Three Novel Mutations of Antithrombin Inducing High-Molecular-Mass Compounds

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Abstract We have identified three novel mutations of the antithrombin (AT) gene in patients with thrombotic complications: a Cys 128 → Tyr mutation, a G → A mutation in the intervening sequence 4 (IVS4) 14 nucleotide 5' to exon 5, and a 9 bp deletion in the 3' end of exon 6 resulting in a short aberrant sequence after Arg 425. The latter mutation was associated with an Arg 47 → His mutation in two compound heterozygous brothers. These three mutations led to the expression in the circulation of small amounts of inactive molecules with a high molecular mass in immunoblot analysis. In reducing conditions, these variant molecules had a normal molecular mass, which led us to postulate that these mutations prevent the formation of one intramolecular disulfide bond and allow the formation of intermolecular disulfide bonds. Plasma from a heterozygous patient bearing the Cys 128 → Tyr mutation and from a compound heterozygote bearing the Arg 47 → His mutation and the 9 bp deletion in exon 6 were passed through a heparin-sepharose column. In both cases a population of high-molecular-weight AT molecules with no binding affinity and no AT activity was separated from a population of normal molecules in the first patient, together with a population of molecules with a reduced binding affinity for heparin due to the substitution of Arg 47, in the compound heterozygote. The common feature of these three mutations is that they lead to partial misfolding and to the formation of intermolecular disulfide bonds with other plasma components, inducing the pleiotropic phenotypes observed. (Arterioscler Thromb. 1994;14:1958-1965.)

Key Words • antithrombin • mutation • disulfide bond • pleiotropic effects

Antithrombin (AT) is the main natural inhibitor of thrombin and other coagulation proteases, and its physiological importance was first shown in 1965 by the description of a hereditary AT deficiency in a family with unexplained thrombosis.1 The inhibitory effect of AT, due to the almost irreversible trapping of target proteases, is strongly enhanced by natural glycosaminoglycans such as heparin.

The AT gene, the nucleotide sequence of which has recently been elucidated,2 maps more than 13 480 bp, comprises 7 exons (1, 2, 3a, 3b, 4, 5, and 6), and encodes a polypeptide of 464 amino acids. Several intracellular modifications occur before the secretion of the folded protein, such as the formation of three intramolecular disulfide bonds and the formation of intermolecular disulfide bonds. Plasma from a heterozygous patient bearing the Cys 128 → Tyr mutation and from a compound heterozygote bearing the Arg 47 → His mutation and the 9 bp deletion in exon 6 were passed through a heparin-sepharose column. In both cases a population of high-molecular-weight AT molecules with no binding affinity and no AT activity was separated from a population of normal molecules in the first patient, together with a population of molecules with a reduced binding affinity for heparin due to the substitution of Arg 47, in the compound heterozygote. The common feature of these three mutations is that they lead to partial misfolding and to the formation of intermolecular disulfide bonds with other plasma components, inducing the pleiotropic phenotypes observed. (Arterioscler Thromb. 1994;14:1958-1965.)

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mutations being described as having a pleiotropic effect (PE).\textsuperscript{22}

We report three AT gene mutations responsible for pleiotropic effects: (1) A substitution of Cys 128 by Tyr; (2) a G → A mutation in the intervening sequence 5' to exon 5, probably creating a new splice site; and (3) a 9 bp deletion in the 3' end of exon 6 associated with an Arg 47 → His mutation in two compound heterozygous brothers. These three genomic abnormalities were associated with detectable amounts of cross-reacting material in plasma, with a molecular mass = 100 kD.

**Methods**

**Blood Sampling**

Plasma was obtained from venous blood collected in 0.11 mol/L sodium citrate (1:9), centrifuged for 15 minutes at 2300g, and stored at −30°C in small fractions. For heparin-Sepharose chromatography, larger amounts of plasma were collected with the informed consent of the affected individuals in two of the three families. For DNA studies, leukocytes were isolated from blood collected on EDTA, as described elsewhere.\textsuperscript{23}

**AT Assays**

Heparin cofactor was measured in terms of bovine plasma thrombin inhibitory activity in the presence of heparin (AT-prest, Diagnostica-Stago). AT activity, ie, the total capacity of plasma to neutralize bovine thrombin, was assayed as previously described.\textsuperscript{24} Plasma antigen was quantified by electroimmunodiffusion with an immune serum from Diagnostica-Stago. The standard was pooled normal plasma obtained from 30 donors. Normal ranges were 80% to 120% in the three assays.

To evaluate AT in the elution fractions after heparin-Sepharose chromatography, we used an ELISA assay developed by Dr Paolucci (Sanofi-Recherche), using goat anti-AT immunoglobulins (IgGs) and goat anti-AT peroxidase-labeled IgGs. Briefly, plates (Maxisorb) were coated overnight at 4°C with 20 μg/mL anti-AT IgG in PBS buffer, then washed with PBS containing 0.1% Tween 20 (PBS-Tween). The samples were diluted 1:100 in PBS and incubated in the wells for 2 hours at room temperature. After they were washed with PBS-Tween, the peroxidase-labeled IgGs (1:1000 in PBS-Tween) were allowed to react for 30 minutes. After washing, a mixture of 1 ng/mL OPD (orthophenylenediamine, Sigma) in 0.1 mol/L Na2HPO4, pH 4.8, and 0.055 mol/L H2O2 was allowed to react for 20 minutes in darkness. The reaction was stopped by the addition of 4 N H2SO4, and absorbance was read at 492 nm on a Titertek Multiskan (Flow).

Crossed immunoelectrophoresis was performed in the absence and presence of heparin.\textsuperscript{25} Immunoblot analysis was performed using a mini Protein apparatus (Biorad). Plasma samples were diluted 1/50 before electrophoresis on SDS 10% polyacrylamide gel (SDSPAGE). Protein was transferred to a nitrocellulose membrane. The blots were treated with 5% dry nonfat milk, as recommended by the manufacturer, reacted with a 1:2000 dilution of rabbit AT immune serum (Diagnostica-Stago), extensively washed, and finally reacted with a 1:3000 dilution of alkaline phosphatase-labeled goat anti-rabbit IgG (Biorad).

**AT Purification**

Plasma was adsorbed on dextran sulfate according to McKay,\textsuperscript{26} dialyzed against 0.02 mol/L Tris buffer, pH 7.4, and passed through a column of 1.5 mL heparin sepharose equilibrated with the same buffer. The column was washed with buffer until the absorbance (A) at 280 nm returned to baseline, then eluted by application of a gradient of 0 to 1.5 mol/L NaCl over 40 minutes.

A fast protein liquid chromatography (FPLC) system was used (Pharmacia) with a prepacked 1-mL Mono Q column (Pharmacia). The buffer was 0.05 mol/L Tris, pH 7.5, and the column was eluted by using an automatic gradient of 0 to 0.5 mol/L NaCl.

The specific activity of the purified preparation was taken as the ratio of heparin-cofactor activity to the protein concentration, determined according to Peterson\textsuperscript{27} and expressed in U/mg, one unit being the activity of 1 mL of normal plasma.\textsuperscript{28}

**DNA Studies**

**Polymerase Chain Reaction (PCR) Amplification**

Genomic DNA was isolated from peripheral blood leukocytes of each subject. All 7 exons and flanking intron sequences were amplified using seven sets of primers.\textsuperscript{29} The PCR reactions were performed in a final volume of 100 μL containing 1 μg of genomic DNA, 50 picomoles of each primer, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 7.5, 1.5 mmol/L MgCl2, 200 μmol/L of each dNTP, 2.5 U of Taq polymerase (Perkin-Elmer Cetus Instrument), and 0.01% (wt/vol) gelatin. After 5 minutes of denaturation at 94°C, samples were amplified for 30 cycles on a DNA thermal cycler (Hybaid Ltd) as follows: 1 minute denaturation at 94°C, 1 minute annealing at 55°C, and 2 minute extension at 72°C, the extension of the last cycle being prolonged to 7 minutes. Ten microliters of the final product was electrophoresed in 6.5% polyacrylamide gel and visualized under ultraviolet light after ethidium-bromide staining.

**Sequencing of the Single-Strand PCR Products**

Asymmetric amplification of the 7 exons was performed as described,\textsuperscript{30} using 50 picomoles of one primer and 1 picomole of the other, to obtain preferential amplification of the noncoding strand. This single-strand product was desalted, and excess dNTPs were removed by spin-dialysis on a Centricon 100 microconcentrator (Amicon). One fifth of this material was sequenced by the dideoxynucleotide chain termination method using the Sequenase kit (US Biochemical Corp) according to the manufacturer's protocol.

Subcloning and sequencing of exon 6 were performed in the case of family C, as previously described.\textsuperscript{31} Briefly, 50 μL of amplified genomic DNA was 5' end phosphorolyzed with 1 μL of 10 mmol/L ATP, using T4 polynucleotide kinase. Blunt-end ligation of 5 μL of the phosphorylated sample with 50 ng of bacteriophage M13 mp 18 digested with SmaI was performed for 16 hours at 14°C. Single-stranded templates were prepared from positive recombinant bacteriophages and sequenced as above.

**Dot-Blot Analysis**

After alkaline denaturation, amplified DNA was dot blotted onto nylon filters and hybridized with [32P]-labeled allele-specific oligonucleotide probes corresponding to either the normal or the mutated sequence (Table 1). Probe labeling, hybridization, and washing were carried out as previously described,\textsuperscript{32} except that temperatures were adapted to the mutations analyzed (see legend of Fig 3). Filters were then exposed to x-ray film for 1 hour at −80°C.

**Results**

**Case Report**

In family A, only two subjects could be explored. The propositus, a 42-year-old man, developed deep vein thrombosis (DVT) associated with pulmonary embolism. His son had the normal AT phenotype. The pedigrees of the other two families are shown in Fig 1.
The proposita in family B (II2) suffered her first DVT when she was 22 years old, after giving birth. She then had an iliac DVT at the age of 36. Her mother reported phlebitis after delivery. The other family members were free of thrombotic complications. Laboratory investigations revealed AT levels <60% in three assays used, ie, apparently a type I phenotype in the propositus (II2), her mother (II), and her son (III).

In family C, the propositus (II2) was 18 years old when he had his first DVT (bilateral phlebitis). Three years later, he developed vena cava thrombosis, which was treated by thrombectomy and oral anticoagulants. A new DVT occurred after oral anticoagulation was stopped 2 years later. The patient, now 35 years old, has been on oral anticoagulants since the last thrombotic episode and has a postphlebitic syndrome. The propositus' older brother (III) underwent cardiac valve replacement when he was 20 years old and had two strokes with neurological sequelae. The mother is free of thrombotic complications. Laboratory investigations revealed AT levels <60% in three assays used, ie, apparently a type II phenotype in the propositus. The same genotype was observed in the brother of the propositus, who had a similar phenotype.

In family B, as in family A, no change in the migration of the PCR products was found. Direct sequencing of exon 5 and intron-exon junctions revealed a G → A transition 14 bp upstream from the beginning of the coding sequence, at position 9788, in intron 4. The propositus, her mother, and her son were found to be heterozygous for this mutation. To eliminate the possibility of a polymorphism, we sequenced 60 normal subjects and failed to find the same genotype. This mutation may create a new exon 5 acceptor splice site AG, with a subsequent modification of the coding sequence. It may induce the insertion of four extra amino acids (Val, Phe, Leu, Pro) between proline at position 352 and glycine at position 353 (Fig 2). The propositus being heterozygous for AT III, this mutation was confirmed by dot-blotting (Fig 3) and detected by PCR. Furthermore, direct sequencing of exon 2 revealed the presence of a G → A transition at position 2694, inducing an Arg → His mutation at position 47, confirming the compound heterozygosity of the propositus. The same genotype was observed in the brother of the propositus, who had a similar phenotype (III). In the mother (II), we found only the Arg 47 → His mutation in exon 2. Because the father (I2) was dead, we were unable to confirm that he bore the mutation found in exon 6.
The other high-molecular-mass compounds with molecular masses >100 kD were detected (Fig 4). Such compounds were never detected in numerous samples of control plasma. To further characterize the variant proteins, we subjected dextran sulfate-treated plasma to heparin-Sepharose chromatography after verifying that the preliminary step did not eliminate the high-molecular-mass molecule. The experiment was performed with plasma from the affected subject in family A and from subject III in family C.

The elution profile of the plasma from the family A member bearing the Cys 128 —> Tyr mutation is shown in Fig 5. The ELISA assay detected trace amounts of AT in the flow-through, suggesting the presence of variant molecules with no binding affinity for heparin. This unbound AT migrated as a series of high-molecular-mass molecules on immunoblots (Fig 5, top). In reducing conditions, the molecular mass returned to 58 kD, ie, the normal AT molecular mass in this technique (not shown), suggesting disulfide bonding of variant AT molecules to other proteins. When normal plasma was passed through heparin-Sepharose chromatography, no high-molecular-mass molecules were detected on immunoblot for AT in the flow-through (not shown). The second peak of AT eluted with heparin cofactor activity after 20 mL of the NaCl gradient and migrated as a single component with a normal molecular mass on immunoblots (Fig 5, top). These apparently normal AT molecules to other proteins. When normal plasma was passed through heparin-Sepharose chromatography, no high-molecular-mass molecules were detected on immunoblot for AT in the flow-through (not shown). The second peak of AT eluted with heparin cofactor activity after 20 mL of the NaCl gradient and migrated as a single component with a normal molecular mass on immunoblots (Fig 5, top). These apparently normal AT molecules were further purified on a mono Q column. The purified preparation was homogeneous in SDS-PAGE chromatography and had a specific activity of 5 U/mg, within the range observed for AT purified from normal plasma by the same procedure. 

The elution profile for plasma from the compound heterozygous subject in family C is shown in Fig 6. There was no heparin cofactor activity in the fractions containing AT, which eluted in two peaks, in the flow-through and 15 mL after the NaCl gradient was started, respectively, the latter corresponding to a lower NaCl concentration than that required for the elution of
normal AT. Immunoblot analysis of the fractions showed that AT with no affinity for heparin migrated as a high-molecular-mass molecule, while AT bound to the column had a normal molecular mass (Fig 6, top). The first peak, i.e., AT with no binding affinity for heparin, was probably due to the expression of mutant molecules by the allele bearing the 9 bp deletion in exon 6. The concentration of these variant molecules, thought to have a modified carboxy-terminal sequence, was evaluated in the ELISA assay and accounted for approximately 15% of the circulating AT. The AT molecules with reduced binding affinity for heparin (bearing the Arg 47 → His substitution) were further purified by chromatography on a mono Q column. The purified preparation was homogeneous in SDS-PAGE chromatography and had a specific activity of 1.5 U/mg, similar to that observed in another case of Arg 47 → His substitution and accounting for approximately 15% of the circulating AT.

Discussion

We present three novel AT gene mutations characterized by reduced circulating levels of AT, with a molecular mass ≈100 kD instead of 58 kD.

The first mutation was a substitution of Cys 128 by Tyr, which suppresses the first disulfide bond. Several high-molecular-mass molecules were separated from normal AT by passing the patient’s plasma through a column of heparin sepharose. In reducing conditions, the molecular mass of these ATs became normal, suggesting the availability of Cys 8 to react with other proteins. The absence of heparin-binding affinity of the molecules bearing the Cys 128 → Tyr mutation confirms the important role of the first disulfide bond in the conformation of the heparin binding site. Cys 128 is located on helix D and links this domain (rich in surface basic amino acids) to the amino-terminal domain. Heparin, through a specific pentasaccharide sequence, probably binds to residues in both domains, the approximation of which is ensured by the disulfide bond. This feature is absent in other serpins lacking heparin-binding affinity. However, the main effect of the mutation was a large reduction in the circulating AT level. Whether the misfolding of AT lacking a disulfide bond leads to defective intracellular processing or to increased turnover in the circulation remains to be established by use of transfected mammalian cells or in vivo kinetic studies.

The second mutation was a G → A transition that might create a cryptic splice site at the intron 4/exon 5 boundary. The deleterious effect of the mutation, i.e., modification of the exon 5 splice site, was suggested by cosegregation of the mutation with the deficient phenotype in the three affected members of the family and by the absence of the mutation in 120 normal chromosomes, ruling out a neutral polymorphism. This missense mutation would be responsible for the inclusion of four extra amino acids (Val-Phe-Leu-Pro) between residues 352 and 353 of exon 4, which could disrupt the tertiary structure of AT and prevent the formation of the third disulfide bridge. This bond between residues 247 and 430 links the C-terminal domain of the molecule (strands s1C, s4B, and s5B) to its core. The presence of a high-molecular-mass molecule that dissociated in reducing conditions argues for the expression of low circulating levels of a mutant protein resulting from the abnormal splicing. Unfortunately, the propositus and affected family members refused further explorations, including RNA analysis. A second case of this mutation has been described recently as a type I muta-
The 9 bp deletion induces a substitution of Val 426 by Ala, a deletion of the tripeptide Ala 427-Asn 428-Pro 429, and a shift of the Cys 430 to position 427, which probably impairs the formation of the last disulfide bond. This could account for a combined defect in the amount of circulating AT and its ability to inactivate thrombin and bind heparin. The replacement of Pro 429 by Leu in AT Budapest results in the synthesis of normal levels of incompetent molecules, and the deletion of the same residue in the case we report may have similar consequences on protein function. The domain encompassing amino acids 426 to 429, the sequence of which was modified by the deletion, is highly conserved among serpins. Some variants of \( \alpha \)-1 AT with frameshift mutations modifying the amino acid sequence of the homologous domain are not secreted. The importance of the C-terminal end of \( \alpha \)-1 AT has been shown by the expression of mutated proteins, which were prevented from moving from the endoplasmic reticulum (ER) to the Golgi apparatus when truncated before Pro 391, which is equivalent to Pro 429 in AT. The sequence Ala-Asn-Pro deleted in our AT variant probably plays an important role in the secretion of both serpins, and this further suggests that it is part of a signal sequence required for transport from the ER to the Golgi apparatus.

The aberrant sequence of AT resulting from the 9 bp deletion observed in our patient may also result in significant changes in three-dimensional structure, explaining an effect on both circulating levels and the function of the inhibitor. AT normally undergoes subtle conformation changes that permit activation by heparin and protease inhibition. The modification of the carboxy-terminal end could reduce the flexibility of the protein, precluding recognition of the heparin binding site and formation of tight complexes between AT and proteases. Glycoproteins such as \( \alpha \)-1 AT bind to a receptor on the endoplasmic reticulum membrane and through the endoplasmic reticulum membrane and is part of a signal sequence required for transport from the ER to the Golgi apparatus. This could account for a combined defect in the amount of circulating AT and its ability to inactivate thrombin and bind heparin. The replacement of Pro 429 by Leu in AT Budapest results in the synthesis of normal levels of incompetent molecules, and the deletion of the same residue in the case we report may have similar consequences on protein function. The domain encompassing amino acids 426 to 429, the sequence of which was modified by the deletion, is highly conserved among serpins. Some variants of \( \alpha \)-1 AT with frameshift mutations modifying the amino acid sequence of the homologous domain are not secreted. The importance of the C-terminal end of \( \alpha \)-1 AT has been shown by the expression of mutated proteins, which were prevented from moving from the endoplasmic reticulum (ER) to the Golgi apparatus when truncated before Pro 391, which is equivalent to Pro 429 in AT. The sequence Ala-Asn-Pro deleted in our AT variant probably plays an important role in the secretion of both serpins, and this further suggests that it is part of a signal sequence required for transport from the ER to the Golgi apparatus.

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can be retained in the ER by resident proteins after improper folding. This can apply to misfolded AT molecules, whose dissociation from the receptor could be delayed. Another explanation for the low levels of the abnormal proteins described in this study is increased catabolism of the abnormal high-molecular-mass species.

Reported mutations of the AT gene causing a pleiotropic effect all involve missense mutations of amino acids located in the domain encompassing residues 402 to 407. The mutations reported here generate high-molecular-weight species, probably resulting from misfolding and the formation of intermolecular disulfide bonds with other plasma components. These important structural changes are responsible for the multiple or pleiotropic effects observed.

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