Antithrombin-III–Hamilton: A Gene With a Point Mutation (Guanine to Adenine) in Codon 382 Causing Impaired Serine Protease Reactivity

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Antithrombin-III–Hamilton is a structural mutant of antithrombin III with defective serine protease reactivity, demonstrable in three members of a French Canadian family. The propositus, a 54-year-old man with a history of recurrent thromboembolic events, and his two asymptomatic grown children are heterozygous for the mutant antithrombin III gene. In all three individuals, the immunoreactive antithrombin III level is normal, while the antithrombin antithrombin III gene. In all three individuals, the immunoreactive antithrombin III level is normal, while the antithrombin antithrombin III gene.

Human antithrombin III is a 432 amino acid plasma glycoprotein, with a molecular weight of approximately 64,000 daltons. It is the major physiologic inhibitor of blood coagulation in vivo involved in the inhibition of several activated serine proteases of the coagulation cascade including factors XIIa, XIa, Xa, IXa, and thrombin. The most important function of antithrombin III is the inhibition of thrombin, which is relatively slow under physiologic conditions, but is greatly accelerated in the presence of heparin.

Elucidation of the mechanism of action of antithrombin III requires that the specific amino acids that interact with both thrombin and heparin be clearly defined. The serine protease reactive site of antithrombin III has been identified as the Arg-393–Ser-394 bond near the carboxyl terminal end of the protein. When a serine protease, such as thrombin, interacts with antithrombin III, it hydrolyzes this Arg-Ser bond and then interacts stoichiometrically at this site to form a covalent antithrombin III-thrombin complex. With the formation of this complex, thrombin loses its protease activity. Available evidence indicates that heparin produces a conformational change in the antithrombin III molecule to enable it to interact more readily with the serine protease. Although the heparin reactive site on antithrombin III has not been clearly delineated, Arg-47, Trp-49, and several other lysine residues in this region have been postulated to be involved in heparin binding. Sequence homology between the amino terminal region of histidine-rich glycoprotein and antithrombin III has led to the hypothesis that the heparin-binding domain of antithrombin III is located near the amino terminal end of the molecule, specifically the Lys-39 to Arg-57 region. Other putative heparin-binding sites near the carboxy terminus have also been postulated. Other evidence indicating separate thrombin-binding and heparin-binding domains has been obtained from families with point mutations in the antithrombin III molecule affecting only a single activity.

Many gene probes have become available over the past decade to analyze the genetic basis of inherited disorders and to define their molecular defects. Such DNA probes enable rapid access to any gene; even the mapping of the whole human genome is a realistic possibility. Recently, the human antithrombin III gene has been isolated, cloned, and assigned to the long arm of chromosome 1. DNA polymorphisms have been used to identify parental antithrombin III alleles and to study the basis for quantitative antithrombin III deficiencies.
We have recently identified a family with a hereditary abnormal antithrombin III molecule with impaired thrombin and factor Xa interaction. Using previously described polymorphisms, we have identified the mutant allele. Genomic DNA was isolated from the propositus and the mutant gene cloned. Sequence data from two clones of the mutant antithrombin III gene indicate a point mutation of guanine to adenine in the first base of codon 382. This mutant antithrombin III gene, which we have called antithrombin-III–Hamilton, has threonine substituted for alanine at amino acid residue 382.

METHODS

Sample collection and coagulation assays. Routine blood samples were obtained atraumatically using a two-syringe technique with 9 vol of blood taken into 1 vol of sodium citrate. Coagulation tests including prothrombin time, partial thromboplastin time, and thrombin time were measured using standard methods. Immunoreactive antithrombin III levels were measured by radial immunodiffusion with commercially available rabbit anti-human antibodies to antithrombin III (Atlantic Antibodies; NCS Diagnostics Inc, Mississauga, Ontario). Functional antithrombin III activity was measured with both thrombin and factor Xa. Thrombin, normal antithrombin III, and factor Xa were prepared as previously described. Control plasma was a pool of normal human plasma.

Identification of the allele carrying the mutant antithrombin III gene. Peripheral blood leukocytes were obtained from family members from 20 mL of whole blood collected into disodium EDTA and the DNA extracted. Ten to fifteen micrograms of DNA was completely digested using the restriction endonuclease enzyme PstI (Bethesda Research Laboratories, Gaithersburg, Md) and subjected to electrophoresis in 0.8% agarose gels. DNA fragments were transferred to nitrocellulose by the blotting procedure of Southern and hybridized with the previously described 32P-labeled antithrombin III probes pAT-III-1.2 and pAT-III-2.26-32 Probe pAT-III-1.2 consists of a 1.2 kilobase (kb) insert carrying 304 base pairs (bp) of the 5'-flanking region, the first exon and part of the first intervening sequence of genomic antithrombin III.26 Probe pAT-III-2 consists of a 1.4 kb cDNA insert from codon 10 through the termination codon following amino acid 432.26

Construction of a human genomic library from the propositus. Genomic DNA from the propositus was partially digested with the restriction endonuclease MboI (Bethesda Research Laboratories) to produce a population of DNA fragments from which 15 to 20 kb molecules could be purified by centrifugation through linear 10% to 40% sucrose gradients. The size-selected fragments were ligated to the arms of the phage vector EMBL 3 (Stratagene Cloning Systems, San Diego). The genomic library was screened using the in situ plaque hybridization technique of Benton and Davis. A total of 4 × 10^7 plaques were screened using the 32P-labeled pAT-III-2 cDNA probe. Five phage plaques carrying antithrombin III gene sequences were identified and isolated.

DNA sequencing of mutant antithrombin-III–Hamilton. Restriction endonuclease EcoRI (Bethesda Research Laboratories) was used to create fragments from two of the mutant clone phages. The DNA from these clones was shotgun cloned into the pUC12 plasmid. Four kilobase EcoRI fragments carrying exon 6 sequences of the mutant antithrombin III clones were identified and then sequenced using the M13 sequencing system and the dideoxy chain termination method. Sequencing was done on two different mutant clones.

CASE REPORT

The propositus, J.G., a 54-year-old French Canadian man, presented in 1981 with bleeding esophageal varices and acute deep venous thrombosis of the right leg. During the 12 previous years, the patient had manifested many episodes of hematemesis due to bleeding esophageal varices. The patient's case history also included three documented previous episodes of deep vein thrombosis of the legs at the ages of 17, 37, and 48 years. Angiography in 1980 showed the esophageal varices to be due to portal vein thrombosis. Also demonstrated by this procedure were thromboses of the splenic and superior mesenteric veins. Subsequent to the angiogram, the patient underwent a ligation of the splenic artery. A splenectomy was not done due to difficulty dissecting the spleen free from the surrounding structures. Liver biopsies done, both in 1970 and repeated in 1980, were entirely normal. The family history revealed that the patient had five siblings, two of whom had also experienced major venous thrombotic episodes. These two siblings are now deceased. Measurements of functional antithrombin III in the patient's plasma with both chromogenic and fluorometric techniques, consistently assayed at 50% of normal pooled plasma. These results were obtained with both thrombin and factor Xa as substrate. Serial determinations of immunoreactive antithrombin III levels consistently assayed at values varying from 80% to 110% of normal pooled plasma. Two dimensional immunoelectrophoresis of the patient's plasma and serum, both in the presence and absence of heparin, showed normal antithrombin III electrophoretic mobility. No additional peaks were found when compared with normal samples. This indicates normal binding of antithrombin III with heparin. The propositus' two children showed identical findings. The three available siblings of the propositus and his wife had normal functional and immunoreactive antithrombin III levels. In 1981, the patient underwent an esophageal transection with paraesophagogastric devascularization—the Sugiuira procedure. A liver biopsy done at the time of this procedure again showed normal histology. Beginning in 1981 the patient has been anticoagulated with coumadin, approximately 2.5 mg daily. He has since been well and has had no further evidence of thrombotic episodes.

RESULTS

Thrombin-antithrombin III complex formation. A 2:1 molar ratio of purified thrombin and normal antithrombin III showed thrombin-antithrombin III complex formation.
after two minutes incubation with almost complete use of the normal antithrombin III. The same molar ratio of thrombin to the propositus' antithrombin III, containing approximately equal quantities of antithrombin-III–Hamilton and normal antithrombin III, showed only an approximate 50% use of the antithrombin III. Incubation of a 2:1 molar ratio of thrombin to purified antithrombin-III–Hamilton for up to 30 minutes, with or without heparin, showed no thrombin-antithrombin III complex formation and no use of antithrombin-III–Hamilton following incubation with thrombin was identical to normal antithrombin III.

Identification of the antithrombin-III–Hamilton allele. From the various functional and immunoreactive antithrombin III assays, an autosomal dominant inherited functional abnormality of antithrombin III was shown to be present in the propositus and his two children (Fig 2). Southern blot analysis of PstI digested genomic DNA from leukocytes revealed the presence of the intragenic PstI polymorphism using the pAT-III-2 probe in this family. Both the propositus and his wife are heterozygous with respect to the intragenic PstI polymorphic site. Their children lack this intragenic PstI polymorphic site in both alleles. Because the mutant antithrombin III allele is known to be transmitted from the propositus to both children, the mutant antithrombin III allele could be differentiated from the wild type antithrombin III allele by linkage to the 10.5 kb allele of the intragenic PstI polymorphism. Similarly, the antithrombin-III–Hamilton allele could be linked with the 1.7 kb allele of the PstI length polymorphism using the pAT-III-1.2 probe. Thus, the antithrombin-III–Hamilton allele in the propositus was shown to be linked to the 10.5 kb allele of the PstI intragenic polymorphism and the 1.7 kb allele of the PstI length polymorphism.

Identification of the molecular defect. Genomic DNA from leukocytes of the propositus was partially digested with Mbol. Conditions suitable to produce a maximum number of fragments in the 15 to 20 kb range were determined in pilot studies. These DNA fragments were ligated into the EMBL 3 vector arms and packaged in vitro. A total of 4 x 10^3 recombinant phages were thus obtained. These were screened using the nick translated 32P-radiolabeled pAT-III-2 cDNA probe. Five phage plaques were identified as carrying antithrombin III gene sequences. DNA was isolated from a single pure plaque of each of the five recombinant clones, which were labeled A through E. Clone A carried a 20 kb insert of which 11.5 kb contained sequences from the propositus' normal antithrombin III allele. Clone B carried a 21.5 kb insert of which only 2.5 kb were antithrombin III-specific sequences. The remainder contained 3'-flanking sequences. Clone C contained a 21.0 kb insert carrying the full length normal antithrombin III allele characterized by the presence of 1.2, 1.8, 2.5, 5.0, and 5.5 kb PstI fragments. Clones D and E both contained 18 kb inserts. Both carried mutant antithrombin III sequences characterized by the presence of a 10.5 kb PstI restriction fragment length polymorphism (RFLP). Clone D contained the 10.5 kb fragment and the 3' 2.5 kb fragment. Clone E carried the 10.5 kb fragment as well as the 5' 1.8 and 1.7 kb fragments.

EcoRI fragments from clones D and E phage DNA were then shotgun cloned into the pUC12 plasmid. EcoRI digestion of clone D phage DNA produced restriction fragments, two of which were large. These carried the complete vector arm sequences as well as antithrombin III insert sequences. These fragments were either 1.5, 3.0, or 4.0 kb fragments. The fragment of interest for sequencing was the 4.0 kb...
EcoRI fragment, which carried exon 6 sequences of the mutant antithrombin III gene. Twenty plasmid preparations of clone D were prepared. Of these, five were found to carry the 4.0 kb EcoRI fragments of interest. Similarly, clone E plasmid preparations were found to carry the 4.0 kb EcoRI fragments. Plasmids from both clones D and E carrying the appropriate 4.0 kb fragments were amplified and sequenced using the dideoxy chain termination method. Exon 6 of both clones D and E containing 375 bp of antithrombin III coding sequence was sequenced in the 5' to 3' direction (Fig 3). The sequence data obtained were compared with the known sequence data obtained were compared with the known sequence of the wild type antithrombin III exon 6. In doing so, a single G → A nucleotide change was observed in the mutant sequence of both clones, corresponding to the nucleotide position of the first base of codon 382 in the wild type cDNA sequence. This results in the substitution of threonine for alanine at position 382 of the normal antithrombin III amino acid sequence.

DISCUSSION

Initial characterization of antithrombin III in the family of the propositus revealed that immunoreactive protein was present in normal quantities but that functional activity was reduced to 50% of the control value. That this was a familial defect was indicated by the presence of mutant antithrombin III protein in the propositus' two children. Analysis of genomic DNA from the propositus and his family indicated that both the PstI intragenic polymorphism and the PstI length polymorphism could be used to distinguish the antithrombin-III–Hamilton allele from the normal allele. Such linkage analysis indicated that the antithrombin-III–Hamilton mutation was linked to the antithrombin III allele that lacked the PstI intragenic polymorphism (the 10.5 kb allele) and, at the same time, carried the PstI length polymorphism (the 1.7 kb allele).

Linkage of the antithrombin-III–Hamilton allele to two PstI RFLPs allowed us to characterize this mutant antithrombin III gene. Two genomic clones containing nearly complete copies of the mutant gene were obtained and sequenced. Both clones indicated that the antithrombin-III–Hamilton gene had a G → A point mutation at the first base of codon 382. Codon 382, which normally encodes alanine, codes for threonine in antithrombin-III–Hamilton. The mutant alleles of antithrombin III that have been characterized, including antithrombin-III–Hamilton, are summarized and shown schematically in Fig 4. Mutant antithrombin III proteins with defective heparin binding include antithrombin-III–Basel (Pro-41 → Leu); antithrombin-III–Toyama (Arg-47 → Cys); and antithrombin-III–Rouen (Arg-47 → His). The identical molecular defect to antithrombin-III–Toyama has also been described in a family from France, in whom the mutant gene has been called antithrombin-III–Tours. Yet another family with the same amino acid substitution as antithrombin-III–Toyama has been described recently. This family showed a C → T substitution in the first base of codon 47. This mutant has been called antithrombin-III–Alger. Two families with point mutations causing defective thrombin interaction have recently been characterized. These are antithrombin-III–Utah (Pro-407 → Leu) and antithrombin-III–Denver (Ser-394 → Leu).

The main biologic function of antithrombin III is the irreversible inactivation of various serine proteases, particularly thrombin.7 When thrombin interacts with antithrombin III, it catalyzes the hydrolysis of the Arg-393–Ser-394 bond.
and the primary binding site. This domain of thrombin is located within a hydrophobic pocket. Thus, for antithrombin III to interact with thrombin, the thrombin-binding domain of antithrombin III must approximate the catalytic center of thrombin within that hydrophobic pocket. Mutations at, or near, the thrombin-binding domain of antithrombin III could result in the abnormal conformation of the antithrombin III molecule causing impaired thrombin binding.

Antithrombin-III–Hamilton, in which there is a substitution of leucine for serine-394, is such a mutation. Similarly, antithrombin-III–Hamilton is a mutant antithrombin III molecule that does not react with thrombin (Fig 1). The molecular weight of purified antithrombin-III–Hamilton, subsequent to incubation with thrombin, with and without heparin, remained identical to normal antithrombin III. This observation is consistent with the concept that the Arg-393–Ser-394 bond of antithrombin-III–Hamilton is not cleaved by thrombin.

Antithrombin-III–Hamilton has a mutation at amino acid residue 382. Alanine-382, 12 residues from the reactive center, is a highly conserved amino acid in the family of inhibitors of coagulation and fibrinolysis known as the serpins. All eight members of the serpin family that are active serine protease inhibitors have an alanine, 12 residues from the reactive center toward the N-terminus (Fig 5). This very high degree of conservation of an alanine residue, 12 residues from the reactive center, indicates that this alanine must be highly important to the normal function of antithrombin III and the other serine protease inhibitors. Further evidence that this molecular region of the serpin family is important is provided by the α2-antiplasmin mutation known as α2-antiplasmin–Enschede. In this mutation, there is an insertion of an alanine residue between residues P9 and P12 that causes inactivation of the serpin. Thus, the substitution of threonine for alanine-382 in antithrombin-III–Hamilton is very unlikely to be a silent polymorphism.

The impaired serine protease reactivity that occurs as a result of the mutation of threonine for alanine-382 may be explained in two ways. First, the substitution of threonine for alanine may cause a change in the tertiary structure adjacent to the reaction center, since threonine is larger and more polar than alanine, interfering with formation of the beta-sheet adjacent to the reactive center. This beta-sheet is required for the formation of the stressed S configuration of the molecule. Thus, alteration of the tertiary structure could result in a conformational change unfavorable to thrombin interaction. Second, the critical Arg-393–Ser-394 thrombin hydrolysis site on the antithrombin III molecule is located between two hydrophobic regions. Hydrophobic regions of a protein molecule are known to cluster to produce a loop structure; it is probable that such is the case with antithrombin III and that such clustering adjacent to the reactive center exposes the critical Arg-393–Ser-394 residues. The substitution of threonine for the hydrophobic alanine could therefore result in reduced affinity between these two hydrophobic regions, changing the shape of the loop structure, thus reducing the affinity between thrombin and the active center of antithrombin-III–Hamilton.

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### REFERENCES

11. Kress LF, Catanesi JJ: Identification of the cleavage sites...
15. Koide T, Foster D, Odani S: The heparin-binding site(s) of histidine-rich glycoprotein as suggested by sequence homology with antithrombin III. FEBS Lett 194:242, 1986
44. Benton WD, Davis RW: Screening lambda-gt recombinant clones by hybridization to single plaques in situ. Science 196:180, 1977
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