in families with hereditary ATIII deficiency. Table 1 summarizes the results of our work on 16 such families.

**Absence of gross abnormalities in the ATIII gene.** Southern blot analysis (experiment A on flowchart, Fig 1) can resolve gross alterations in a locus of interest, since the deletion, insertion, or rearrangement of a substantial amount of DNA (>~100 bp) will alter the distance between adjacent restriction sites and generate new hybridizing fragments with unusual mobilities. A battery of restriction enzymes (BamHI, EcoRI, HindIII and PstI) known to cleave collectively at fairly regular intervals throughout the ATIII locus\(^2\) was used to digest normal and patient DNAs. Southern blots were prepared and hybridized to an ATIII cDNA probe. In the blot shown in Fig 2 and others, the patterns of hybridizing fragments obtained from DNAs of affected individuals in all 16 deficiency kindreds were qualitatively identical to those produced by DNAs from normal relatives and controls after variations caused by commonly occurring DNA polymorphisms had been accounted for. These data indicate that ATIII deficiency in these families is not caused by insertion or rearrangement of a substantial amount of DNA in the ATIII locus or partial deletion of the gene but suggest (1) complete deletions, (2) point mutations, or (3) trans-acting, unlinked mutations as possible causes of hereditary thrombosis.

**Antithrombin II gene dosage in ATIII deficient families.** The next stage in our analysis of ATIII deficiency was to determine ATIII gene dosage in the 16 families with hereditary thrombosis (experiment B on flowchart, Fig 1). The issue of whether hemizygosity of the ATIII locus was responsible for reduced antithrombin levels was assessed (1) by using ATIII DNA polymorphisms as linkage markers for specific antithrombin III alleles and (2) through quantitative Southern blot experiments.

Two two-allele DNA polymorphisms\(^{31,32}\) have been identified with ATIII cDNA hybridization probes. The "+" and "-" alleles of the PstI sequence polymorphism generate a hybridizing 5-kb doublet and a 10-kb band, respectively (Fig 3 A), while the "F" and "S" alleles of the length polymorphism produce 1450 and 1550 nucleotide BamHI fragments, respectively (Fig 3 B). Figure 3 C shows blots prepared with PstI- (top) and BamHI-cleaved DNA (bottom) from two affected members of the RUS52/56 kindred. The DNA sample from patient 1 produced a 10-kb PstI band and a 1450 nucleotide BamHI fragment. This pattern is consistent with the presence of one or two -,-F haplotype alleles. However, polymorphism analysis of DNA from another

### Table 1. AT III Levels and AT III Gene Status in 16 Families With Hereditary Antithrombin III Deficiency

<table>
<thead>
<tr>
<th>Kindred</th>
<th>ATIII Functional Activity</th>
<th>ATIII Antigen Level</th>
<th>ATIII Gene Dosage</th>
<th>Type of Gene Defect</th>
<th>Polymorphism Haplotype</th>
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<tr>
<td>RU 51</td>
<td>R</td>
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<td>2*</td>
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<td>-,-F</td>
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<td>R</td>
<td>1*</td>
<td>CD</td>
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Abbreviations: R, reduced; N, normal; ND, not determined; DG, dysfunctional gene; UL, unlinked, transacting mutation; CD, complete deletion.

†Method of gene dosage determination: DNA polymorphism heterozygosity in affected individual.
§Method of gene dosage determination: Family study using DNA polymorphism.

[Fig 1. Flow chart showing experimental procedures used for molecular genetic analysis of hereditary antithrombin III deficiencies and their possible outcomes.](www.bloodjournal.org)
affected member of the family, patient 2, showed that deletion of one ATIII gene has not occurred in this kindred. The presence of two ATIII genes can be directly visualized in individual 2 as heterozygosity at both the sequence and length polymorphisms. Identification of affected individuals who are heterozygous at one or both ATIII DNA polymorphisms was also used to exclude hemizygosity as the cause of antithrombin III deficiency in CH2,4 CH4, CH5, CH6, and RU90.10 Similarly, inheritance of DNA polymorphisms was used to demonstrate hemizygosity of the ATIII locus in another family. The presence of a null allele was deduced by monitoring the segregation of the PstI sequence polymorphism in CH1 and established that the hereditary tendency to thrombosis in this kindred is due to complete deletion of one ATIII allele.11

Quantitative blotting experiments were used to determine ATIII gene dosage in the families in which analysis of ATIII DNA polymorphisms was not productive due to the lack of marker heterozygosity in available affected patients. Southern blots prepared from HindIII-digested DNAs were hybridized to a mixed probe containing ATIII cDNA and C1 inhibitor cDNA sequences. The latter probe, which maps to chromosome 11,12 is unlinked to ATIII (which maps to chromosome 1) and served as a DNA dosage and transfer control in this experiment. Autoradiograms of the blots were scanned on a densitometer and normalized so that the intensities of the 4-kb bands resulting from control probe hybridization equaled 100%. Some traces are shown in Fig 4. When samples from affected individuals in the RU51, RU53, RU54, RU55, RU57, RU58, RU59, RU60, and CH3 pedigrees were analyzed, intensities of the 2.2-kb and 2.5-kb ATIII fragments were equal to those of the corresponding bands in samples from unaffected, normal controls and double those observed in a sample from the ATIII deletion control GM2025 (del 1q21-1q25). Therefore, two ATIII genes are present in patients from these nine families, and hemizygosity of the antithrombin III locus is not the cause of anticoagulant deficiency. Analysis of a sample from CH1, however, reconfirmed that affected patients in this family have only one ATIII gene.31

Fig 2. Southern blot of DNAs from normals and ATIII-deficient patients. The blot was prepared with EcoRI-digested genomic DNAs and hybridized to 32P-labeled ATIII cDNA insert sequences. Each lane shown contains DNA from a different individual. N, normals; a, affected patient from RU51; b, four affected patients from RU52/56; c, two affected patients from RU53; d, two affected patients from RU54; e, affected patient from RU55; f, two affected patients from RU90.

Fig 3. Use of ATIII DNA polymorphisms to determine antithrombin III gene dosage. Southern blots were prepared from PstI- (a and c, top panel) and BamHI- (b and c, bottom panel) digested DNAs and hybridized to 32P-labeled ATIII cDNA sequences. (a) Sequence polymorphism. "-/-" homozygotes produce a 10-kb band (left), while "+/+" homozygotes generate a 5-kb doublet (center), and "-/+" heterozygotes produce a composite pattern (right) in which bands are approximately one half as intense as in homozygotes. (b) Length polymorphism. "S/S" homozygotes produce a 1550-bp band (left), "F/F" homozygotes a 1450-bp band (center), and "S/F" heterozygotes show both fragments at reduced intensity (right). (c) Affected individuals in kindred RU52/56 have two copies of the ATIII gene. Sequence polymorphism data are shown in the top panel and length polymorphism data in the bottom panel. The haplotype of patient 1 could be "-.F/-,-F" or "-.F/null". However, the haplotype of patient 2 is "-.F/+,-S", indicating that deletion of one ATIII allele has not occurred.
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have reduced functional activity and immunologic levels of
normal

GM2025

RU53

RU60

CH3

Fig 4. Quantitative ATIII gene dosage experiment. Southern blots were prepared from the indicated HindIII-digested genomic DNAs and hybridized to a mixed probe containing ATIII and C1 inhibitor cDNA sequences. Following autoradiography the film was scanned on a densitometer. Peaks a and b are 2.2- and 2.5-kb bands resulting from hybridization to the ATIII cDNA probe. Peak c is a 4-kb band resulting from hybridization to the C1 inhibitor control probe, and its height has been normalized to 100% in the tracings shown. Intensities of peaks a and b from patient samples of RU53, RU60, and CH3 correspond to those observed in normals and indicate that two copies of the antithrombin III gene are present. Complete deletion of one ATIII allele in the deletion control sample (GM2025, del 1q21-q25) and in ATIII deficiency kindred CH1 is indicated by reduced intensity of peaks a and b in these scans.

DISCUSSION

Table 1 summarizes the results of our series of 16 ATIII deficiency families. Complete deletion of the ATIII locus has occurred in one case (CH1). Affected members of this family have reduced functional activity and immunologic levels of ATIII, and one individual has experienced deep vein thrombosis. Half normal levels of antithrombin III have also been observed in patients with large, cytologically visible deletions of 1q (del 1q22-q25 and del 1q21-q25).37

Major structural gene alterations (partial deletions, rearrangements, and duplications) have been established as the molecular causes of genetic disease in certain patients with thalassemia,38 Lesch-Nyhan syndrome,39 apolipoprotein AI deficiency,40 and hemophilia.41 However, among the families we studied there were no examples of large gene alterations in the ATIII locus causing anticoagulant deficiency. The hybridization patterns of normal and patient DNAs were determined using four different restriction enzymes that are known to cleave collectively at fairly regular intervals throughout the ATIII gene, and we believe that changes affecting ≥100 bp of DNA would have been detected.

Affected individuals in 15 of the 16 families surveyed here had two ATIII genes. In these cases ATIII deficiency may be caused by a small mutation in the ATIII gene itself or by a defect in a transacting factor involved in the synthesis, maturation, or secretion of the functional anticoagulant molecule. These two alternatives can be distinguished by linkage tests (experiment C on flowchart in Fig 1). In the case of dysfunctional ATIII genes, ATIII deficiency will cosegregate with a given ATIII allele (which can be recognized by its DNA polymorphism haplotype). The disorder and specific ATIII alleles will show unlinked inheritance in the cases in which anticoagulant deficiency is caused by the dysfunction of a transacting factor or the gene encoding it. Since only two two-allele DNA polymorphisms have been identified in the ATIII locus (combined polymorphism information content = 0.4519), extensive family studies are required to confidently distinguish the dysfunctional gene and unlinked mutation alternatives and have been completed in only one case so far (RU 90).

RU 90 is a large Utah kindred in which 13 members in 3 generations have had ATIII deficiency. This family was originally described as a type I deficiency, since ATIII antigen and activity levels are both reduced to approximately 50% of normal.33 However, subsequent immunoblotting experiments revealed the presence of very small amounts of a variant ATIII molecule in the plasma of Utah patients, and a maximum likelihood linkage study established that a dysfunctional gene residing on a chromosome of the +S ATIII DNA polymorphism haplotype encodes this electrophoretically abnormal inhibitor.19 The ATIII-Utah allele has been isolated by molecular cloning, and a C to T mutation has been identified in the second position of the codon for proline-407.24,42 A highly conserved residue in the serine protease inhibitor gene family.43 The resultant leucine for proline substitution may cause rapid turnover of the mutant gene product (due to incorrect folding) and/or loss of the ability to form functional inhibitor-protease complexes with thrombin (since proline-407 is located only 14 residues away from the reactive site arginine44). Molecular genetic techniques have been used to survey ATIII gene status in a series of 16 kindreds with ATIII deficiency. Knowledge of the genetic heterogeneity and mutations underlying ATIII deficiencies will allow improve-
ments in the diagnosis and treatment of affected individuals. Additionally, characterization of functionally abnormal ATIII molecules and the genes encoding them will contribute to our understanding of how this anticoagulant molecule works and how it is activated by heparin.

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