Molecular mechanisms of antithrombin deficiency in two Chinese families

One novel and one recurrent point mutation in the antithrombin gene causing venous thrombosis

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Summary

We investigated the molecular mechanisms responsible for type I congenital antithrombin (AT) deficiency in two unrelated Chinese pedigrees manifesting multiple site venous thrombosis. Phenotype analysis showed both probands had almost 50% of normal AT levels. Direct sequencing of amplified DNA revealed 2757C>T in proband 1 and 13328G>A in proband 2, predicting a heterozygous Thr98Ile (T98I) and Ala404Thr (A404T), respectively. No proband had 20210A allele or Factor V Leiden mutation. Transient expression of complementary DNA coding for the mutations in COS-7 cells showed impaired secretion of the mutant molecules. Real-time quantitative PCR indicated that the mutant AT mRNA was transcribed at a similar or even higher level as that of wild-type (wt). Pulse-chase labeling studies suggested both AT variants did not accumulate, but degraded intracellularly. Immunohistochemical staining of the transfected cells revealed that CHO cells expressing the AT-I98 mutant were stained diffusely without perinuclear enhancement and cells expressing AT-T404 mutant mainly in the whole cytoplasm with weaker perinuclear enhancement. We conclude that the impaired secretion of the mutant AT molecules, due to intracellular degradation, is the molecular pathogenesis of AT deficiency caused by T98I and A404T mutation for the two families, respectively.

Keywords

Antithrombin deficiency, gene mutation, venous thrombosis

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Introduction

Antithrombin (AT) is the major plasma inhibitor of thrombin and other coagulation serine proteases, and plays a vital role in maintaining normal hemostasis (1). The gene for human AT is located on chromosome 1q23–25 and contains 7 exons and 6 introns (2). Mature human AT protein has two functional domains: NH2-terminal heparin-binding domain and COOH-terminal protease-binding domain (3).

Individuals with AT deficiency have a predisposition for venous thrombosis. Congenital AT deficiency is most often inherited as an autosomal dominant trait and is classified into type I (quantitative) and type II (qualitative). Type II AT deficiency is further classified into three subclasses as follows: HBS, functional abnormalities limited to the heparin binding site; RS, functional abnormalities limited to the reactive site; and PE, pleiotropic effect-type (1).

In the present study, the AT gene was analyzed in 2 unrelated Chinese pedigrees with thrombotic episodes and a novel missense mutation (2757C>T in proband 1) and a previously described mutation (13328G>A in proband 2) was identified. We expressed wt and mutant ATs corresponding to the respective genetic abnormalities of each proband to elucidate the molecular mechanisms of the secretion of AT wt and impaired secretion of AT variants.
Patients, materials and methods

Patients
Informed consent was obtained from family members involved in the study. Proband 1 was a 21-year-old male who had deep vein thrombosis (DVT) and mesenteric venous thrombosis at 20 years. Proband 2 was a 42-year-old male with a history of DVT and pulmonary embolism at 41 years. There was no family history of thrombosis in the two pedigrees (Fig. 1A and 1B).

Plasma AT assays
The AT activity (AT:A) was measured using chromogenic substrate assay (DadeBehring, Germany). The AT antigen (AT:Ag) level was determined using enzyme-linked immunoabsorbent assay (ELISA) with Antithrombin Kit from Beckman Coulter, USA.

DNA sequence analysis and enzymatic detection of mutation
All seven exons including exon-intron boundaries of the AT gene were amplified by polymerase chain reaction (PCR), as described previously (4), except for exon 2. The sense primer for amplifying exon 2 was 5’-AATCCTCTGCTTTACTGGGG-3’ and the antisense primer 5’-TGCTCCTAACAAGGTGGC-3’. PCR products were purified and sequenced using the ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotides and amino acids are numbered according to Oldset al (2).

The PCR product of exon 2 was digested with restriction enzyme BseGI (MBI, Fermentas, Vilnius, Lithuania) and electrophoresed in an agarose gel. 100 normal individuals were simultaneously analyzed using the same procedure.

Construction of expression vectors and site-directed mutagenesis
The pCRII/ATcDNA, containing the full-length AT cDNA, was kindly provided by Dr. Alison Fitches (University of Otago, New Zealand). The expression vector, pcDNA3.1(-)/ATcDNA (AT-wt) had been obtained in our laboratory (5). The mutant version of AT containing I98 or T404 was constructed using a “mega-primer” method (6) from pCRII/ATcDNA. The resultant plasmid pcDNA3.1(-)/AT-T98I and pcDNA3.1(-)/AT-A404T were designated as AT-T98I and AT-A404T, respectively.

Cell culture and transfection assays
Monkey COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum under 5% CO₂ at 37°C. Transient expression assays using polyFect® reagent (Qiagen, Hilden, Germany) were performed with 1.5 µg of expression vector (pcDNA3.1 (-), ATwt, AT-T98I and AT-A404T) per well dish. Conditioned media and cells were processed and measured for AT:Ag by ELISA, as described previously (5).

Real-time quantitative PCR analysis
Transfected cells were subjected to AT mRNA expression level analysis by real-time quantitative PCR with SYBR Green Dye. Total RNA was extracted from transiently transfected cells using TRIzol® reagent (GibcoBRL, USA) and cDNA was obtained with reverse transcription system kit (Promega, USA). Real-time quantitative PCR analysis was performed (5) and the data were statistically analyzed using Student’s t-test.

Pulse-chase and immunofluorescence analysis
To examine the secretion rates and the subcellular localization of recombinant AT and AT mutants, pulse-chase experiments and immunofluorescence analysis were performed as described previously except for transfection with polyFect® reagent (5).

Results

Phenotype analysis
Proband 1 (III-1, Fig. 1A) had parallel reduced AT:Ag (174mg/L) and AT:A (53.5%) (normal range, 250 to 360mg/L and 90 to 108%, respectively), suggesting type I AT deficiency. Five other members from the family also showed similar reduction in plasma AT levels. Proband 2 (II-1, Fig. 1B) had an approximately 50% reduction of both AT:Ag and AT:A, also suggesting type I AT deficiency.

Genotype analysis
Sequencing of the AT gene showed that members with reduced plasma AT levels from pedigree 1 were heterozygous for an novel mutation (C2757>T) in exon 2 (Fig. 1A). This mutation alters the normal Thr98 to Ile (T98I). In pedigree 2, a heterozygous mu-
tation (G13328>A) in exon 6 was identified in 4 members, of whom III-2 had normal plasma AT levels (Fig. 1B). This mutation results in Ala404 to Thr (A404T). Neither factor V Leiden mutation nor prothrombin G20210A mutation was found in members of the two pedigrees.

PCR-enzymatic analysis Since C2757>T mutation created an other BseGI restriction site in PCR fragment of exon 2, the wild-type one was cleaved by BseGI into 2 fragments of 252bp and 246bp, whereas 252bp, 246bp, 188bp and 64bp fragments were present in proband 1. The mutation was absent in 100 normal individuals, suggesting that the mutation is not a common polymorphism (data not shown).

**Transient transfection assays in COS-7 cells**

To investigate the influence of the T98I and A404T substitutions on AT biosynthesis, transient transfections were performed in COS-7 cells. In lysates of cells expressing the mutant alleles, AT:Ag levels were reduced to 48±4.3% and 68±5.2% (mean±SD, n=3) (in cells transfected with the AT-T98I or the AT-A404T plasmid, respectively) of those measured in cells expressing the ATwt, whereas AT:Ag levels in conditioned media were decreased to 0 and 40±3.6% of the wt, suggesting that the two mutations lead to reduced secretion, and mutants were degraded partially in cells with little, if any, intracellular accumulation of these mutants.

Real-time quantitative PCR was performed to investigate the mRNA expression levels of AT wt and mutations in transient transfectants. The difference in the threshold cycle between ATwt and AT-I98 or AT-T404 mutant was approximately 1.273 and 1.520, indicating a slightly increased mRNA expression level of AT-I98 and AT-T404 compared with that of ATwt (P<0.01, Fig. 2), respectively. Because our in vitro studies utilized a transient expression system, however, the meaning and significance of the slightly elevated mRNA expression level in AT-I98 and AT-T404 mutant should not be overestimated.

**Metabolic labeling studies in COS-7 cells**

To further compare the secretion rates of wt and mutant antithrombins, pulse-chase experiments were performed. After a 30 min pulse with [35S] methionine, the recombinant ATwt in cell lysates was maximal at 0 to 30 min and decreased as the protein was secreted and the total amount of radioactivity remained unchanged during the chase period (Fig. 3A). In contrast, AT-I98 mutant was only detected in cell lysates and the total amount of radioactivity decreased during the chase period (Fig. 3B). For AT-T404 mutant, a band of 52kDa corresponding to premature AT was detected in cell extracts, which reduced slower than ATwt with the chase (Fig. 3C). Only 47.9% of the pulse-labeled AT-T404 mutant with an band of 58kDa corresponding to mature

**Figure 2**: Comparison of mRNA expression levels between wild-type antithrombin (ATwt) and mutant ATs (ATmuts) by real-time quantitative polymerase chain reaction (RT-PCR). Total RNA was isolated from transiently transfected COS-7 cells, and after reverse transcription, real-time quantitative PCR was performed using SYBR Green. The relative quantification of target RNA was achieved by the comparative threshold cycle (CT) method, and the target CT number was normalized to GAPDH. A representative result was shown from three independent transfection assays. The difference between ATwt and AT-I98 or AT-T404 was approximately 1.273 and 1.520, indicating a slightly increased mRNA expression level of AT-I98 and AT-T404 compared with that of ATwt (P<0.01), respectively.

**Figure 3**: Pulse-chase analysis of wild-type and mutant antithrombins (AT) protein in COS-7 cells. COS-7 cells, transiently transfected with ATwt and ATmuts, were pulse-chase with [35S]-methionine for 0.5 h, and then chased by methionine for various periods of time up to 3 h. At the specified chase period (0, 0.5, 1, 2 and 3 h), labeled AT proteins from cells lysates, and conditioned media were immunoprecipitated and analyzed by 10% SDS-PAGE. On kinetic analysis, the amount of radioactivity in the pulse-labeled cell lysates was taken as 100%, and the relative radioactivity of the intracellular and secreted fractions were shown by closed diamond and square respectively. The sum of the radioactivities of the two fractions at each time is shown by the closed triangle.
AT was secreted into medium in the 3h chase period, and about 30% decrease in the total amount of radioactivity was observed. The results indicated partial intracellular degradation and impaired secretion for AT-I98 and AT-T404 mutants.

Using immunohistochemical techniques to detect intracellular AT mutants, we found that staining of ATwt (Fig. 4A) was mostly perinuclear, suggesting these molecules were localized primarily in the Golgi apparatus. In contrast, AT-I98 (Fig. 4B) staining was predominantly diffuse without perinuclear enhancement. AT-T404 (Fig. 4C) staining was similar to ATwt, but mostly diffuse with weaker perinuclear enhancement, suggesting that parts of the mutant molecules were localized in the Golgi apparatus.

Discussion

We investigated two Chinese pedigrees with type I AT deficiency and discovered two heterozygous mutations in exon 2 and exon 6 of the AT gene, respectively. Proband 1 had a novel mutation (C2757>T), resulting in T98I, and we designate the mutant Thr98>Ile as AT Shanghai. Proband 2 had a reported mutation (G13328>A), resulting in A404T, which had previously been named as AT Oslo or Paris 3 (1, 7).

Transient expression studies revealed that the secretion of the two mutant proteins was impaired and that the intracellular levels of AT mutants were reduced, whereas the mutant mRNAs were present inside of the cells at an equivalent or even higher level compared to wt, suggesting that AT-I98 and AT-T404 proteins are normally transcribed and synthesized at the endoplasmic reticulum. Pulse-chase experiments and immunohistochemical staining of transfected cells further confirmed the results of transient expression studies and suggested that mutant AT proteins did not accumulate but partially degraded intracellularly in spite of major secretion defects.

Most type I AT deficiencies are caused by a small insertion or deletion of bases in the gene, resulting in a frameshift mutation and usually a premature stop signal (1). T98I mutation found in proband 1, however, is a missense one. T98 is located on the C helix of the AT protein and lies underneath the region proposed to be involved in heparin binding. The small polar T98 is a hydrophilic residue in AT protein and is also a highly conserved one among serpins superfamily, whereas nonpolar I98 is a hydrophobic one. We postulate that the substitution of T98 with I98 would break the interactions of T98 with internal hydrophobic residues, alters the conformation of the helix, thereby affecting AT protein folding, resulting in a secretion defect and undergoing intracellular degradation by proteasome rather than lysosome pathway (data not shown). It has also been reported that AT mutants of 13387–9delG, ΔE313, and P429>stop had similar fates in COS-7 and BHK cells, respectively (5, 8).

Although Proband 2 was classified as having type I deficiency, the A404T mutation detected in this family is associated with type II PE deficiency. It has been reported that AT:Ag level for proband with A404T mutation was 48% (9), 72% and 100% (1), all with ~50% functional activity, suggesting that this mutation may not always result in a low plasma concentration of AT (10). To date, there are twelve distinct PE-type cases being reported (1, 11), of which, R425T, P407L, and L409P have approximately 50% the normal levels of AT antigen and activity in plasma, suggesting that the phenotype and genotype of AT deficiency is not always consistent with each other. Interestingly, the member III-2 showed normal AT levels, but she carried the mutation (Fig. 1B). The reason for this phenomenon is unknown.

In the study, results of transient expression and pulse-chase experiments showed that the secretion of mutant AT-T404 was impaired and the mutant partially degraded intracellularly, which is consistent with the results of Sheffield et al (12) but not with that of Shirotani et al (10). Sheffield et al (12) reported that the

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Figure 4: Immunofluorescence localization of (4A) wild-type antithrombin (ATwt) molecules, (4B) AT-I98 molecules, (4C) AT-T404 molecules, and (4D) negative control in CHO cells. CHO cells that transiently expressed ATwt and ATmuts were stained with rabbit anti-human AT polyclonal antibody, followed by fluorescein isothiocyanate-labeled goat-anti-rabbit IgG.
media levels of mutant AT-T404 were reduced by 50%, compared with wt in COS-1 cells. Shirotani et al (10) reported that the mutation of A404T did not cause a secretion defect in BHK cells. The reason for this discrepancy is not known, probably due to the cell lines used in these experiments. A404 is located in s1C region of AT protein containing the 402–407 amino acids. Integrity of the 402–407 region may be required for AT to fold correctly and the normal conformational linkage between the reactive site and heparin binding regions of the molecule (7). The substitution of very small nonpolar hydrophobic A404 with polar hydrophilic T404 would result in the change of local conformation, disturbing the packing at buried sites (13, 14) and affecting the hydrogen bonding between E414 on strand 4B and R129, one of the major heparin-binding sites, on the D-helix (13). It seems likely that the A404T mutation not only causes secretion impairment but also affects both the heparin-binding and thrombin inhibitory abilities of AT as a mutation P407L (10, 12, 15). Verpy et al. (16) reported that a V451M mutant in s1C, corresponding to the mutation site of mutant AT-T404, was partially secreted in COS-7 cells, but the secreted C1 inhibitor similar to mutant AT T404 was dysfunctional, which is similar to our studies.

In conclusion, our data showed that heterozygous C2757>T and G13328>A mutation in AT gene is the cause of AT deficiency in two unrelated Chinese pedigrees, respectively. The two mutations result in impaired secretion and partially intracellular degradation of AT mutants. The C2757>T mutation is a novel one, and we named the mutant AT Thr98>Ile as AT Shanghai.

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