Molecular Basis for Type 1 Antithrombin Deficiency: Identification of Two Novel Point Mutations and Evidence for a De Novo Splice Site Mutation

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Inherited type 1 antithrombin (AT) deficiency is characterized by a reduction in both immunologically and functionally detectable protein. The disorder is associated with a high risk of thromboembolic disease. We have investigated the molecular basis of type 1 AT deficiency in three unrelated families. We have used the polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis, followed by direct sequencing of the seven exons and the intron-exon junctions of the AT gene. Two novel point mutations were identified. A T to C single-base substitution was found in codon 421 in exon 6 (nucleotide position 133801), leading to an AT 421 isoleucine to threonine substitution.

ANTITHROMBIN (AT) is the major physiologic inhibitor of thrombin and other proteases of the coagulation system. The rate of inhibition is markedly increased in the presence of heparin. The single-chain plasma glycoprotein consists of 432 amino acids and belongs to the serine protease inhibitor (serpin) superfamily of proteins.

Hereditary AT deficiency was first described by Egeberg as an autosomal dominant inherited disease. Affected individuals have an increased risk of venous thrombosis and embolic complications. Immunologic and functional assays allow the identification of different types of inherited AT deficiencies. In the first update of the AT mutation data base, a preferred revised classification is proposed. Classical type 1 or quantitative deficiency is characterized by low functional and immunologic levels of normal AT protein. Type 2, or qualitative deficiency, represents a group of defects resulting in the presence of variant AT proteins. Depending on the localization of the mutation, there is an effect on the reactive site or heparin binding site, or a pleiotropic effect. The gene for human AT maps on chromosome 1q23-25 and contains 7 exons distributed over a 14-kb DNA sequence. The complete nucleotide (nt) sequence shows a gene spanning 13477 bp from the transcription start site to the poly(A) addition signal. The AT mutation data base reports 39 distinct molecular defects and nine whole or partial gene deletions in type 1 AT deficiency. We have investigated the genetic basis for type 1 AT deficiency in three unrelated families, using polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis and direct DNA sequencing. We have detected a novel point mutation in exon 6 (AT 421 Ile to Thr) in one kindred and another kindred, one of three Cs at nucleotide (nt) positions 5448 to 5450 in exon 3A (codon 151 or 152) was deleted, resulting in a frameshift mutation and predicting premature termination of protein translation at codon 251. In a third family, a previously reported G to A substitution, at nt position 9788 in intron 4, 14 bp in front of exon 5, was found. We have demonstrated the creation of a de novo exon 5 splice site by ectopic transcript analysis of lymphocyte mRNA. In all cases, the affected individuals were heterozygous for the mutation and no variant AT protein was detected.

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MATERIALS AND METHODS

Patients

Family dM (The Netherlands). The proposita was born in 1936. At the age of 27 years, she developed deep venous thrombosis (DVT) and pulmonary embolism (PE) during the postpartum period of her first pregnancy. Similar episodes occurred after a second and third delivery. A fourth and fifth pregnancy were complicated by thrombosis before the partus. Her daughter suffered from DVT with PE after appendectomy at the age of 25 years. Six other relatives were found to be AT-deficient; four of them had venous thrombotic episodes involving the legs.

Family H (Belgium). The proposita, 10 weeks’ pregnant, presented with sudden abdominal pain at the age of 26 years. At laparotomy, mesenteric venous thrombosis was diagnosed. Resection of a part of the small bowel was necessary, and the pregnancy is currently continued under heparin therapy. AT activity and antigen levels determined on plasma samples, taken during heparin therapy, were 26% and 25%, respectively. At the age of 19 years, she had already developed a DVT with PE, without a search for AT deficiency at that time. Her sister had a DVT after an episode of immobilization, but had no problems during her two pregnancies. AT activity levels were 56%. The mother developed DVT during nearly every pregnancy (four of six). Her AT levels were 56%. Further family studies are in progress.

Family dV (The Netherlands). The propositus is a 51-year-old man with a history of DVT at the age of 19 years. A second DVT developed 25 years later, complicated with PE. His daughter presented with a first thrombotic event at the age of 18 years, after taking oral contraceptives. In the large kindred, we diagnosed six more AT deficiencies. All affected members suffered from recurrent thromboembolic events, such as DVT after pregnancy or immobilization, or cerebral thrombosis after pregnancy. An episode of PE in a 20-year-old woman had resulted in death.

Hemostatic Tests and AT Assays

Investigation of different hemostatic parameters was performed on plasma samples from patients of the three families. Activated partial thromboplastin time and prothrombin time, and fibrinogen...
protein C, protein S, and plasminogen levels were assayed as described previously. Functional AT activity was measured using an amidolytic heparin cofactor assay with Chromogenix, Mölndal, Sweden. Immunoreactive AT levels were evaluated using the Laurell technique with Assera-Plate AT III (Stago, Gennevilliers, France). Crossed immunoelectrophoresis (CIE) of plasma in the absence and presence of heparin (20 U/mL), was performed according to the method reported by Sas et al.

DNA Studies of the AT Gene

General. PCR-SSCP analysis and DNA sequencing of the seven AT exons, as well as Southern blot analysis of the AT gene, have been previously described in full detail. Restriction enzyme digestion of PCR products was performed without prior purification. For each individual enzyme, the optimal salt concentrations, as indicated by the manufacturer, were adjusted by addition of a 10X concentrate, taking into account the concentration of the different components already present in the PCR mixture. All products used in this study were from Amersham, Buckinghamshire, UK, except for the Taq polymerase (Perkin Elmer Cetus Instrument, Norwalk, CT), the restriction enzyme BsaAI (New England Biolabs, Beverly, MA) and the First-Strand cDNA Synthesis Kit (Pharmacia, Brussels, Belgium).

Study of the exon 4 exon 5 boundary by ectopic PCR. Total RNA was isolated from Percoll-purified white blood cells from heparinized blood of patient dV and a normal control. One microgram of RNA was used in reverse transcription reactions (First Strand cDNA Synthesis Kit) in a total volume of 35 μL. Five microliters of both patient and control reverse-transcribed RNA was used then in the first of two consecutive PCR reactions with nested primers. The first oligonucleotide primer pair consisted of AT3.82 (5'-TGG-TCTCTATCTTGGCCCAAG-3'; positions 7553-7572 in exon 4) and PS12B in exon 6. Five microliters of the first PCR was used in the second PCR with nested primers AT3.83 (5'-AGGGGAGAAGGG- AAACATACC-3'; positions 7592-7611 in exon 4) and AT3.81 (5'- AACACACAGGTTGCTACTTG-3'; positions 13407-13390 in exon 6). The composition of the PCR mixtures was the same as previously described for sequencing of the AT exons, except for the DNA template and the thermal cycling conditions: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes for 30 cycles, and a final extension at 72°C for 5 minutes. The fragment lengths of the PCR products using primer combinations AT 3.82/PS12B and AT3.83/AT3.81 are 485 bp and 413 bp, respectively. For DNA sequencing of the exon 4/ exon 5 boundary of patient dV, the PCR-amplified AT3.83/AT3.81 fragment was incubated with 10 U of restriction enzyme BsaAI for 2 hours at 37°C, loaded on a 5% nondenaturing polyacrylamide gel, and electrophoresed for 4 hours at 200 V. The gel was stained with ethidium bromide, and the undigested fragment was cut out and eluted overnight in 100 μL of water. Ten microliters was used to reamplify the fragment with primers AT3.83/AT3.81. This PCR mixture was then purified on a Centricron 100 microconcentrator (Amicon, Beverly, MA) and directly sequenced as described previously with an internal primer AT3.5' (5'-AGCAATCACAAC-A GGCGTAC-3'; positions 13291-13327 in exon 6). For DNA sequencing of the same region of the control, the PCR product obtained after the second PCR was immediately purified on a Centricron 100 microconcentrator and sequenced as described for the patient.

RESULTS

Hemostatic Tests and AT Assays

Immunoreactive and functional AT activity levels, determined in affected individuals, were equally decreased (Table 1). The results in the three unrelated families were consistent with type 1 AT deficiency. AT-CIE, in the absence and the presence of heparin, was performed on plasma samples of AT-deficient subjects in the three kindreds. In family dM (Fig 1A), family H (Fig 1B), and family dV (Fig 1C), the CIE with heparin in the first dimension showed no abnormal migrating component. The characteristic fast-moving anodal peak was present in reduced amounts as compared with the normal control. All other hemostatic parameters were normal (data not shown).

DNA Studies

Southern blot analysis of Psfl and BamHI digests of the AT gene of all individuals described in Table 1 did not show any abnormalities (data not shown). In PCR-SSCP analysis of the seven AT exons, abnormal migrating bands, as compared with the patterns observed in two non-AT-deficient controls, were found for exon 6 in family dM, for exon 3A in family H, and for exon 5 in family dV. For each family, the corresponding exon was sequenced.

In Family dM, a T to C substitution was found at position 13380 in one of the alleles (Fig 2A), predicting an isoleucine (ATC) to threonine (ACC) substitution at position 421 (I421T) of the AT gene product. The presence of this mutation was confirmed by digestion of the PCR product of exon 6 (210 bp) of both affected family members with restriction enzyme MboII. In normal individuals, this enzyme generates four fragments of lengths 114, 59, 24, and 13 bp, whereas the mutation described in this family destroys one site, generating fragments of 173 (114 bp + 59 bp), 24, and 13 bp. The two patients were indeed heterozygous for the 173-bp and the 114-bp and 59-bp fragments, whereas in 115 normal unrelated controls only bands of 114 and 59 bp were found (data not shown). To further exclude that the T to C substitution represents a natural polymorphism, the six other exons of the proposita were sequenced, but no other abnormalities compared with the published sequence were found.

In family H, sequencing of exon 3A showed a deletion of a C in one allele; the exact location of this deletion cannot be determined because it occurs in a stretch of three Cs at positions 5448 to 5450 (Fig 2B), amino acid positions 151 or 152. The deletion results in a frameshift, predicting a
highly truncated protein from amino acid position 153, and a premature stop codon at position 251. This mutation does not create or destroy a restriction enzyme site.

In family dV, a G to A substitution was found in one allele, 14 bp in front of exon 5 (position 9788; Fig 2C). The mutation destroys the unique Mspl restriction site in the 181 bp exon 5 PCR fragment. The propositus and his symptomatic daughter were heterozygous for Mspl fragments of 181 bp, and 135 bp and 46 bp, whereas normal controls only showed 135-bp and 46-bp fragments (data not shown). This mutation has been described previously.\(^{13,14}\) The creation of a new splice site in front of the usual exon 5 splice site has been suggested. To verify this hypothesis, we have developed an ectopic PCR, using RNA from white blood cells, which allows the amplification of part of exon 4, exon 5 and part of exon 6. The new splice site would predict the insertion of 12 bp of intron 4 into the AT cDNA. In the normal cDNA, a unique BsaI site (CCNGG; two Cs and two Gs, interrupted by two random bases) is present in the AT3.83/AT3.81 PCR fragment, spanning the exon 4/exon 5 boundary (CCAGG exon 4/G exon 5). In the alternatively spliced boundary, this site would be destroyed by the insertion of 12 bp of intron 4 DNA (CCAGG exon 4/ttccttcag intron 4/G exon 5). PCR amplification of the fragment, followed by digestion with BsaI, indeed confirmed that the patient was heterozygous for fragments of 413 bp, and 175 bp and 238 bp, instead of being homozygous for the 175-bp and 238-bp fragments, as normally predicted (Fig 3).

Densitometric scanning of the bands from the patient and the normal control showed that almost equal amounts of uncut (48%) and cut (52%) fragments were present in the patient. The uncut fragment was isolated from a polyacrylamide gel, reamplified, and sequenced. The sequencing showed that the 12 bp from intron 4, just in front of exon 5, were present, whereas in the normal control the expected sequence was found (Fig 4).

**DISCUSSION**

Hereditary AT deficiency is a well recognized cause of thrombophilia.\(^{15}\) It is associated with a high risk of recurrent venous thrombosis and PE. Type I or classical type is characterized by low functional and immunologic protein activity without variant AT protein on CIE. This type accounts for the majority of clinical problems in affected individuals. We studied three unrelated kindreds with type I AT deficiency and found a high incidence of thrombotic disease.

In 1991, an AT mutation data base was created, which compiled the molecular defects responsible for AT deficiency.\(^{16}\) During the following years, several new genetic abnormalities have been described. The content of the first update of the data base\(^5\) shows an expansion of the listings of type I deficiencies, and more recent reports can be added.\(^9\) These data indicate that type I deficiency is caused by heterogeneous molecular defects, with few partial or whole gene deletions, and a majority of point substitutions, minor insertions, or minor deletions. Most of these mutations are unique events that are characteristic for one kindred. We studied three different families, and two of the three mutations identified have not been described before now.

Using PCR-SSCP analysis, followed by direct DNA se-
MOLECULAR BASIS FOR TYPE 1 AT DEFICIENCY

Fig 2. Direct genomic sequencing of PCR-amplified exon 6(A), exon 3A (B), and exon 5(C) from the propositae of the families dM, H, and dV, respectively. (A) The presence of both a T (normal) and a C (mutant) in the second position of codon 421 is indicated by an arrow. (B) Arrow indicates the start of the frameshift in this family: deletion of a C in one allele. Below the arrow the sequences of both alleles are identical, whereas above the arrow the normal and the deleted sequences are intermixed. (C) Arrow at the left points at the presence of both a G (normal) and an A (mutant) at a position 14 bp in front of exon 5.

sequencing of the seven exons and intron/exon junction regions, we were able to detect the different abnormalities. SSCP analysis has proven to be a rapid and sensitive screening method for the detection of base changes in given sequences of genomic DNA. This technique is based on the detection of conformational changes of single-stranded DNA in nondenaturing gels, caused by modifications of size or sequence. In our patients, SSCP analysis disclosed aberrant patterns in exon 3A, intron 4, and exon 6.

In family dM, the SSCP gels showed abnormal bands in exon 6 not found in healthy controls. On DNA sequencing, we detected a single-base substitution in codon 421. At nt position 13380, a heterozygous T to C transition was demonstrated, which converted an ATC codon for isoleucine to an ACC codon for threonine. This T to C substitution removes a cutting site for the enzyme MboII. Restriction analysis with this enzyme allowed confirmation of the sequencing data. The substitution has not been described previously, neither as a mutation nor as a polymorphism, and it was not found in the AT gene of 115 normal unrelated controls. DNA sequencing of the other six exons showed no further abnormalities. Referring to the proposed three-dimensional structure of the serpins, isoleucine 421 is located in the AT protein in sheet strand 5B. At the carboxy-terminal end of the molecule, sheet strands 4B and 5B represent highly hydrophobic regions, which are close together in the tertiary structure of
the AT protein. Consensus sequences become apparent by aligning the amino acids of the serpin superfamily members. Highly conserved nonpolar residues are present in both strand 4B (positions 408 to 410) and strand 5B (positions 422, 424, and 426). Frameshift mutations, deletions, and two single–amino acid substitutions (Gly 424 Arg and Ala 427 Asp) have been identified in this part of the gene, and all are associated with type 1 AT deficiencies. Apparently, the substitution of isoleucine at position 421 by threonine belongs to the same group of mutations. Antigenic and functional AT assays were decreased to the same extent and no variant AT protein could be detected by CIE. The three single–amino acid substitutions, so far detected in this region, drastically influence the nature of the SB sheet strand: hydrophobic nonpolar side groups are replaced by polar side groups. Probable, these changes have an important influence on the tertiary structure of the molecule by disturbing the alignment of sheet strand 4B and SB, with consequent impairment of the stability of the mutant AT protein. Lane et al and Watton et al studied different mutations in or around this part of the gene. These investigators demonstrated that point mutations, leading to single–amino acid substitutions in the 402 to 407 region of AT strand 1C, have pleiotropic effects on the resulting variant proteins. They decrease the amount of circulating variant protein in most cases, and they affect both the reactive site and the heparin-binding properties. The P429L substitution in the AT Budapest variant exhibits the same pleiotropic effect. The proline at position 429 lies just out of sheet strand 5B and is involved, together with N428, in the initiation of a one-turn C-terminal helix (h13). The reported defect maps close to 142IT, G424R, and A427D in the primary structure of the AT molecule. The difference in expression between the mutations underlying the pleiotropic defects, and the substitutions in sheet strand 5B, can probably be explained by their position in the native molecule. Moreover, in the AT Budapest variant, the nonpolar proline is substituted by another nonpolar leucine.

A second novel mutation was found in family dV. The proposita presented with a major thrombotic event during her first pregnancy. She developed a mesenterial thrombosis, which necessitated partial resection of the small bowel. The fetus remained in good condition and the pregnancy is currently continued under subcutaneous heparin therapy. The patient had a history of previous DVT and PE, without diagnosis of AT deficiency. Very low levels of functional and immunologic AT activity were found, probably influenced by the continuous heparin therapy. The mother and a sister, both with a history of DVT, had equally deficient levels of functional and immunologic AT protein. CIE assays, in the absence and the presence of heparin, showed a single, small, fast-moving peak, with normal AT protein mobility. No variant protein was demonstrated. Studying the molecular basis for this type 1 AT deficiency, PCR-SSCP gels demonstrated a disturbed pattern for exon 3A. DNA sequencing showed a single-base deletion in this exon. In the normal nucleotide sequence of the AT gene, three consecutive Cs are found at positions 5448 to 5450 (codon 151 TGC and codon 152 CTT). In our case, a deletion of one C was found, generating a frameshift. In protein translation, codon 151 remains unaffected (TCC), whereas codon 152 changes from CTT to TTA, both coding for a leucine. Starting from codon 153, the translation of an altered amino acid sequence of approximately 100 residues is predicted. In the new reading frame, a premature stop codon (TGA) is encountered at amino acid position 251 in exon 4. There was no detectable truncated protein in the plasma. As suggested previously, the absence of the gene product in the plasma may result from different mechanisms: transcription of unstable mRNA, translational or posttranscriptional defects, or intracellular degradation of the translated polypeptide.

Family dV presented with an interesting mutation, recently reported by Vidaud et al and Chowdhury et al, located in the intron 4/exon 5 junction region. In this large kindred, we diagnosed type 1 AT deficiency in several members, all of whom had a history of thromboembolic events at a young age. PCR-SSCP analysis demonstrated an abnormal pattern for exon 5. DNA sequencing of the seven exons and

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**Fig 3.** BsaJl digestion of PCR product, spanning exons 4 through 6. The PCR product, generated after reverse transcription of leukocyte RNA from a control and the propositus of family dV, followed by nested PCR, was cut with restriction enzyme BsaJl and run on a 2% agarose gel (lanes 1 and 3). Uncut fragments are shown in lanes 2 and 4. Lanes 1 and 2, normal control; lanes 3 and 4, propositus dV family.

**Fig 4.** Sequencing of the exon 4/exon 5 boundary in the propositus of the family dV (P) and a normal control (C). The cDNAs were sequenced from the antisense strand (direction from exon 6 to exon 4). In the control, the lower arrow indicates the last base of exon 4, and exon 5 is to the bottom of the figure. In the patient, the upper and lower arrows indicate the 12-bp insertion of intron 4 DNA between exon 4 (top) and exon 5 (bottom).
the flanking regions showed a single-base substitution in intron 4, in one allele. At nt position 9788, 14 nt 5' to exon 5, a G to A substitution was detected. This mutation occurs in a CpG dinucleotide. The localization of an A at this position leads to a new nt sequence, mimicking in a perfect way the 3' splice site consensus sequence. This finding suggests that the mutation might create an abnormal splice site for exon 5. If so, 12 bp would be added to the coding part of the gene, resulting in an altered mRNA. This mRNA would code for four extra amino acids in the polypeptide chain. These ectopic transcripts facilitate the study of the effect of certain mutations on the transcript structure. Berg et al also used ectopic transcript analysis to investigate a putative splicing defect involving the exon 3A donor splice site and demonstrated the splicing out of exon 3A. We isolated total RNA from the propositus' peripheral blood lymphocytes and reverse-transcribed it. PCR was repeatedly performed with nested primers, spanning the studied region. Restriction enzyme analysis of the obtained cDNA showed the heterozygous absence of the normal BsaII restriction site. Subsequently, the fragment was isolated and sequenced, confirming the insertion of 12 bp in exon 5. Our studies indicate that, although apparently no mutant protein is formed, there is expression of the mutant allele at the mRNA level with nearly exclusive utilization of the new splice site. At the protein level, glycinine at position 353 is normally coded in part by exon 4 (G) and exon 5 (GT). If the insertion of 12 bp would happen, this glycinine would be lacking and be replaced by valine-phenylalanine-leucine-proline-glycine. In the tertiary structure, glycinine 353 is close to glycinine 93, located at the end of helix B. Addition of side chains at these positions would disrupt this structure. The substitution of glycinine 353 by valine, together with the insertion of four extra amino acids, probably leads to major alterations of the normal tertiary structure, which results in an unstable protein.

In the present report, we studied three unrelated kindreds with hereditary type 1 AT deficiency, associated with familial occurrence of serious thrombotic complications. We identified two novel point mutations. A single-base substitution (nt position 13380, T to C) in exon 6 resulted in a single-amino acid change at a highly conserved residue, probably inducing an important change in the tertiary structure of the molecule. A single-base deletion in exon 3A (nt position 5448, 5449, or 5450) introduced a frameshift with premature stop at codon 251. The third point mutation, in intron 4 (nt position 9788, G to A) was suggested to create a new splice site for exon 5. Using ectopic transcript analysis, we determined the phenotypic consequences of this genetic defect at the mRNA level by demonstrating the presence of an abnormal mRNA, including 12 extra bp between exon 4 and 5, predicting an unstable protein with four extra amino acids.

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