

Met 358 to Arg Mutation of Alpha₁-Antitrypsin Associated with Protein C Deficiency in a Patient with Mild Bleeding Tendency

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Abstract

The molecular defect responsible for a dramatic prolongation of all standard clotting tests discovered in a 15-yr-old boy has been identified. Initial investigations revealed the presence of an activated Factor X (Factor Xa) and thrombin inhibitor which copurified with alpha₁-antitrypsin (α_1 -AT), thereby suggesting the occurrence of an α_1 -AT variant similar to α_1 -AT Pittsburgh. This was confirmed by dot-blot analysis and direct sequencing after amplification by the polymerase chain reaction. A G to T transition at nucleotide 10038 results in the substitution of Met to an Arg, converting α_1 -AT into an Arg-Ser protease inhibitor (serpin) that inhibited thrombin and Factor Xa more effectively than antithrombin III. Surprisingly, there was no bleeding history in the proband. The common mutation Z, which may explain a reduced expression of the allele bearing the Arg 358 Met mutation, was not observed in the proband's DNA. To exclude the presence of another mutation, the coding regions and intron/exon junctions were sequenced. No other mutation was found. Recently, the patient experienced his first hemorrhagic episode at the age of 17. The level of the abnormal inhibitor had increased twofold 2 mo before. The large decrease in protein C concentration may account for the mild bleeding tendency in this case, despite the presence of the α_1 -AT Pittsburgh mutation. An abnormal protein C pattern was observed in patient's plasma, suggesting that the circulating deficiency might be due to a deleterious effect of the abnormal inhibitor on both intracellular processing and catabolism of protein C. (*J. Clin. Invest.* 1992; 89:1537-1543.) Key words: α_1 -antitrypsin Pittsburgh • protein C • bleeding • DNA • mutation

Introduction

In 1983, a molecular defect responsible for the development of a potent circulating antithrombin activity in a young boy with a severe bleeding disorder was characterized as Met 358 to Arg substitution at the reactive Met-Ser site of alpha₁-antitrypsin (α_1 -AT),¹ thereby modifying the target of this protease inhibitor (1).

This mutant, designated α_1 -AT Pittsburgh, or recombinant Arg 358 α_1 -AT, was shown to inhibit other clotting proteases (2-4), thus displaying strong functional analogy with the physiologic clotting inhibitor antithrombin III (AT III), whose reactive site consists of an Arg-Ser bond. This mutation provided an explanation for the patient's recurrent and ultimately fatal hemorrhagic diathesis (1, 5), and confirmed the critical role played by a single amino acid at the reactive site of serine-protease inhibitors (serpins). Serpins are a superfamily of proteins that have evolved from a primitive ancestral inhibitor and to which α_1 -AT and AT III both belong (6).

Recently, α_1 -AT was described as a physiological inhibitor of activated protein C (7), a natural anticoagulant playing a major role in the regulation of blood coagulation (for review see 8). Moreover, the Met 358 to Arg substitution was shown to multiply by 4,400 the rate of activated protein C inhibition (9). A protease responsible for the intracellular processing of precursor polypeptides is also inhibited by the mutated α_1 -AT (10). Its coexpression with complement C3 and albumin in transfected cells led to the inhibition of propeptide cleavage (11). Such an inhibition confirmed the mechanism proposed to explain the presence of proalbumin in the Pittsburgh patient's plasma (12).

We suspected the presence of a similar abnormality in a 15-yr-old patient whose routine tests before surgery revealed markedly impaired coagulation, and were able to confirm the existence of a Met 358 to Arg mutation by genomic DNA analysis. This patient had, however, never experienced any hemorrhagic complication. The association of the α_1 -AT mutation with a profound decrease in plasma protein C concentration suggests that Arg 358 α_1 -AT interferes with the in vivo metabolism of this anticoagulant regulatory protein.

Methods

Coagulation factor assays. All plasma clotting assays were performed using commercial reagents from Diagnostica Stago (Gennevilliers, France) for Factors XII, XI, IX, VIII, X, and VII, or from bioMérieux (Lyon, France) for Factors II and V.

Protein C was evaluated by amidolytic assays after Protac activation (Diagnostica Stago), and after thrombin activation of an aluminum hydroxide eluate according to Bertina (13), using Coatest protein C (Kabi, Möhndal, Sweden). AT III was measured using an amidolytic assay (AT-prest, Diagnostica Stago). Fibrinogen was evaluated in a chromometric assay, using Fibrinogen kit from bioMérieux.

Immunoassays were performed using immune sera from Diagnostica Stago and rocket immunoelectrophoresis, except for protein C and Factor VII, which were measured by immunoenzymatic techniques (Diagnostica Stago).

Bleeding time was measured using a simplate device (Organon Teknika, Turnhout, Belgium).

Purification of the variant. Plasma from the patient was first adsorbed with bentonite (Sigma Chemical Co., St. Louis, MO), which eliminates numerous proteins, including AT III. The supernatant was

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1. Abbreviations used in this paper: α_1 -AT, α_1 -antitrypsin; α_1 M, normal ASO probe; α_1 N, wild-type ASO probe; α_1 P, mutant probe; α_1 Z, mutant ASO probe; ASO, allele-specific oligonucleotide; AT III, antithrombin III; Factor Xa, activated Factor X; PCR, polymerase chain reaction.

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then passed through a 50-ml column of Q Sepharose (Pharmacia, Uppsala, Sweden) at pH 7.4, using a FPLC system (Pharmacia), and eluted by a sodium chloride gradient. The fractions containing both α_1 -AT and strong thrombin inhibitory activity were applied to the same column and eluted using a pH gradient. The two final steps were performed by chromatography on a Mono Q column (Pharmacia), the first at pH 7.4 and the second at pH 6.

Thrombin and Factor Xa inhibition rates. The inhibitory activity of plasma or purified fractions was evaluated toward purified proteases at 37°C in 0.02 M Tris, pH 7.4, 0.15 M NaCl, PEG 0.5%. Briefly, 0.035 ml of the sample was added to 0.35 ml of purified human thrombin or Factor Xa (Diagnostica Stago). After a 1-min incubation, the residual protease amidolytic activity was determined and compared with a control incubated in the same conditions, but without inhibitor. The chromogenic substrates were *H*-D-phenylalanine-L-pipecolyl-L-arginine-D-nitroanilide (S 2238; Kabi Vitrum, Stockholm, Sweden) and methane sulfonyl-D-leucyl-glycyl-arginine-paranitroanilide (CBS 31.39; Diagnostica Stago). The first- and second-order rate constants for thrombin and Factor Xa inhibition were determined by incubating less than 10 nM of the protease for 1 min with a large molar excess of the inhibitor.

Enzymatic gene amplification. Genomic DNA was isolated from peripheral blood leukocytes, and target sequences from position 9912 to 10190 in the numbering system of Long et al. (14) were amplified according to Saiki et al. (15).

The polymerase chain reaction (PCR) was performed for 30 cycles on an Intelligent Heating Block (model 101; Hybaid, Teddington, UK), with each cycle comprising a 1-min denaturation step at 94°C, a 2-min annealing step at 57°C, and a 2-min extension step at 72°C. The first cycle was preceded by a 5-min denaturation step, and the last cycle was prolonged to 7 min.

The specificity of the PCR fragments containing the sequence encoding for amino acid 332 to the TAA stop codon was checked on 6% polyacrylamide gel using a minisystem (Miniprotean; Biorad Laboratories, Richmond, CA).

Dot-blot analysis. After alkaline denaturation, each amplified DNA was applied to nylon filters (Biotrace RP; Gelman Sciences, Inc., Ann Arbor, MI) and assessed by dot-blot analysis utilizing ³²P-labeled allele-specific oligonucleotide (ASO) probes. After hybridization, filters were washed at a temperature depending on the T_m of each ASO probe and exposed to x-ray film for 1 h at room temperature.

Direct sequencing. The 278-bp amplified DNA fragment was recovered from 6% polyacrylamide minigel, electroeluted in accordance with the standard protocols (16), precipitated with ethanol and again amplified in the presence of a large excess of one of the PCR primers (50 pM α_1 B – 1 pM α_1 A) to obtain preferential amplification and enrichment of the noncoding strand. The single-strand product was desalted and concentrated by spin-dialysis on a Centricon 100 micro-concentrator (Amicon, W.R. Grace and Co, Beverly, CA). 7 μ l of the resulting product was then sequenced by the dideoxynucleotide chain termination method (17), using a Sequenase kit (US Biochemicals, Cleveland, OH) in accordance with the recommendations of the supplier. The same method was used to sequence exons II, III, and IV of the α_1 -AT gene.

Immunoblotting procedure. Electrophoresis of samples containing 0.5 μ l of the patient's or normal plasma were performed on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). The proteins were transferred to a nitrocellulose membrane using a minisystem (Biorad Laboratories). The blot was treated as recommended by the manufacturer, made to react with an antiprotein C or anti- α_1 -AT immune serum diluted 1/1000 (Diagnostica Stago), thoroughly washed, and incubated with a 1/3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit-IgG IgG (Biorad Laboratories). Protein C was activated by incubating the plasma with Protac (Behring, Rueil-Malmaison, France) as recommended by the manufacturer. Aluminium hydroxide adsorption and subsequent elution were performed according to Bertina (13).

Results

Case report. The hemostatic abnormality was discovered during a routine preoperative laboratory investigation of a 15-year-old-boy suffering from kyphoscoliosis. The patient had no personal or family history of spontaneous bleeding. Moreover, a traumatic humeral fracture sustained at the age of 12 was not associated with any bleeding complication. Apart from orthopedic disease, his clinical examination was entirely normal.

Laboratory evaluation included immunoelectrophoresis, complement, tumoral markers, antinuclear factors, immune complexes, blood and urinary amino acids, and bone marrow aspirate, all of which were normal. No hematuria was detected. Chest x-ray, abdominal echography, bone scintigraphy, and abdominal and thoracic CAT scan were also normal.

The results of the coagulation tests (Table I) suggested the presence of a potent coagulation inhibitor, because they showed no correction by mixing with normal plasma. Coagulation Factors XII, XI, IX, and VIII all had activities of less than 10%. The activities of Factors II, V, VII, and X were 40, 65, 42, and 17%, respectively.

Protein S, AT III, and fibrinogen were within the normal range. Bleeding time was 10 min (normal values < 9 min).

Protein C activity, measured in plasma by an amidolytic assay after activation by Protac, was less than 10%. In aluminium hydroxide eluates, protein C amidolytic activity measured after activation by thrombin according to Bertina (13) was 18%.

The concentrations of some of the factors measured by immunoassays were normal or slightly reduced (Factor II: 50%; V: 75%; X: 46%; IX: 72%; VII: 70%). The protein C antigen concentration, measured on several plasma dilutions, was greatly reduced to 13% of normal values.

The parents and siblings of the propositus all had normal protein C and clotting factor concentrations in both the activity and immunologic assays (not shown).

The patient came 19 mo later (June 1991) for a routine clinical examination. Laboratory investigations showed the same coagulation profile with a protein C concentration of 26%. During August 1991, he went to an Eastern European country for vacation. He suddenly suffered from swelling and pain in the right buttock. An intramuscular injection of an unidentified drug was performed. 4 d later, he was hospitalized in our department. His coagulation factors were similar to the levels previously observed, including protein C concentration which was 18% of normal value. A CAT scan confirmed the diagnosis of buttock hematoma measuring 20 × 8 cm. After two plasmapheresis of 3 liters, the hematoma was operated on and drained without any complication.

Table I. Patient's Coagulation Tests

	Patient's plasma	Normal plasma	Normal + patient's plasma (1:1)
Prothrombin time (s)	30	11.5	14
Activated partial thromboplastin time (s)	>120	30	>120
Thrombin time (s)	>100	25	>100
Reptilase time (s)	28	26	

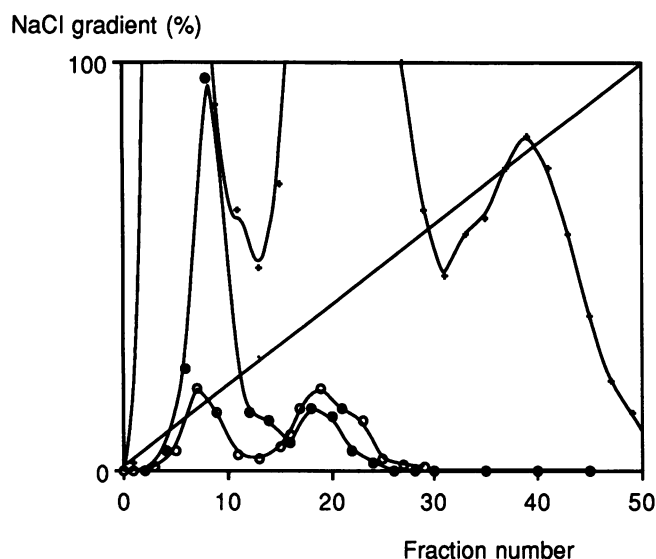


Figure 1. Q Sepharose chromatography of bentonite adsorbed patient's plasma. The column was eluted by a sodium chloride gradient in Tris 0.02 M, pH 7.4, NaCl 0.05 M. (—) A 280 nM. (○ — ○) Immunoreactive α_1 AT. (● — ●) Thrombin inhibitory activity. (—) % of 0.35 M NaCl buffer. Fractions 4 to 11 were further purified.

Characterization of the inhibitor and comparison with the Pittsburgh case. The inhibition of coagulation persisted after adsorption of the plasma with protein G Sepharose, as well as in the presence of protamine sulfate or polybrene. This excluded the possible presence of a pathologic immunoglobulin G or a heparin-like substance. Electrophoresis of the patient's serum on agarose gel at pH 8.6 revealed the presence of an abnormal component migrating as a fast α_1 -globulin, together with the presence of proalbumin. This pattern was identical to the one observed in the original Pittsburgh case (1, 5). Further confirmation that α_1 -AT inhibited clotting protease activity was obtained by its purification. Plasma was first adsorbed by

bentonite to remove AT III whose antithrombin activity could interfere with the other steps of purification. The first anion exchange chromatography column was eluted with a sodium chloride gradient. Two peaks of immunoreactive α_1 -AT were observed (Fig. 1). The first peak presenting high antithrombin activity was further purified, as described in Methods. The resulting product was homogeneous in SDS-PAGE, and reacted with anti- α_1 -AT serum but not with anti-AT III serum.

The purified protein formed high molecular mass complexes with purified human thrombin, of molecular masses 95 and 80 kD in SDS-PAGE. To determine the stoichiometry of the reaction, 15 nM of the purified abnormal inhibitor were incubated with an equimolar amount of thrombin. 80% of the thrombin amidolytic activity were neutralized, indicating that the stoichiometry of the reaction was $\sim 0.8:1$. The deviation to the theoretical stoichiometry (1:1) may be due to experimental errors in determination of the protein concentration. The patient's plasma diluted in order to adjust the abnormal inhibitor concentration to 15 nM also neutralized 80% of the thrombin amidolytic activity (Fig. 2, left). In pseudo first-order conditions (large excess of inhibitor upon protease), the rate of thrombin inhibition was a linear function of the inhibitor concentration (Fig. 2, right). The second-order rate constants for the inhibition of human thrombin and human Factor Xa were of the same order of magnitude as the rate constants observed with the Pittsburgh mutant (2). Comparison with purified human AT III prepared as described (18) confirmed that the abnormal α_1 -AT was more potent than the natural thrombin and Factor Xa inhibitors (Table II).

In order to evaluate the circulating amount of the abnormal inhibitor, we measured the rate of thrombin inhibition by patient's plasma samples obtained during the 22 mo of follow up. In the experimental condition of the assay (plasma diluted 1:10 and 1:20), no thrombin inhibition was observed in normal subjects or in the patient's family members. The purified inhibitor was used as a standard. The levels of the abnormal thrombin inhibitor increased from 8 to 17 μ M within the first 19 mo of

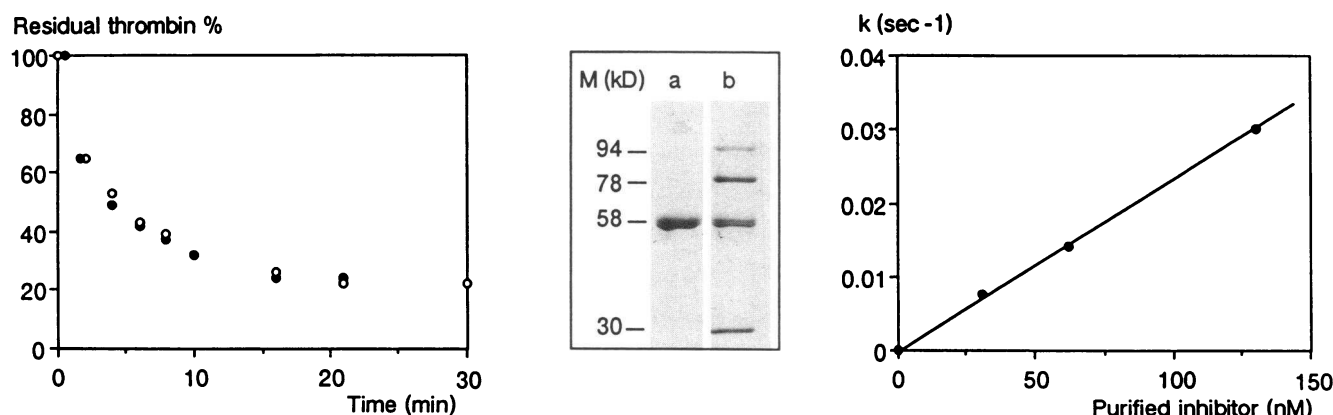


Figure 2. Characterization of the purified thrombin inhibitor. (Left panel) Thrombin inhibition by purified inhibitor (●) or diluted plasma (○). Equimolar concentrations (15 nM) of thrombin and inhibitor were incubated at 37°C. At different time intervals, the residual amount of thrombin was measured by the amidolytic activity upon the synthetic substrate. (Middle panel) SDS-PAGE of purified inhibitor (1.25 μ M) incubated for 10 min at 37°C with buffer (lane a) or equimolar amount of thrombin (lane b). The molecular masses were calculated from standards (Biorad Laboratories). (Right panel) Kinetic analysis of the inactivation of thrombin by the purified inhibitor. The inhibition of amidolytic activity of 10 nM human thrombin was measured after 1 min at different inhibitor concentrations. The pseudo first-order rate constant of the inhibition of thrombin was plotted as a function of the purified inhibitor concentration. The second-order rate constants were determined from the slope of the curve.

Table II. Second Order Rate Constants Observed for Inhibition of Human Thrombin and Human Factor Xa Activity

	α_1 -AT	AT III	α_1 -AT
	Variant purified from the patient's plasma	Purified from normal plasma	Pittsburgh (data from Travis, 2)
Thrombin ($s^{-1} M^{-1}$)	2.3×10^5	4.7×10^4	3.1×10^5
Factor Xa ($s^{-1} M^{-1}$)	1.6×10^4	3.7×10^3	1.9×10^4

survey. 2 mo later, when the patient experienced his first hemorrhagic episode, the level was 38 μM before any treatment, and returned to 16 μM within 2 wk after the operation (Fig. 3).

Frozen plasma from the original Pittsburgh patient, obtained by plasma exchange at the time of the ultimate hemorrhagic episode, presented standard coagulation tests similar to those observed at the time of sampling. This suggests that most protein activities were unaffected by prolonged storage. The protein C level using the immuno-enzymatic technique was 62%. The abnormal inhibitor concentration, evaluated by the rate of thrombin inhibition as described above, was 42 μM .

Genomic DNA analysis. Molecular characterization of the α_1 -AT Pittsburgh abnormality, i.e., a Met 358 to Arg mutation, was performed on amplified exon V, which encoded for the α_1 -AT reactive site, using dot-blot analysis and direct sequencing. In an attempt to explain the reduced expression of the mutant gene product, we also searched for a mutation frequently involved in quantitative deficiencies in Caucasians: the Z mutation, also located in exon V.

The conversion of Met 358 to Arg requires substitution of a G for T at position 10038 of the α_1 -AT gene in the numbering system of Long et al. (14). The specific wild-type ASO probe (α_1 N) and the mutant probe (α_1 P) were synthesized in accordance with this change, and the samples were hybridized with both α_1 N and α_1 P. We also analyzed the amplified DNA with the corresponding normal and mutant ASO probes (α_1 M and α_1 Z) (19), with the Z mutation in the same exon at position 9991 (G to A substitution). A schematic representation of the α_1 -AT exon V with the positions of the different PCR primers and ASO probes is shown in Fig. 4.

The pedigree of the patient's family and the complete results of exon V analysis are displayed in Fig. 5. Only DNA from

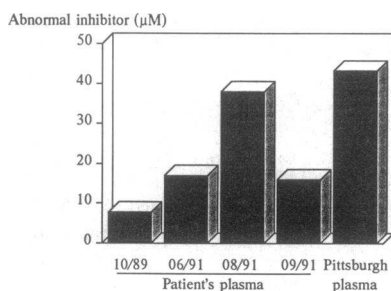


Figure 3. Abnormal inhibitor concentrations observed in the patient's plasma and in the original Pittsburgh's plasma. The plasma samples were assayed diluted 1:10 and 1:20 (and 1:40 in the Pittsburgh plasma), and the pseudofirst constant (k) for thrombin inhibition

were determined, as described under Methods. A calibration curve was constructed with the purified inhibitor as depicted in Fig. 2 (right panel). The data presented here are the mean values of determinations performed by testing two different dilutions in duplicate.

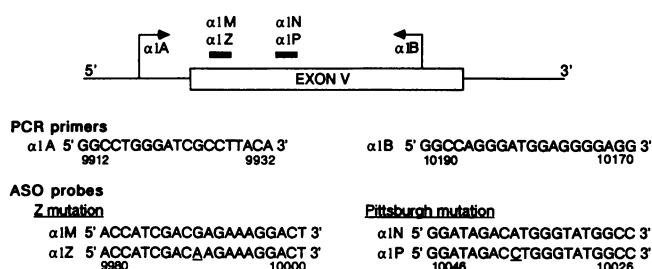


Figure 4. Schematic representation of exon V of the α_1 -AT gene. Horizontal arrows indicate the position and orientation of the PCR primers α_1 A and α_1 B. Horizontal bars indicate the positions of the wild-type ASO probes α_1 M and α_1 N and of the mutant α_1 Z and α_1 P probes.

the propositus (II₃) hybridized with both α_1 N and α_1 P. This result confirmed the existence of a heterozygous Met to Arg substitution at position 358 of the reactive site of the protein. No amplified DNA hybridized with the mutant α_1 Z ASO probe, demonstrating that the propositus and members of his family exhibited a normal homozygous M genotype.

To confirm the dot-blot results and search for another putative mutation in this part of the α_1 -AT gene, we determined the nucleotide sequence of exon V after single-strand PCR amplification between primers α_1 A and α_1 B, using α_1 A for the sequencing reaction. The results (Fig. 6) show that our patient bears a heterozygous G to T substitution at position 10038 that generates an AGG codon instead of an ATG codon at position 358 of the mature protein. No other nucleotide substitution was observed in exon V.

Furthermore, all the coding regions of the α_1 AT gene, as well as the intron/exon junctions, were sequenced after asymmetric PCR, as described in Methods, using the oligonucleotides listed in Table III. No other mutation was found.

SDS-PAGE and immunoblot analysis of protein C. As stated in the case report, a decrease in the concentration of several vitamin K-dependent factors was observed in our patient. The extremely low level of protein C was of particular interest, as it may provide an explanation for the mild hemorrhagic tendency. As a defect in the expression of one or both alleles encoding for protein C was extremely unlikely, we suspected that the α_1 -AT mutant was the cause of the defect, and therefore analyzed patient's plasma for the presence of abnormal forms of protein C.

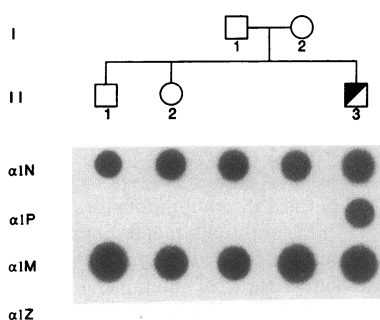


Figure 5. Dot-blot analysis of amplified DNA from the B family. Samples were hybridized with the wild-type ASO probes α_1 M and α_1 N, and the mutant α_1 Z and α_1 P probes. The propositus' sample (II₃) is the only one that hybridized twice with α_1 N and α_1 P, thus indicating the presence of

the heterozygous Pittsburgh mutation. Nobody bore the Z mutation, as shown by the absence of hybridization from all DNA samples amplified with α_1 Z.

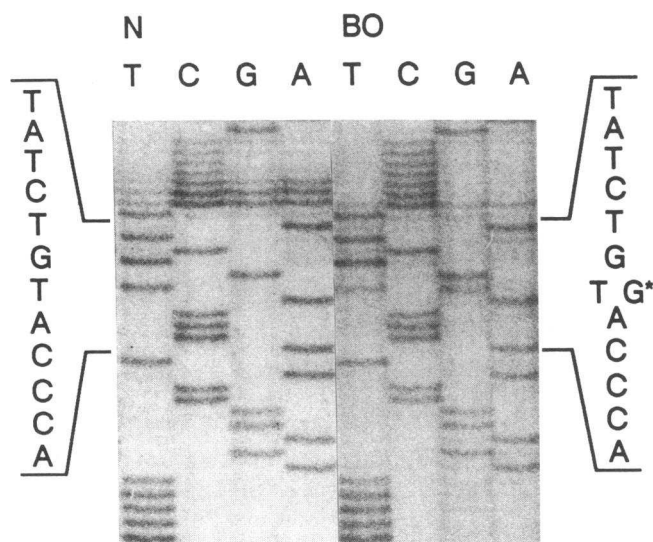


Figure 6. Direct sequencing of the Pittsburgh mutation. N, normal sequence; Bo, patient's sequence with both T and G present in position 10038. *Substituted nucleotide.

The blots for protein C antigen obtained from 10% and 7% SDS-PAGEs clearly showed an abnormal migration in the plasma obtained from our patient, or from the original Pittsburgh case (Fig. 7). In normal plasma, protein C migrates as a triplet, as described (20), with molecular masses of 68, 58, and 54 kD, respectively, due to variations in the carbohydrate chains (21). Patient's plasma yielded a single band at 68 kD, and another compound migrating as a doublet with a mass of 97 and 102 kD. This abnormal pattern was also observed in the original Pittsburgh case. In normal plasma, activation by Protac led to the appearance of a high molecular mass compound which probably results from the formation of complexes between activated protein C and its two inhibitors, protein C inhibitor and α_1 -AT (22, 23). Activation of our patient's plasma resulted in an almost complete disappearance of the 68-kD band. The activated protein C-inhibitor complexes, if formed, could not be distinguished from the 97-kD compound observed before activation.

To investigate whether the high molecular mass bands resulted from complex formation between activated protein C

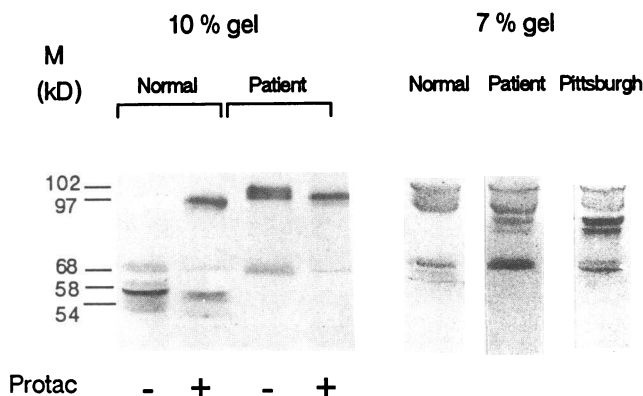


Figure 7. Immunoblots for protein C antigen in plasma samples. Left panel is from a 10% SDS-PAGE. The patient's plasma was compared to a normal plasma. Protein C was activated by incubating the plasma (1:4) with snake venom Protac for 1 h at 37°C. Right panel is from a 7% SDS-PAGE. The normal and patient's plasma were electrophoresed together with a sample of plasma from the original Pittsburgh case.

and the mutated α_1 -AT, we made additional experiment on semipurified samples obtained from plasma processed by aluminum hydroxide adsorption (Fig. 8). Vitamin K-dependent zymogens normally bind to aluminum hydroxide, and thus can be separated from the physiologic inhibitors that remain in the supernatant. Protein C eluates prepared from normal plasmas were activated by Protac and added to the purified abnormal inhibitor. High molecular mass complexes of 98 and 105 kD were formed, confirming a strong inhibitory effect of the

Table III. Base Sequences of the Primers Used to Amplify and Sequence the Exons II, III, and IV

Exon II
AT21 <u>5'GGCACAGGCTGGTTTAATAATC3'</u>
AT22 <u>5'GCATTGCCAAGGAGAGTTCA3'</u>
AT23*5'CACCCAACCTTCAACAAGATC3'
AT24*5'TACCCTCAACCAGCCAGACAG3'
Exon III
AT31 <u>5'CACTCTTCCAAACCTTCACTCAC3'</u>
AT32 <u>5'TTACCCCTCCTCAGCCCTCT3'</u>
Exon IV
AT41 <u>5'GGTCCCAGAAGAACAAGAGG3'</u>
AT42 <u>5'CCCCACACATTCTTCCCTACA3'</u>

Primers used for both amplification and sequence are underlined. Primers used only for sequencing are identified by an asterisk.

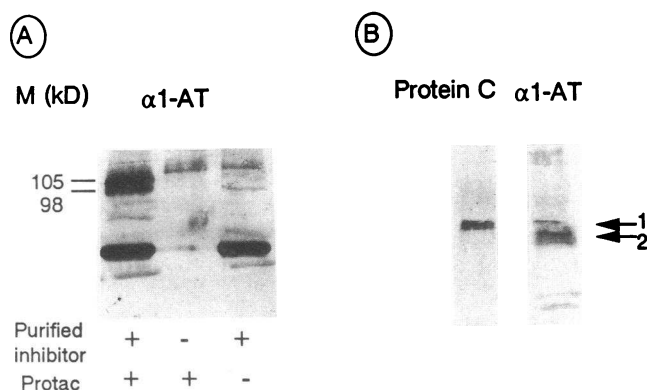


Figure 8. Immunoblots for α_1 -AT or protein C antigen in aluminum hydroxide eluates obtained from normal plasma (A) and the patient's plasma (B). (A) The normal plasma eluate was adjusted to plasma initial concentration, then activated by Protac and incubated with the purified abnormal inhibitor (7.5 μ M). Addition to each mixture is indicated under each line. Blots were made to react with α_1 -AT antiserum. The molecular masses of the complexes formed between the inhibitor and activated protein C were 105 and 98 kD. (B) Two samples of the patient's plasma were submitted to electrophoresis in a 10% SDS-gel, blotted, and then separately made to react with protein C antiserum and α_1 -AT antiserum. After washing, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit-IgG IgG. Arrow 1 indicates the compound migrating exactly as protein C high molecular mass complexes. Arrow 2 indicates another unidentified compound of high molecular mass reacting with α_1 -AT antiserum.

mutated α_1 -AT on activated protein C. Such complexes were not observed in the absence of Protac or of the purified inhibitor (Fig. 8 A). When protein C eluates were prepared from the patient's plasma, high molecular mass complexes were observed in the absence of any additional components (Fig. 8 B). One of them (arrow 1) reacted with both protein C and α_1 -AT antisera.

These data strongly suggest the presence in the patient's circulation of activated protein C- α_1 -AT Pittsburgh complexes.

Discussion

Screening tests performed in a 15-yr-old patient with abnormal coagulation tests revealed the presence of a strong coagulation inhibitor. The abnormal inhibitor copurified with α_1 -AT and presented several characteristics of the mutated protein called α_1 -AT Pittsburgh. The identification of this mutation was initially done when the patient had no bleeding history. 22 mo later, he experienced a first bleeding episode.

Direct sequencing of amplified DNA allowed us to rapidly identify a G to T substitution in exon V of the α_1 -AT gene encoding for the reactive site of the serpin in this second case of α_1 -AT Pittsburgh variant. Both parents of our patient had normal DNA sequence for exon V, which suggests the existence of a spontaneous mutation during gamete formation in one of the genitors or during the first steps of embryogenesis.

This conversion of a Met serpin to an Arg serpin resulted in marked inhibition of thrombin and Factor Xa. These proteases were both inhibited by the variant protein purified from our patient's plasma, with second-order constant rates similar to those observed for the first α_1 -AT Pittsburgh variant (2).

Other coagulation factors were found to have markedly reduced activities in clotting assays performed with the patient's plasma, probably due to the presence of the mutant inhibitor. The virtual absence of protein C activity from the patient's plasma might have been due to the strong inhibitory activity of Arg 358 α_1 -AT toward activated protein C *in vitro* (9). However, this does not explain the markedly reduced activity of protein C measured in the absence of inhibitors, according to Bertina (13), or the reduced concentration observed *ex vivo* with an immunoenzymatic assay. As the patient's parents had normal protein C antigen, a homozygous deficiency can be excluded. The immunologic concentration of two other vitamin K-dependent zymogens (Factors X and II) were also decreased, but to a lesser extent.

The presence of abnormal molecular forms of protein C in our patient's plasma, as well as in the plasma provided to us from the original Pittsburgh case, suggests that this mutation of α_1 -AT has unexpected effects on protein C metabolism. The 68-kD compound might be incompletely processed protein C, and the high molecular weight compounds that reacted with α_1 -AT immune serum probably reflect the presence of activated protein C-Arg 358 α_1 -AT complexes in the circulation. As the mutant α_1 -AT Pittsburgh has a strong affinity for thrombin, it should block protein C activation within the circulation. However, the *in vivo* activation is mediated by thrombin bound to thrombomodulin at the vascular endothelium surface (24, 25), which may reduce the affinity of the enzyme for the mutated inhibitor and makes possible the activation of protein C. The presence of proalbumin in the original Pittsburgh case (also observed in our patient) was shown to be due to an inhibitory effect of the α_1 -AT variant on propeptide cleavage

(12). Protein C undergoes several intracellular modifications, including endoproteolytic processing to remove the propeptide and the dipeptide that connect the heavy and light chains in the precursor (26, 27). An inhibition of the endoprocessing might reduce the secretion of protein C in the extracellular medium. The removal of protein C from the circulation may also be accelerated by the rapid formation of complexes with the mutated α_1 -AT.

Comparison of the present case with the original case of α_1 -AT Pittsburgh shows a strong discrepancy in the clinical expression of the mutation. The first patient died at the age of 14 after more than 50 episodes of bleeding in an acute-phase state, while our patient displayed a mild bleeding tendency with a first episode at the age of 17. The protein C deficiency observed in our patient might explain the maintenance of the *in vivo* hemostatic balance. Since the importance of protein C as a physiologic inhibitor has only been known since 1981 (28), protein C was not measured when the first Pittsburgh variant case was reported in 1978 (5). In a plasma kept frozen for more than 10 yr, we found the same abnormal molecular forms of protein C as those observed in our patient. The protein C level at the lower limit of the normal range (62%) does not allow conclusion, as the assay was performed on a single sample kept frozen for a long time. As the α_1 -AT gene is pleiomorphic (29, 30), our first hypothesis to explain why our patient was asymptomatic was the presence on the same allele of a second mutation leading to a reduced expression of the gene. We used dot-blot analysis with ASO probes to exclude the presence of the Z mutation frequently observed in Caucasians. We also analyzed the α_1 -AT genomic DNA coding sequences and intron/exon junctions, and did not find an additional mutation that could explain the reduced amount of mutated protein initially observed in our patient.

Taking advantage of the strong antithrombin effect of the mutant α_1 -AT, we developed an assay to quantitate the abnormal inhibitor in plasma. In several samples obtained in November 1989 at the time of the diagnosis, we found levels between 8 and 10 μ M which were around 30% of the total α_1 -AT. This was consistent with a reduced expression of the mutant protein, at variance with the original Pittsburgh case found to express 60% of α_1 -AT as the mutant protein (1). Using our assay, we found 42 μ M (65% of the total α_1 -AT), which was consistent with the published results. However, this difference in the level of the respective mutated and normal α_1 -AT in the two patients might have been due to the fact that the Pittsburgh patient was always explored during acute hemorrhagic episodes (J. Lewis, personal communication). The recent data obtained in our patient in June 1991 when he was in good health showed an increased level of the abnormal inhibitor concentration (17 μ M) and a ratio of 65% to the total α_1 -AT. Similar values were observed when he left the hospital in September, 2 wk after his hematoma was drained surgically. This might result from a change in the expression and/or secretion of the mutant versus the normal α_1 -AT. It is tempting to speculate that the first hemorrhagic accident occurred because the concentration of the abnormal α_1 -AT had recently increased. Indeed, the abnormal inhibitor level (38 μ M) during the acute phase was similar to the level observed in the original Pittsburgh case in such a situation. However, as the fluctuations of the mutant protein expression were not explored before the age of 15 in our patient, it is difficult to conclude. Our assay will allow us to clarify this issue in the future.

It is also important to elucidate the consequences of the inhibition by the mutated α_1 -AT of endoproteases specific for dibasic peptide-bound cleavage. Many proteins of great physiologic significance require the action of this type of endocellular proteases (31). Furin, recently characterized as an endogeneous protease expressed in mammalian cells, was shown to be inhibited by Arg 358 Met α_1 -AT (32). This second case of α_1 -AT Pittsburgh provides new insights in the consequence of this single amino-acid substitution, and suggests that there are in vivo consequences of the mutation that may contribute to maintain a normal hemostatic balance.

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