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Antithrombin deficiency in three Japanese families: One novel and two reported point mutations in the antithrombin gene

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ABSTRACT

Introduction: Inherited antithrombin (AT) deficiency is associated with a predisposition to familial venous thromboembolic disease. We analyzed the AT gene in three unrelated patients with an AT deficiency who developed thrombosis.
Materials and Methods: We analyzed the *SERPINC1* gene in three patients. Additionally, we expressed the three mutants in the COS-1 cells and compared their secretion rates and levels of AT activity with those of the wild-type (WT).
Results: We identified three distinct heterozygous mutations of c.2534C>T: p.56Arginine → Cysteine (R56C), c.13398C>A: p.459Alanine → Aspartic acid (A459D) and c.2703C>G: p.112 Proline → Arginine (P112R). In the *in vitro* expression experiments, the AT antigen levels in the conditioned media (CM) of the R56C mutant were nearly equal to those of WT. In contrast, the AT antigen levels in the CM of the A459D and P112R mutants were significantly decreased. The AT activity of R56C was decreased in association with a shorter incubation time in a FXa inhibition assay and a thrombin inhibition-based activity test. However, the AT activity of R56C was comparable to that of WT when the incubation time was increased.
Conclusions: We concluded that the R56C mutant is responsible for type II HBS deficiency. We considered that the A459D and P112R mutants can be classified as belonging to the type I AT deficiency.

Introduction

Antithrombin (AT) is a plasma serine protease inhibitor (serpin) that inactivates a number of proteases in the coagulation cascade, particularly thrombin and factor Xa (FXa) [1]. The human AT gene measures 13.5 kb in length, comprises seven exons and six introns and is located on human chromosome 1 at q23.1-23.9 [2]. Plasma AT is synthesized by hepatocytes as a 464 amino acid precursor with a 32 amino acid single peptide that is cleaved off before secretion as a 432 amino acid mature inhibitor into the plasma. It is a single chain glycoprotein with a molecular weight of approximately 58 kDa [3].

AT is a globular protein composed of three β-sheets (A, B and C), nine α-helices and a reactive center loop (RCL). The RCL protrudes above the core of the serpin molecule and has a sequence of amino acids at the reactive center that is complementary to binding pockets in the active sites of target proteases [4,5]. The AT activity is accelerated approximately 1,000-fold by the binding of heparin to arginine (Arg) residues in the D-helix of the AT protein, which occurs via two different mechanisms [6,7]. A conformational change induced by binding to a specific pentasaccharide sequence within heparin allosterically activates the inhibitor, thereby increasing the inhibition rate. Moreover, a bridging effect, by which heparin brings AT and the protease into a ternary complex, further increases this rate [8]. The individual contributions of these two mechanisms to the heparin-related acceleration of AT

Abbreviations: AT, Antithrombin; serpin, Serine protease inhibitor; FXa, Factor Xa; RCL, Reactive center loop; Arg, Arginine; Lys, Lysine; VTE, Venous thromboembolism; type II RS, Type II reactive site defects; type II HBS, Type II heparin binding site defects; type II PE, Type II with pleiotropic defects; cDNA, Complementary DNA; WT, Wild-type; COS-1 cells, Green monkey kidney cells; Cys, Cysteine; Pro, Proline; Ala, Alanine; Asp, Aspartic acid; PMSF, Phenylmethyl sulfonylfluoride; ELISA, Enzyme-linked immunosorbent assay; RFLP, Restriction fragment length polymorphism analysis; CM, Conditioned media; CL, Cell lysates; PE, Pulmonary thromboembolism; DVT, Deep vein thrombosis.

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inhibition vary for different proteases, the conformational change being of major importance for FXa and IXa, and the bridging effect dominating for thrombin [9]. The X-ray structures of AT and its complex with a synthetic variant of the heparin pentasaccharide indicate that this change involves the elongation of the A- and D-helices, the formation of a new short P-helix at the base of the D-helix and the contraction of the A-sheet. These changes lead to a higher level of accessibility of the reactive bond and exposure of exosites surrounding the loop, thus promoting the binding of target proteases [10]. The region of AT to which the heparin pentasaccharide binds with high affinity and specificity consists primarily of positively charged lysines and arginines within the A- and D-helices and the N-terminal region, including Lys43, Arg45, Arg78, Arg79, Lys146, Lys157 and Arg161 [11]. Lys43, Arg45, Arg78 and Arg79 make lesser contributions to the binding affinity since mutations of any one of these residues result in the loss of binding energy, which, in most cases, approximates the loss of a single ionic interaction. In contrast, Lys146, Lys157 and Arg161 represent binding hotspots since mutations of any one of these residues cause major losses in binding energy that are much greater than would be expected from the loss of a single ionic interaction [12].

An inherited AT deficiency is an autosomal dominant thrombotic disorder associated with a 1.7–4.0% overall annual incidence of venous thromboembolism (VTE) [13,14]. The two primary AT deficiency phenotypes are defined based on the plasma levels of functional and antigenic AT. Type I AT deficiency is characterized by equally low functional and antigenic AT levels, whereas type II deficiency covers all variants with reduced functional but normal AT levels. Depending on the localization of the mutation, type II deficiency is further subdivided into three groups: i) type II reactive site defects (type II RS) are characterized by a low serine protease reactivity in both the presence and absence of heparin; ii) type II heparin binding site defects (type II HBS) are associated with an impaired heparin binding capacity, but normal serine protease reactivity in the absence of heparin; and iii) type II defects associated with pleiotropic defects (type II PE) [15,16]. In type II PE deficiency, the amino acid substitutions affect the highly conserved C-terminal hinge region (strand 1C-5B) of AT, resulting in a decreased circulating concentration of abnormal AT molecules, with impaired heparin binding and serine protease inhibition capacity.

In this study, we evaluated the AT deficiency in three Japanese patients and identified three distinct mutations, including one novel mutation in the AT gene. Additionally, we expressed the three mutants in green monkey kidney cells (COS-1 cells) and compared their secretion rates and levels of AT activity with those of the wild-type (WT) gene product.

Materials and Methods

Materials

The pcDNA3.1/AT expression plasmid containing the full-length AT complementary DNA (cDNA) was kindly provided by Dr. Tsuneo Imanaka (Toyama University, Japan). The Big Dye Terminator v3.1 Cycle Sequencing Kit was purchased from Applied Biosystems Japan, Ltd. Dulbecco's modified Eagle medium (DMEM) was obtained from Nissui Seiyaku (Tokyo, Japan), and fetal bovine serum (FBS) was purchased from Serum Source International, Inc. (Charlotte, NC). The TaKaRa Ligation kit Ver. 2 was obtained from TaKaRa Bio, Inc. (Ohtsu, Japan). HilyMax Transfection Reagent was purchased from DOJINDO (Kumamoto, Japan). Amicon ultra was obtained from Nihon Millipore, Inc. (Tokyo, Japan). A Matched-Pair Antibody Set for the enzyme-linked immunosorbent assay (ELISA) of human AT antigens was purchased from Affinity Biologicals, Inc. (Ontario, Canada). Thrombin was purchased from Sigma Aldrich (St Louis, MO), and heparin was purchased from Novo Industry (Copenhagen, Denmark). The FXa and chromogenic substrates S2238 and S2222 were provided by Sekisui Medical (Tokyo, Japan).

Sample Preparation

Ethical approval for the study was obtained from the Ethics Committee of Kanazawa University School of Medicine, Japan. After obtaining informed consent, venous blood samples were collected from patients with AT deficiency in a 1:10 volume of 3.13% (wt/vol) trisodium citrate. The plasma was separated via centrifugation at × 2,000 g for 20 minutes, and genomic DNA was isolated from peripheral blood leukocytes.

Identification of Abnormalities in the Patients' AT Genes

All seven exons, including the splice junctions of the AT gene, were amplified via PCR using the primer sets listed in Table 1. The DNA was subjected to the following PCR conditions: 30 cycles of denaturation at 94 °C for 60 seconds, annealing at 55 ~ 60 °C for 30 seconds and extension at 70 °C for 30 seconds. The obtained PCR product was directly sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 310 genetic analyzer. The initial Met residue was denoted as amino acid +1.

Site-Directed Mutagenesis of AT

The Arg56 → Cysteine (Cys) (R56C), Proline (Pro) 112 → Arg (P112R) and Alanine (Ala) 459 → Aspartic acid (Asp) (A459D) mutations were introduced into the full length AT cDNA in the pcDNA3.1(+) plasmid using the TaKaRa Ligation kit Ver.2. The primers used for mutagenesis are listed in Table 2. The AT cDNA was sequenced to confirm the presence of a mutation.

Transient Expression of Recombinant AT

COS-1 cells were grown in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2. The cells were cultured in 60-mm dishes and transiently transfected using HilyMax Transfection Reagent. Following incubation in FBS-free DMEM for 24 hours, the cells were lysed in buffer comprising 50 mmol/l Tris–HCl (pH 7.5), 1% BSA, 2 mmol/l of EDTA, 1% Triton X-100, 0.1 mol/l of NaCl, 100 U/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstain and 200 mmol/l of phenylmethyl sulfonylfluoride (PMSF). The conditioned medium (CM) was concentrated with Amicon ultra. The recombinant proteins were not purified. The following experiments were performed using the CM.

AT Antigen and Activity Measurements in the Conditioned Medium (CM) and Cell Lysates (CL)

The antigen levels of the recombinant AT molecules were measured using ELISA with a Matched-Pair Antibody Set for ELISA of the human AT antigen. The AT activity levels were measured using the FXa inhibition activity assay and the thrombin inhibition activity assay with the CM containing each recombinant AT protein. The reaction of recombinant AT with 4 U/ml of thrombin or 3.55 nkat/ml of FXa was studied at 37 °C in the presence of 60 IU/l of heparin. Recombinant AT was incubated with thrombin in Tris buffer (50 mmol/l of Tris–HCl, 100 mmol/l of NaCl, pH 7.4). At different

Table 1
Oligonucleotide primers used for the amplification of the *SERPINC1* gene.

Exon	Forward primer (5' → 3')	Reverse primer (5' → 3')
1	TCAGCCTTTGACCTCAGTTC	AGGTCACAAAACCCATGAGG
2	GGCAGTGGGGCTAGGGGTT	TTGAGGAATCATTGGACTTGGG
3a	ACCACCCATGTTAACTAGGC	AGCAGCAAAGCAGTGTGAAT
3b	TAGCACAGGTGAGTAGGTTTATT	GAAGAGCAAGAGGAAGTCCCT
4	CAATAACTATCCTCCTATGAATG	CTTTCTCCAACTCTTCCACTTTT
5	AGCCAACTTTCTCCCATCTC	CAGAAGAGGTAGTGGGAGGG
6	TCTGTGGATGATTTACCTGC	TTCACAAACCAAAAATAGGA