circumstances [36–38], and the C-alpha-H bond dissociation energy at Asn is lower than that at the other amino acid residues [39]. Generally, the O-H bond dissociation energy of the water molecule is much higher than the C-alpha-H bond dissociation energy of Asn and the hydroxyl radical would react nonselectively with Asn; nevertheless, the hydroxyl radical would first react with the side chain Tyr or Trp, and the side chain radical could then selectively react with C-alpha-H in the main chain [40–42]. The disufide bond is not chemically inert and radical transfer possibly occurs from Tyr to Cys [43], thus Asn127 can be an intermediate with radical transfer from Tyr to Cys resulting in the racemization. In fact, Asn127 could interact with Tyr124 through the hydrogen bond network in the vicinity of Cys128-Cys6, and racemization of hydroxyproline in *Homo tirolensis* relates to Tyr oxidation and hydroxyl radical generation [44]. Additionally, from the solution structure of ML using NMR [19] and molecular dynamics simulation (data not shown here), Asn127 was suggested to locate at an exposed position of loose structure in which it contacts with bulk water. Thus, the loose structure allows the specific racemization at Asn127. Therefore, if the racemization in ML is induced by the oxidation described above, scavengers might prevent the racemization and prevent protein aging and conformational disease. In summary, as an alternative mechanism, we propose that the racemization at Asn127 is induced by an oxidation mechanism, and we are now verifying the validity of the alternative mechanism using theoretical calculations.

On the other hand, the racemization of Asn127 is also similar to that of other amino acid residues, rather than through the succinimide pathway, during the aging process [14, 44, 45]. Furthermore, amyloid beta peptides in Alzheimer disease plaques show racemization at Ser as well as Asp [14], and the amyloid beta peptide 1–42 generates hydroxyl radicals during incubation under physiological conditions [46]. Fujii et al. [5, 47] reported that site-specific racemization and isomerization at Asp151 in αA-crystallin of the lens protein in rat and bovine are related to UV-B irradiation or gamma-irradiation which might be relevant to the generation of hydroxyl and hydrogen radicals. Notably, in bovine αA-crystallin, the isomerization is reported to increase but the racemization decreases at Asp151 [47], which could suggest that racemization is independent of succinimide formation.

In this study, we experimentally elucidated that the specific racemization of Asn127 occurred without formation of succinimide, the typical obligatory intermediate for racemization, in ML after incubation for 8 weeks in phosphate buffer at pH 7 and 37 °C. To date, it has commonly been accepted that the racemization at Asn in a protein under physiological conditions was accompanied with the formation of Asp, through the deamidation of Asn. The present finding may lead us to pay more attention to the racemization at Asn in proteins. This may contribute to a better understanding of aging disorders at the atomic level. As for the biological significance of the racemization: it has been found in amyloid beta peptide in senile plaques of Alzheimer patients [14, 15] and in αA-crystallin of lens protein in rat and bovine [5, 47]. According to the study on amyloid beta peptide, racemization is related to the protein aging process and conformational disease, depending on the modified product and intermediate of the reaction [15, 42, 48]. Furthermore, modification of an amino acid residue in a protein has been suggested to cause an immunological response [1]. So far, there has been no report on the effect of the racemization in a protein on the immunological response. Since the present racemization occurred under physiological conditions, we are interested in the effect of the racemization on autoimmune disease. The investigation is in progress in our laboratory.

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Case report

Successful therapy with argatroban for superior mesenteric vein thrombosis in a patient with congenital antithrombin deficiency

Muta T, Okamura T, Kawamoto M, Ichimiya H, Yamanaka M, Wada Y, Urata M, Kayamori Y, Hamasaki N, Kato K, Eto T, Gondo H, Shibuya T. Successful therapy with argatroban for superior mesenteric vein thrombosis in a patient with congenital antithrombin deficiency. Eur J Haematol 2005: 75: 167–170. © Blackwell Munksgaard 2005.

Abstract: A 38-year-old woman was admitted with superior mesenteric vein (SMV) thrombosis, which was refractory to anticoagulation therapy. The plasma antithrombin activity was decreased and hardly compensated by concentrated antithrombin preparation due to high consumption rate. However, successful anticoagulation was achieved by administration of direct thrombin inhibitor, argatroban. Family studies of antithrombin activity revealed that she had type I congenital antithrombin deficiency. A novel heterozygous mutation in the gene for antithrombin (single nucleotide T insertion at 7916 and 7917, Glu 272 to stop in exon 4) was identified. Argatroban administration would be effective in the treatment of congenital antithrombin deficiency with SMV thrombosis.

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Key words: congenital antithrombin deficiency; superior mesenteric vein thrombosis; argatroban

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It was recently reported that asymptomatic congenital antithrombin deficiency has a prevalence of 1 in 600 in the general population (1). Such deficiency underlied 3–5% in patients with thrombotic diseases (2). Superior mesenteric vein (SMV) is an occasional site of thrombotic manifestation in congenital antithrombin deficiency. The SMV thrombosis causes a short bowel syndrome (3) and sometimes proceeds to a fatal outcome (4).

We describe a congenital antithrombin deficiency patient with SMV thrombosis, who was successfully treated with a direct thrombin inhibitor, argatroban, in combination with concentrated antithrombin preparation.

Case description

A 37-year-old woman was admitted to the Department of Surgery of our hospital due to severe abdominal pain, while she was receiving fertility treatments with a combination of ethinil-estradiol and norgestrel in April 2004. She was diagnosed with SMV thrombosis using computed tomography and magnetic resonance angiography. Intestinal congestion caused extreme swelling of the jejunal wall, resulting in obstructive ileus. Acute pancreatitis and massive ascites were also accompanied. She received continuous infusion of heparin (1200 IU/h), gabexate mesilate (FOY) (1.6 mg/kg/h), and

urokinase (240 000 U/d). The antithrombin activity in the plasma was markedly decreased to 34% (normal, 70–125%), so concentrated antithrombin preparation derived from normal human plasma (Neuart®, Mitsubishi Pharma Co., Tokyo, Japan) was administered at a dose of 60 U/kg/d for five consecutive days. The plasma levels of protein C and protein S activities were within normal limits. Lupus anticoagulant or anticardiolipin antibody was not detected. Her symptoms were alleviated and warfarin was instituted to prevent thrombosis. Heparinization kept an adequate activated partial thromboplastin time (aPTT) between 40 and 100 s. In addition, the thrombo test was controlled around 20% in the short period of warfarinization. However, thrombosis recurred and she developed hypovolemic shock in May. C-reactive protein (CRP) elevated up to 34 mg/dL, and her antithrombin activity markedly decreased to 16.8%. Heparin, FOY, and urokinase were restarted. Soon after administration of concentrated antithrombin preparation, plasma antithrombin activity was elevated to 85.5% but quickly decreased 45.6%. Severe abdominal pain continued and CRP was re-increased. The consumption rate of antithrombin was too fast to control the activity of thrombosis, so argatroban was started at the dose of 1 μg/kg/min and she was referred to our department. Since the consumption rate of antithrombin was too high to control the activity of thrombosis, argatroban was instituted at a dose of 1 μg/kg/min (Fig. 1) after informed consent was obtained. Argatroban was administered to keep the aPTT between 37 and 60 s, although the aPTT was increased transiently. Thereafter, clinical findings gradually improved. The plasma levels of plasmin-α2-plasmin inhibitor complex (PIC), thrombin-antithrombin complex (TAT), d-dimer, and CRP decreased below the estimation limit. However, the plasma activity and the plasma concentration of antithrombin were still decreased to 35.4% and 9.7 mg/dL (normal, 23.6–33.5 mg/dL), respectively. Family studies revealed that her mother had received the treatment of cerebral infarction, and her sister had a history of deep vein thrombosis of the leg during pregnancy. The plasma activity and the antigen concentration of antithrombin of her mother were 60.4% and 16.9 mg/dL, her sister 49% and 12.2 mg/dL, and her nephew 51.4% and 15.7 mg/dL, respectively. The DNA sequence analysis of the patient was performed using the methods described elsewhere (5, 6), which demonstrated a novel insertion mutation, CTG to CTTG at nucleotide positions 7916–7917 in one allele of exon 4 in the antithrombin gene (Fig. 2). This mutation caused deletion of the C-terminal domain of antithrombin and loss of thrombin binding domain. There were no other alterations in the exons and exon–intron boundaries of this gene. As a result, the patient was diagnosed as a heterozygote for type I congenital antithrombin deficiency. Argatroban was administered for 20 d and then replaced by warfarin. When we stopped the administration of argatroban, we introduced

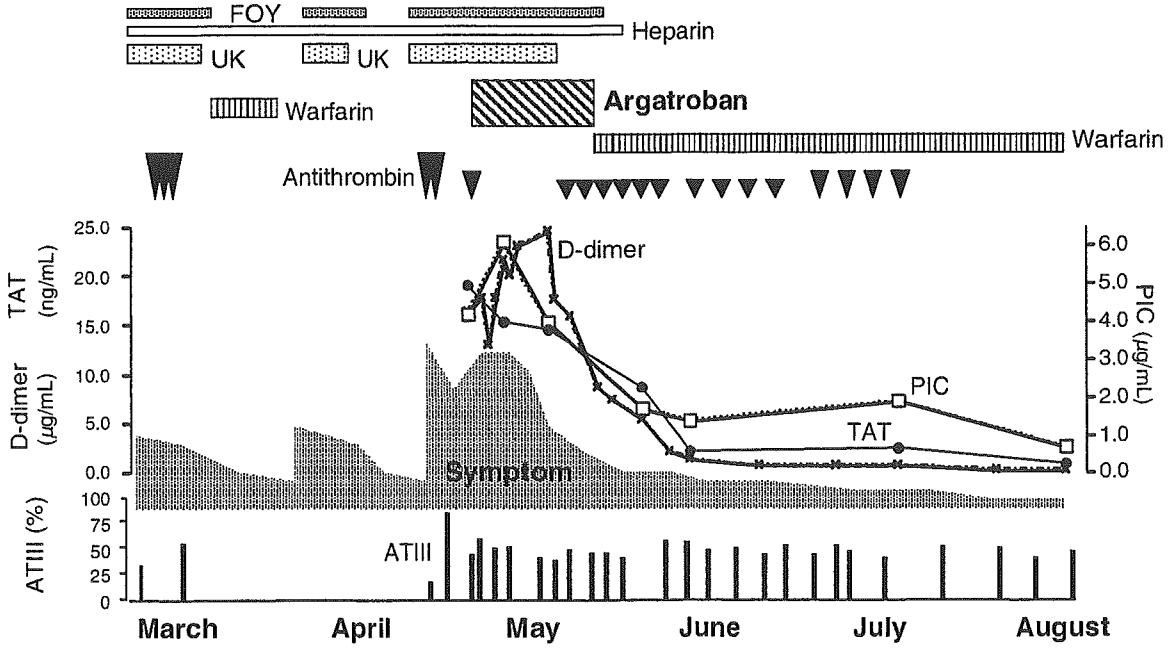

Fig. 1. Clinical course. TAT, thrombin-antithrombin complex; PIC, plasma-α2-plasmin inhibitor complex; FOY, gabexate mesilate; UK, urokinase; antithrombin, concentrated antithrombin preparation; ATIII, antitrhombin activity (trough).

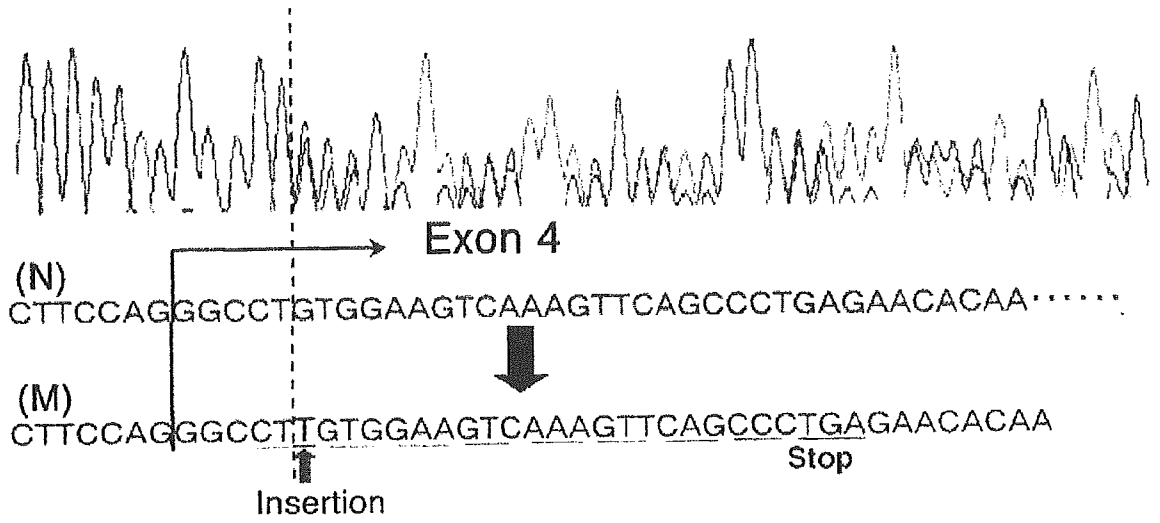

Fig. 2. Direct sequence analysis of the antithrombin gene demonstrated an insertion of T at nucleotide positions 7916–7917 in one allele of exon 4, which caused frameshift and changed the codon 272 to stop. The blue curve indicated the nucleodide 'C', the red curve 'T', the black curve 'G', and the green curve 'A'. The upper line (N) corresponded to the normal allele and the lower line (M) indicated the mutational allele.

concentrated antithrombin preparation at a dose of 30 IU/kg to keep the antithrombin activity up to 70%. Two months later, concentrated antithrombin preparation was combined and she received jujunal anastomosis for obstructive ileus. Thrombosis did not recur by prophylactic administration of warfarin alone thereafter. She discharged with a perfect performance status.

Discussion

Argatroban is a synthetic direct thrombin inhibitor that specifically but reversively binds to the catalytic site of thrombin (7). Argatroban can bind and exert an effect on both free thrombin and fibrin clot-bound thrombin (7). Argatroban is an effective drug for the treatment of acute cerebral infarction (8) and peripheral arterial obstructive disease (9). As for a patient with congenital antithrombin deficiency, argatroban is used during dialysis (10) to prevent clotting in the extracorporeal circuit. In a case report, argatroban was effective for SMV thrombosis caused by acquired antithrombin deficiency due to heavy alcohol consumption (11).

In our patient, a novel point mutation was demonstrated in the gene for antithrombin, which had not previously been reported in the database (12) or in other reports (13–19). Our patient maintained an innate half dose of functional antithrombin, and did not have a history of thrombosis from birth. Fertility treatments with estradiol and norgestrel might have triggered the thrombosis as indicated in the literature (20, 21). The third thrombotic episode was quite severe, and a life threatening. Although a high dose of concentrated antithrombin preparation was introduced, the plasma antithrombin activity promptly decreased and we could not control the progression of thrombosis. However, argatroban administration led to a rapid improvement of thrombosis. Concentrated antithrombin preparation was less needed to keep antithrombin activity. Argatroban may have delay antithrombin consumption and increase antithrombin activity, as suggested by Matsuo *et al.* (7). As for adverse effects of argatroban in our patient, serum levels of transaminase, ALP and gamma GTP were slightly elevated, which did not require specific treatments or discontinuation of argatroban administration. In the literature, 1–3% of patients showed liver dysfunction as adverse events (7).

Our patient indicates that argatroban administration should be considered in congenitally antithrombin-deficient patients complicated with thrombosis who may require immediate anticoagulation. More experience and trials are needed to define the efficacy and safety of argatroban in congenital antithrombin deficiency complicated with thrombosis.

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Detection of Point Mutations in the HBV Polymerase Gene Using a Fluorescence Intercalator in Reverse Micelles

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We report a novel and simple method for mutation detection in DNA oligonucleotides using a double-stranded DNA specific dye (SYBR Green I) in nanostructured molecular assemblies, called reverse micelles. The intercalation of SYBR Green I into the duplex DNA exhibits fluorescent emission in a CTAB/isooctane reverse micellar system as well as in an aqueous solution. We found marked differences in the fluorescence intensity between perfectly matched and mismatched 52-mer synthetic oligonucleotides, which were designed to contain the YMDD motif of the hepatitis B virus (HBV) polymerase gene, in a reverse micellar solution. Using this method, we successfully detected a mutation in PCR-amplified oligonucleotides of the HBV polymerase gene in sera of four patients with chronic hepatitis B. This detection method does not require DNA immobilization, chemical modification of DNA, or any special apparatus; it only needs a normal fluorescence spectrophotometer, an inexpensive dye, and just 10 pmol of sample DNA.

Introduction

The great progress in genomic studies has demonstrated that variation in the canonical sequence of the human genome causes genetic diseases and genetically influenced traits (*1*). These studies direct our attention to polymorphisms in the human genome. The reliable detection of single nucleotide polymorphisms (SNPs) in individuals is of great importance both clinically and in the biological sciences. SNPs exist not only in the human genome but also in the genes of pathogenic bacteria and viruses. SNPs in viral and bacterial genes sometimes reduce the effectiveness of drug treatments. Extensive study into SNP detection methods has resulted in a number of different approaches (*2*–*11*), some of which are already commercially available. Most employ DNA-immobilized chips (DNA microarrays) with fluorescent or electrochemical labels to track the different amounts of DNA hybridization (*7*, *9*). However, DNA immobilization and fluorescent labeling are often complicated procedures and sometimes require specific apparatus that can be unfamiliar to clinicians. Furthermore, in practical SNP analysis the amount of sample DNA is severely limited. An ideal detection method for SNPs would be simple and fast, use a very small amount of DNA sample, and have high sensitivity.

SYBR Green I is a double-stranded DNA (ds-DNA) specific fluorescent dye (*12*) that is widely used for DNA staining (*13*, *14*). We previously reported a simple method for mutation detection in synthetic DNA oligonucleotides using intercalation of SYBR Green I in an aqueous solution (*15*). We also demonstrated that nanostructural molecular assemblies, called reverse micelles, amplify the difference in the thermal stability between the perfectly matched and the mismatched DNA duplexes (*16*, *17*). The reverse micelles provide nanosized water pools surrounded by an organic solvent, in which a small amount of DNA molecules can be dissolved at a high concentration (*18*). Our previous reports imply that a mismatched base-pair in a DNA duplex could easily be detected using the intercalation of SYBR Green I into a DNA duplex in reverse micelles by fluorescence measurement (Figure 1). Based on this concept, we investigated the applicability of the ds-DNA specific fluorescent dye, SYBR Green I, in the reverse micelles for the detection of mismatch base-pairs in DNA duplexes. A 52-mer nucleotide containing the YMDD motif of the hepatitis B virus (HBV) poly-

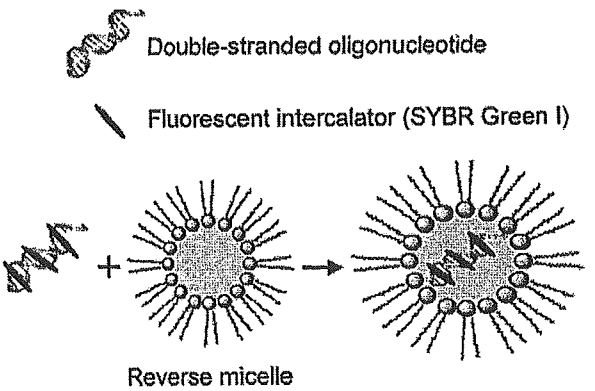

Figure 1. Concept of the SNP detection using the intercalation of SYBR Green I in reverse micelles.

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Table 1. Sequences of Synthetic Probe and Target Oligonucleotides

probe	5′-CATCCATATA-3′
target perfectly matched	3′-CTGAACCGGGGGTTATGGTGTAGTAGGTATATTGACTTTCGGTTTCGGTTTGTCACCC-5′
target C−T mismatched	3′-CTGAACCGGGGGTTATGGTGTAGTAGTTATATTGACTTTCGGTTTCGGTTTGTCACCC-5′
target C−A mismatched	3′-CTGAACCGGGGGTTATGGTGTAGTAGATATATTGACTTTCGGTTTCGGTTTGTCACCC-5′
target C−C mismatched	3′-CTGAACCGGGGGTTATGGTGTAGTAGCTATATTGACTTTCGGTTTCGGTTTGTCACCC-5′
target G−T mismatched	3′-CTGAACCGGGGGTTATGGTGTAGTAGGTGTATTGACTTTCGGTTTCGGTTTGTCACCC-5′

merase gene, taken from patients with chronic hepatitis B and amplified by PCR, was used as the model sequence for SNP detection.

The (−) enantiomer of 3′-thiacytidine (lamivudine) has been found to be a potent inhibitor of HBV (*19*, *20*). The emergence of lamivudine-resistant hepatitis B virus (HBV) was reported in patients with prolonged lamivudine administration (*21*). The majority of lamivudine resistant mutants isolated from such patients have mutations within the YMDD motif of the HBV-DNA polymerase gene (*22*). The simple fluorescence measurement of SYBR Green I in the reverse micelles allowed us to distinguish a mutated HBV polymerase gene DNA sequence from the wild-type.

Materials and Methods

Materials. SYBR Green I was purchased from TaKaRa Bio Co., Shiga, Japan. Surfactants, sodium di-2-ethylhexyl sulfosuccinate (AOT) and cetyl trimethylammonium bromide (CTAB), were purchased from Aldrich Co. (Milwaukee, WI). Another surfactant, tetraethylene glycol dodecyl ether (TGDE), was obtained from Wako Pure Chemicals Ltd., Osaka, Japan. All other chemicals were obtained from commercial suppliers and were of the highest purity available. Probe and target oligonucleotides were synthesized and purified using reversed-phase chromatography by Hokkaido System Science Co. Ltd (Sapporo, Japan). Probe and target oligonucleotides were typically 10-mers and 52-mers, respectively. The perfectly matched target sequence was a 52-mer containing the YMDD motif of the HBV polymerase gene, which included the base likely to be mutated by prolonged lamivudine administration (Table 1). Mismatched target sequences were designed so they had a single-base mismatch (underlined in Table 1) in the perfectly matched sequence. The extinction coefficients of the oligonucleotides were calculated by the software (http://paris.chem.yale.edu/extinct.frames.html). Based on the calculated values, the concentrations of these oligonucleotides were determined by absorbance at 260 nm.

Fluorescence Measurement of a Reverse Micellar Solution Containing Synthetic DNA Oligonucleotides and SYBR Green I. Probe and target DNA oligonucleotides (50 pmol of each) were dissolved in a Tris-HCl buffer (pH 8.0, 10 mM) containing 1 mM EDTA and 0.002 vol % SYBR Green I (i.e., commercially available SYBR Green I solution was diluted 50,000-fold) at 30 °C. The probe and target DNA hybridized to each other and SYBR Green I intercalated between the hybridized oligonucleotides. The resultant solution (3.6 μL) was injected into an *n*-octane solution (0.9964 mL) containing 10 mM cetyl trimethylammonium bromide (CTAB, surfactant) and 5 vol % hexanol. The reverse micellar solution was mixed for several seconds, followed by ultrasonication for several seconds to form a reverse micellar solution containing oligonucleotide duplexes with SYBR Green I. The W_0 (= $[H_2O]$/[surfactant]) was 20. The fluorescence of the reverse micellar solution was then measured at 30 °C using a luminescence spectrometer LS 50B (Perkin-Elmer). Excitation and emission wavelengths were 494 and 530 nm.

To simplify the data analysis and to obtain good reproducibility, an increase of the fluorescence intensity (ΔF) at 530 nm by the intercalation of SYBR Green I into ds-DNA was defined as

$$\Delta F = \frac{F_{ds} - F_{ss}}{F_{ss}} \times 100\ [\%]$$

where F_{ds} is the fluorescence intensity of ds-oligonucleotides, and F_{ss} is the sum of the fluorescence intensity of a single-stranded target and probe oligonucleotides.

As a control experiment, the fluorescence measurement of intercalated SYBR Green I was performed in an aqueous solution. Probe and target DNA oligonucleotides (each 50 pmol) were dissolved in a Tris-HCl buffer (pH 8.0, 10 mM) containing 1 mM EDTA and 0.002 vol % SYBR Green I. The resultant solution (3.6 μL) containing the hybridized DNA was diluted with 0.9964 mL of a Tris-HCl buffer (pH 8.0, 10 mM) and the fluorescence was measured.

PCR Amplification of the Hepatitis B Virus Polymerase Gene from Patients with Chronic Hepatitis B. Samples of venous blood were obtained from four patients with chronic hepatitis B in Kyushu University Hospital, Fukuoka, Japan. The patients had received lamivudine treatment for different periods. Serum samples of 200 μL were mixed with 200 μL of Tris-HCL buffer (pH 8.0, 20 mM) containing 1 mM EDTA, 1% w/v SDS and proteinase K (0.1 g/L). The mixture solution was incubated at 70 °C for 3 h, followed by centrifugation at 30,000 rpm for 5 min. The supernatant was extracted with phenol to remove contaminants and the DNA was precipitated from the supernatant by the addition of ethanol. The precipitated DNA was used as a template for the first step PCR amplification. The sequences of PCR primers were 5′-TCGTGTTACAGGCGGGGTTT-3′ and 5′-CGAACCACTGAACAAATGGC-3′. PCR amplification in the first step was performed in a total volume of 100 μL containing Ex Taq polymerase (0.5 μL), 10 × Ex Taq buffer (10 μL), dNTPmix (10 μL) and 5 μL of DNA sample. The denaturation and extension steps were at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s for 35 cycles. The obtained PCR product was used as a template for the second step PCR. In the second PCR, the primers were biotinyl-5′-CCCACTGTCTGGCTTT-3′ and biotinyl-5′-GACTTGGCCCCCAATA-3′ (called R2-AS). The second step PCR amplification was performed in a total volume of 100 μL containing Ex Taq polymerase (0.5 μL), 10 × Ex Taq buffer (10 μL), dNTPmix (10 μL), primers (20 pmol/μL, 2.5 μL) and 5 μL of DNA sample. The denaturation and extension steps were at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s for 25 cycles. The second PCR product was gently mixed with avidin-immobilized gel beads for 30 min. After washing with phospate buffer saline, NaOH (0.1 M, 160 μL) was added to the gel beads. After mixing for 30 min, the suspension was centrifuged at 12,000 rpm for 3 min and neutralized with HCl. The resultant solution was subjected to gel permeation chromatography using Sephadex G-50. After desalination, the sample solution was freeze-dried. The sequence of the

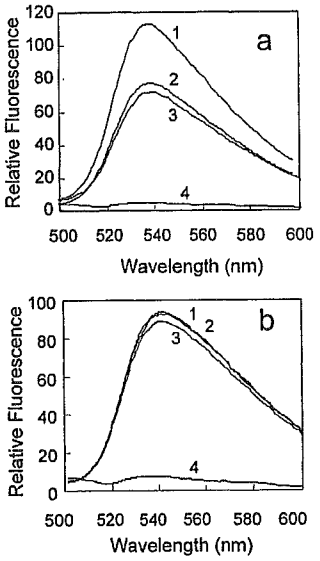

Figure 2. Fluorescence spectra of SYBR Green I intercalated into double-stranded targets in the reverse micelles (a) and in an aqueous solution (b). The reverse micellar solution consisted of *n*-octane containing 10 mM CTAB and 5 vol % 1-hexanol and of Tris-HCl (pH 8.0, 10 mM) containing EDTA 1 mM, 0.002 vol % SYBR Green I, 52-mer probe and 52-mer target DNAs (each 50 nM). W_o was 20. The aqueous solution (b) was a Tris-HCl buffer (pH 8.0, 10 mM) containing EDTA 1 mM, 0.002 vol % SYBR Green I, 52-mer probe and 52-mer target DNAs (each 50 nM). The fluorescence measurements were carried out at 30 °C. Spectrum 1 was the perfectly matched duplex; 2 was the C−T mismatched duplex; 3 was the single-stranded target; and 4 was the single-stranded probe.

PCR product was confirmed by direct sequencing using a primer (5′-GTACAAAACCTATGGACG-3′).

The PCR amplification provided approximately 12.5 pmol of the 52-mer target oligonucleotides. In the assay, 10 pmol of target and 20 pmol of probe were hybridized. The fluorescence of the PCR products was measured as described above after they were dissolved in the reverse micellar solution with SYBR Green I.

Results and Discussion

Fluorescence of SYBR Green I in the Reverse Micelles and in an Aqueous Solution. SYBR Green I intercalated into ds-DNA exhibits strong fluorescence emission at 530 nm with excitation at 494 nm. Figure 2a depicts the fluorescence spectra of SYBR Green I intercalated into target oligonucleotides in the reverse micellar solution consisting of CTAB and *n*-octane. The perfectly matched target DNA hybridized with the probe had high fluorescence intensity compared to that of the single-stranded target and the single-stranded probe oligonucleotides. The presence of a C−T mismatch in the target oligonucleotide obviously reduced the fluorescence intensity. The reduced fluorescence was similar to that of the single-stranded target oligonucleotide. Our previous studies revealed that the reverse micelles were stringent for DNA hybridization and that the reverse micelles induced the marked difference in the melting temperature (T_m) between the perfectly matched and mismatched DNA duplexes (*16*, *17*). These results mean that the probe and perfectly matched target oligonucleotides kept the duplex form in the reverse micelles, and that the probe and single-mismatched target oligonucleotides did not retain a stable duplex in the reverse micelles, probably due to the high stringency of the nanostructured reverse micelles. Under the same conditions, the fluorescence measurement was performed in an aqueous solution (Figure 2b). No obvious difference was found in the fluorescence intensity between the perfectly matched and mismatched oligonucleotides. The change in the thermal stability of DNA duplexes caused by one mismatched base-pair is usually quite small in an aqueous solution, corresponding to as little as 0.5 °C in the melting temperatures (*23*−*25*). Thus under the present experimental conditions, the single-base mismatched target oligonucleotides formed a duplex with the probe as did the perfectly matched target. SYBR Green I intercalated into both the perfectly matched and mismatched duplexes in a similar manner, which resulted in virtually identical fluorescence spectra. In contrast, the reverse micelles provide a highly stringent environment for DNA hybridization (*16*, *17*). The high stringency of the reverse micelles destabilized the single-base mismatched duplex and induced a marked change in the thermal stability of the mismatched double-strand compared to the perfectly matched one. That is why we observed the obvious difference in the fluorescence intensities of SYBR Green I between the perfectly matched and mismatched oligonucleotides.

Selection of Surfactant and Cosurfactant for the Reverse Micelles. The surfactant and cosurfactant are important factors determining the properties of the reverse micelles. To date, AOT (anionic), CTAB (cationic) and tetraethylene glycol dodecyl ether (nonionic) have been reported for use as surfactants for solubilizing DNA in reverse micelles (*18*, *26*−*28*). Here we also examined these surfactants for forming the reverse micelles. SYBR green I did not exhibit enough fluorescence intensity in the reverse micelles based on AOT (data not shown). The reverse micelles based on tetraethylene glycol dodecyl ether, provided similar fluorescence intensity as that found with CTAB, but the reproducibility was not satisfactory. SYBR Green I showed high fluorescence intensity and good reproducibility in the CTAB-based reverse micelles.

Alcohols are well-known as good cosurfactants for stabilizing reverse micelles (*29*, *30*). In the present study, 1-butanol, 1-pentanol and 1-hexanol were examined. 1-Hexanol (5 vol %) resulted in the highest fluorescence intensities and in the marked difference in the fluorescence intensity between the perfectly matched and mismatched oligonucleotides (data not shown). It should be noted that the stable reverse micelles did not form at a 1-hexanol concentration below 4 vol %. The following experiments were conducted using CTAB and 1-hexanol as the surfactant and cosurfactant for the reverse micelles.

Effect of W_o and Surfactant Concentration on the Fluorescence of SYBR Green I in the Reverse Micelles. The size of the water pools in the reverse micelles plays a key role in their stability and in the solubilization of biomolecules. In discussing the size effect of reverse micelles, we often use the parameter W_o, which is defined as the concentration ratio of water to a surfactant, being proportional to the size of the water pools (*31*). Figure 3a shows the fluorescence intensity of SYBR Green I in the reverse micelles as a function of W_o. A W_o of 20 provided the highest intensity. A W_o of 20 corresponds to a reverse micelle with a diameter of 10 nm. The physical length of a 52-mer duplex is 17.7 nm, therefore it was supposed that the duplex was bent in the reverse micelles. Increasing the W_o over 25 destabilized the reverse micellar solution. A smaller W_o reduced the fluorescence intensity, suggesting that the small size

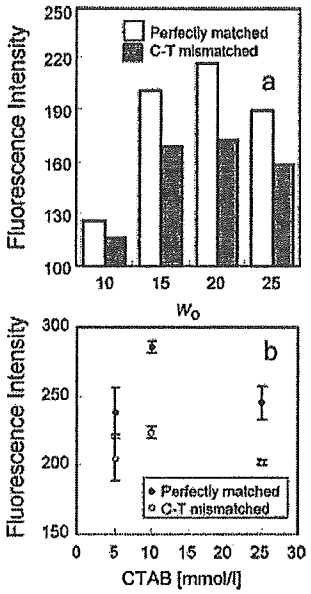

Figure 3. Effects of W_o (a) and CTAB concentration (b) on the fluorescence intensity of SYBR Green I in the reverse micelle. The reverse micellar solution consisted of 0.9964 mL *n*-octane containing CTAB and 5 vol % 1-hexanol and of 0.036 mL Tris-HCl buffer (pH 8.0, 10 mM) containing EDTA 1 mM, 0.02 vol % SYBR Green I, 52-mer probe and 52-mer target DNAs (each 50 nM). The fluorescence measurements were carried out at 40 °C. (a) The CTAB concentration was 25 mM and W_o was 20. (b) W_o was 20.

of the reverse micelles prevented the duplex formation of probe and target oligonucleotides. The surfactant concentration was also investigated (Figure 3b). High fluorescence intensity with good reproducibility was obtained using 10 mM CTAB. Subsequent experiments were carried out at W_o 20 using 10 mM CTAB.

Effect of Sequence Length of the Probe Oligonucleotide on the Mismatch Detection. The sequence length of the probe oligonucleotide is proportional to its cost, therefore a shorter probe is desirable in practical terms. We examined the fluorescence of SYBR Green I in the reverse micelles using probes with varied sequence lengths. The longer the probe length was, the higher the fluorescence intensity was. Although the presence of a mismatched base-pair was detected by fluorescence measurement for all probe length used, the probe with the shortest sequence length allowed us to easily discriminate the fluorescence intensity of the mismatched oligonucleotide from that of the perfectly matched one (Figure 4). The duplex of the target oligonucleotide with the shortest probe had a smaller region of duplex than that with the longer probes. Since SYBR Green I was intercalated mainly into the duplex region, the effect of a single-base mismatch in the duplex on the fluorescence intensity was larger in the shortest duplex than in the longer ones.

Mismatch Detection in Target Oligonucleotides Using the Fluorescence of SYBR Green I in the Reverse Micelles. Based on the above optimization of the fluorescence measurement, we investigated the detection of mismatches in synthetic target oligonucleotides using the reverse micelles. To enhance the difference in the fluorescence intensity between the perfectly matched and mismatched oligonucleotides, an increased rate of the fluorescence intensity (ΔF) was defined as a mismatch indicator. Figure 5a summarizes the results of the mismatch detection in the synthetic target oligonucleotides. The transformation of the fluorescence intensity to ΔF allows the obvious discrimination of the mismatched oligonucleotides from the perfectly matched one. Three types of mismatches, C−A, C−T and C−C, exhibited considerably lower ΔF values, but the T−G mismatch showed only a slightly lower ΔF than the perfectly matched one. The G−T mismatch generally forms a Wobble base pair through double hydrogen bonds, which while not as stable as a Watson−Crick base pair, is stable enough to make it difficult to detect the mismatch (32). We concluded that all the types of mutations in the YMDD motif could be detected using the intercalation of SYBR Green I in the reverse micelles.

Finally, we attempted the mismatch detection in the PCR products (52-mer) of HBV polymerase gene from the sera of four patients with chronic hepatitis B, using reverse micelles. Although four types of mutations in HBV have been reported in the world (A739G, G741T, G741C and G741A) (21), only the G741T mutation of

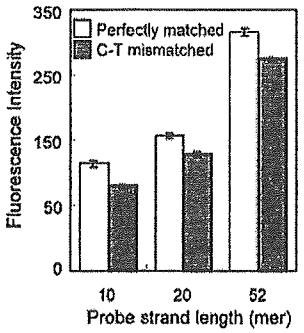

Figure 4. Effect of sequence length of the probe DNA on the fluorescence intensity of SYBR Green I in the reverse micelles. The reverse micellar solution consisted of 0.9964 mL *n*-octane containing 10 mM CTAB and 5 vol % 1-hexanol and of 0.036 mL Tris-HCl buffer (pH 8.0, 10 mM) containing EDTA 1 mM, 0.02 vol % SYBR Green I, probe and 52-mer target oligonucleotides (each 50 nM). The fluorescence measurements were carried out at 30 °C.

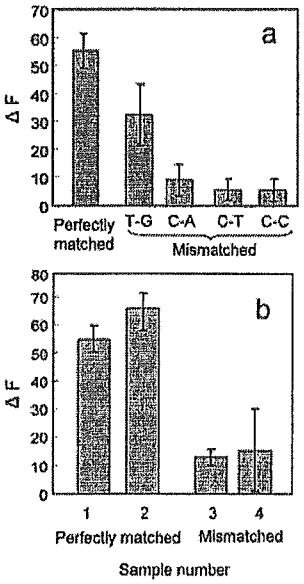

Figure 5. (a) Increased rate of fluorescence intensity induced by synthetic ds-oligonucleotides including various types of single-base mismatches. (b) Detection of a mismatch in the PCR products from patients with hepatitis B using the fluorescence measurement of SYBR Green I in the reverse micelles. Probe DNA (10-mer, 20 pmol) was used.

HBV was found in chronic hepatitis B patients with prolonged lamivudine administration in the Kyushu University Hospital. Two of the four patients possessed wild-type HBV and the others possessed mutated HBV (G741T). In Figure 5b, sample numbers 1 and 2 were the wild-type HBV, and sample numbers 3 and 4 were the G741T mutated HBV (i.e., C−T mismatched). Marked differences were observed in ΔF between the wild type and the mutant. We successfully detected the mutation in the PCR products (10 pmol of DNA sample) of the patients utilizing the fluorescence change of the double-helix specific intercalator SYBR Green I in reverse micelles.

Acknowledgment

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REGULAR ARTICLE

Characterization of two novel mutations of the antithrombin gene observed in Japanese thrombophilic patients

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Structure and function of antithrombin;
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Deep vein thrombosis, 20210A, factor V Leiden

Abstract We investigated the molecular basis of reduced functional levels of antithrombin (AT) in two individuals suffering from thromboembolic events. In each case direct sequencing of amplified DNA revealed 13,260–13,262 del in one patient and 2511C>A in the other patient, predicting a heterozygous E381del and P16H, respectively. Both patients had no 20210A allele and factor V Leiden mutation. To understand the molecular mechanism responsible for antithrombin deficiency, stable expression experiments were performed using HEK293 cells transfected with the expression vector containing the wild-type or the mutated recombinant cDNA. In these experiments, the media levels of the two mutated antithrombins were the same as that of wild type, but the specific activity of the E381del mutant decreased significantly compared with that of wild type. These results showed that the E381del mutation was responsible for type II deficiency, whereas the other mutation, P16H, did not produce any definite abnormality which could contribute to antithrombin deficiency.

Abbreviations: AT, antithrombin; PS, protein S; PC, protein C; PT, prothrombin time; APTT, activated partial thromboplastin time; FDP, fibrin degradation product; TAT, thrombinantithrombin complex; PIC, plasmin–plasmin inhibitor complex; HEK293, human embryo kidney 293 cells.

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Antithrombin (AT) is a 58-kDa plasma glycoprotein which is mainly synthesized in hepatocytes and circulates at a concentration of approximately 112–140 mg/l with a half-life of 2 to 3 days [1,2]. It is composed of 432 amino acid residues with four Asn-linked glycosylation sites [3,4]. AT belongs to the serine proteinase inhibitor (serpin) superfamily and plays a critical role in the regulation of the blood coagulation system by inhibiting the final two proteinases of factor Xa and thrombin [2,5–7]. Inhibition of proteinases by AT involves interaction between the active site of the proteinase and the reactive center loop of the inhibitor, which leads to an inactive and stable proteinase–inhibitor complex [8]. The reactive center of AT is held on an exposed peptide loop that extends from 15 residues (P15) on the amino-terminal side of the P1 residue (Arg393) to five residues (P5′) on the carboxy-terminal side [6–9]. After thrombin cleaves the reactive site of Arg393–Ser394, AT undergoes a dramatic conformational change when the cognate proteinases interact directly with the reactive center loop [6–11]. The reactive center loop is incorporated as strand four in the central β-sheet A when the loop interacts with the proteinases, and induces the massive conformational change of AT [8,12]. Inhibition of thrombin by AT is enhanced at least 1000-fold in the presence of heparin [2,13]. The human AT gene is mapped to chromosome 1q23.1–23.9 and comprises seven exons and six introns spanning a total of 13.5 kb of genomic DNA [14].

Since the existence of AT deficiency was first reported in 1965 [15], there has been an increasing line of evidence that a molecular anomaly of AT is an integral risk factor for thrombosis [16–22]. Our laboratory has been directing efforts to identify the causal factors that generate thrombosis by performing a systematic haemostatic investigation [23]. With respect to the AT anomaly, to date, all the coding exons and intron–exon boundaries of the AT gene were analyzed in 6 out of 22 patients having reduced AT activity. Abnormalities of AT gene were detected in two out of six thrombotic patients, with one a deletion mutation and the other a missense mutation. In the present study, we report on the identification and characterization of two novel mutations of the AT gene.

Materials and methods

Patients

Patient 1: A 51-year-old male patient had acute arterial obstruction from a right common iliac artery to a right thigh artery. He had undergone an operation on his right leg to remove the blood clots and a plasty in the right thigh arteries. The patient had a medical history of deep vein thrombosis in both legs at the age of 38. However, there was no suggestion of a family history of thrombosis.

Patient 2: The patient was a 64-year-old female with deep vein thrombosis in the right leg induced by infectious arthritis. A filling defect and deterioration of blood flow in the right femoral vein were observed by venography. She recovered from the deep vein thrombosis by heparin/warfarin treatment for a month. She has been suffering from myeloma for 10 years and impaired function of the liver due to chronic hepatitis B. There was no suggestion of family history of thrombosis. We were unable to study the hematological profile of her family.

Subjects

The subjects consisted of the proband and his daughter in patient 1 and the proband in patient 2. In addition, 50 healthy individuals recruited from the employees of our institution were subjected to this study as normal controls. Prior to the trial, written informed consent was individually obtained from all of them by the attending physicians following full explanation of the aim of the research and guarantee of privacy.

Plasma

Peripheral blood samples collected in 3.13% sodium citrate were centrifuged at 1500×*g* for 20 min, and the resulting supernatant fraction was used to perform clot-based tests. Aliquots of supernatant fractions divided into smaller portions were stored at −80 °C for future use.

Haemostatic examination

The haemostatic profile involved measurements of AT, protein S (PS), protein C (PC), fibrinogen, plasminogen, α2-plasmin inhibitor, heparin cofactor II, lupus anticoagulant, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombotest, fibrin degradation product (FDP), thrombin–antithrombin complex (TAT), and plasmin–plasmin inhibitor complex (PIC) [24]. In addition, protein levels of AT and progressive AT activity were also assessed. AT activity was assayed by chromogenic substrate as heparin-dependent inhibition of bovine thrombin (heparin cofactor activity) using Testzym AT III 2 kit (Daiichi Kagaku, Tokyo, Japan). The reference interval ranged from 80% to 130%. AT

activity independent of heparin (progressive AT activity) was determined by chromogenic substrate after precipitation of fibrinogen by incubating at 56 °C for 15 min as previously described [23].

DNA extraction

Genomic DNA was extracted from peripheral lymphocytes collected from the patients, their relatives, and the healthy individuals using an automated DNA extraction device (NA-1000, KURABO, Osaka, Japan).

Polymerase chain reaction (PCR)

Early studies suggested that there were only six exons but subsequent analysis revealed a 1-kb intron within exon 3 [14]. Although we follow the old nomenclature of AT gene as consisting of exons 1–6, the primers cover all the exons from 1 to 7. Genomic regions of exons 1–6 of the AT gene were each amplified using appropriate primers in a reaction mixture containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM 4dNTPs, 0.2–0.4 μM primer-set, 0.8–4 ng/μl template DNA, and 0.025 U/μl Extaq (Takara Shuzo, Otsu, Japan). A reaction cycle consisting of sequential incubations for denaturation at 94 °C for 1 min, for annealing at 56–61 °C for 1 min, and for extension at 72 °C for 1 min was repeated twice with denaturation at 94 °C for an additional 4 min being included in the first cycle. Additionally, a reaction cycle consisting of sequential incubations for denaturation at 94 °C for 30 s, annealing at 56–61 °C for 30 s, and extension at 72 °C for 30 s was repeated 30 times, followed by incubation at 72 °C for 10 min. These reactions were performed in an automated device (Gene Amp PCR system 9600R, Roche Diagnostic Systems, Basel, Switzerland). Detection of the G20210A mutation in the prothrombin gene and factor V Leiden was performed as described by Finan et al. [25].

DNA sequencing

The PCR products derived from exons/introns of the AT gene were purified through a Micro Spin™ S-300HR column (Amersham Pharmacia Biotech, Bucks, UK) and processed for pretreatment using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The reaction products were purified through a Centri-Step Spin column (Perkin Elmer Applied Biosystems) and subjected to direct sequencing in an automated sequencer (ABI PRISM 377 sequencer, Perkin Elmer Applied Biosystems). To confirm the presence of mutated AT gene in patient 1, the PCR product of exon 6 was subcloned with the original TA cloning kit (Invitrogen, Carlsbad, CA, USA). For DNA sequencing, the insert DNA was amplified by the colony PCR method. The colony PCR products were then used for sequencing analysis.

Mutagenesis of AT cDNA

The full-length human AT cDNA was prepared from a human liver cDNA library (Uni-ZapRXR Library, Stratagene, CA, USA) by PCR using a mutagenic primer set (sense sequence of 5′-TGTCGACGATTAGCGGCCATGTATTC-3′ and antisense sequence of 5′-AACCCG GGAAGAGGTGCAAAG-3′, mutagenic C, C and CCC are underlined), by which new *Sal*1 and *Sma*1 sites are produced in the amino-terminal and carboxyl-terminal regions of the complete AT coding sequence, respectively. The PCR product was sequenced to check it had a proper sequence. An expression vector for the wild-type AT was constructed as follows by inserting the full-length AT cDNA into pC1neo Mammalian Expression Vector (Promega, WI, USA). The 1466-bp PCR product was restricted with *Sal*1 and *Sma*1 and then ligated to a 5466-bp *Sal*1–*Sma*1 restriction fragment of pC1neo Mammalian Expression Vector with T4 DNA ligase. The wild-type AT cDNA-vector construct was transformed into Epricurian Coli XL1-Blue supercompetent cells (Invitrogen). The sequences of DNA from the resulting colonies were verified to be correct by sequence analysis. Mutations were generated by the overlap extension method [26] using the wild-type AT cDNA-vector as a template. The final mutated PCR fragments were also inserted into pC1neo Mammalian Expression Vector as described above and the mutation was confirmed by sequencing of the resulting vector.

Stable expression of recombinant AT

Human embryo kidney 293 (HEK293) cells (Health Science Research Resources Bank, Osaka, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, USA) supplemented with 10% fetal calf serum (Gibco BRL), penicillin and streptomycin. After 11 μg of expression vector DNA and 3 μl/DNA μg of liposome reagent (Trans FastTM Transfection Reagent, Promega) were combined and incubated for 15 min at room temperature, the mixture was added to HEK293 cells

(3×105 per 60-mm dish). After 24 h, the medium was replaced with DMEM containing serum and antibiotics. After an additional 48 h, the transformed cells were selected in medium to which 1 mg/ml G418 (Gibco BRL) was added. The medium was replaced every 3 days. When the resistant cells were grown at about 50% confluency, the concentration of G418 in the medium was reduced to 0.6 mg/ml. After 2–3 weeks, the resistant cells were grown at 80–100% confluency and the medium was replaced with serum-free DMEM. After 48 h, the media were harvested and centrifuged at 1500×g for 5 min and the resulting supernatant fraction was stored in aliquots at −80 °C for future use.

Measurement of the activity and the antigen of recombinant AT in the culture medium

Assays of AT activity in the culture media were performed by an amidolytic assay using Testzym AT III 2 kit (Daiichi Kagaku). Assays of AT antigen level in the culture media were performed by Western blotting analysis. The supernatant of the culture media and purified plasma AT (Sigma, Missouri, USA) as a standard were electrophoresed on a 10% polyacrylamide gel for 60 min at 25 mA, and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was then immersed in the blocking buffer containing 50 mM Tris–HCl, 100 mM NaCl, 0.05% Tween 20 (pH 7.4) and 5% skim milk for 1 h, and incubated with goat anti-human AT antibody (1:6000) (Enzyme Research Laboratories, Indiana, USA) in the TBS–Tween buffer at 4 °C for 18 h. The membranes were then washed three times for 10 min each with the TBS–Tween buffer, and incubated for 1 h at room temperature with HRP anti-goat immunoglobulin (1:6000) (Amersham Pharmacia Biotech). The membranes were subsequently washed three times for 10 min each with the TBS–Tween buffer. Immunoreactive bands were visualized with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech), and measured using a luminoimage-analyzer (LAS-1000 plus, Fujifilm, Tokyo, Japan).

Results

Haemostatic examination

Patient 1: AT activity in the presence or absence of heparin in blood samples collected from the patient was nearly half of the normal level at 48% and 49%, respectively. The protein level was within the normal range at 117% (Table 1). All other analytes were within the normal range (Table 1). AT activity of the patient's daughter was within the normal range (data not shown).

Patient 2: AT activity in the presence of heparin and protein level of AT were nearly half of the normal level at 64% and 52%, respectively (Table 1). All other analytes were within the normal range with exception of the FDP and D-dimer, which were 0.0153 and 0.0062 g/l, respectively (Table 1).

Nucleotide sequence of the AT gene

Genomic DNA was extracted from peripheral blood cells of individuals. Genomic regions of exons 1–6 and their exon/intron junctions of the AT gene were amplified using appropriate primers and sequenced.

Patient 1: In direct sequence analysis of the AT gene of patient 1, additional aberrant peaks were observed from nt 13,260 in exon 6, suggesting heterozygous deletion or insertion mutation (Fig. 1). As a consequence of subcloning, deletion of AAG from nucleotide position 13,260 to 13,262, the mutation of which produces a deletion of Glu381, was detected in 7 out of 14 subclones. The remaining subclones were normal implying that the patient was

Table 1 Laboratory examination of the patient

	Reference interval	Patient 1	Patient 2
PT (%)	>70	51	72
APTT (s)	24.0–38.0	24.7	38.6
Fbg (g/l)	2.0–4.0	2.49	4.26
TBT (%)	>60	47	90
HPT (%)	60–120	55	87
PLG (%)	85–145	127	113
a2PI (%)	80–130	96	77
LAC	(−)	(−)	(−)
AT (%)	80–120	48	64
PS (%)	59–128	83	93
PC (%)	75–131	67	72
FDP (g/l)	0.0–0.005	0.0034	0.0153
PIC (g/l)	0.0–0.008	0.0003	0.001
D-D (g/l)	0.0–0.005	NT	0.0062
AT activity (%) Heparin (−)		49	NT
AT activity (%) Heparin (+)	80–120	48	64
AT antigen (%)	80–130	117	52

PT: prothrombin time; APTT: activated partial thromboplastin time; Fbg: fibrinogen; TBT: thrombotest; HPT: hepaplastintest; PLG: plasminogen; α2PI: α2 plasmin inhibitor; LAC: lups anticoagulants; AT: antithrombin; PS: protein S; PC: protein C; FDP: fibrinogen and fibrin degradation product; PIC: plasmin-α2PI complex; D-D: D-dimer; NT: not tested.

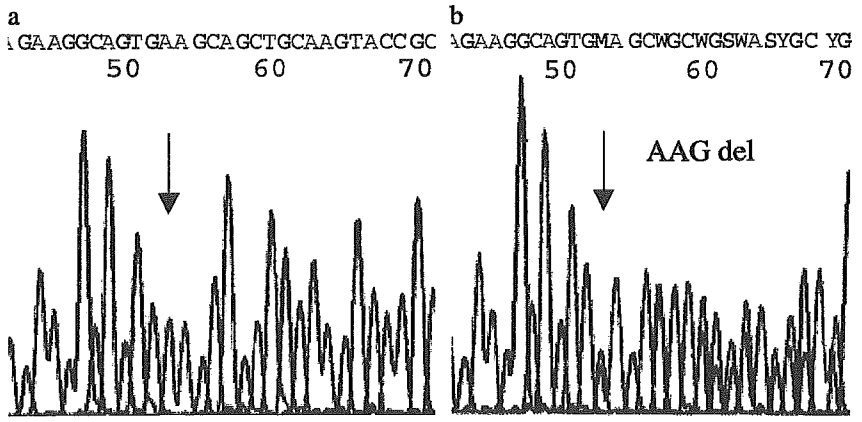

Figure 1 Nucleotide sequences of antithrombin exon 6 from patient 1. A deletion of AAG was observed in the patient's exon 6. (a) Normal control; (b) patient.

heterozygous for the deletion mutation. In his daughter, no mutations were detected in the AT gene.

Patient 2: Direct sequencing of the amplified exon 2 of patient 2 showed a cytosine to adenine transversion mutation at nucleotide position 2511 that converts proline-16 to histidine (P16H). The patient was heterozygous for the mutant (Fig. 2).

We analyzed genomic DNA from 50 healthy individuals for exon 2 and exon 6 of AT molecule to exclude the possibility of the detected mutations being polymorphisms (data not shown).

Secretion of wild-type and mutant ATs in stably transfected HEK293 cells

Wild-type and mutant ATs were expressed in cultured HEK293 cells to confirm whether the identified deletion mutation or amino acid substitution causes an AT deficiency. The AT activities and antigen levels in the culture supernatants were examined by an amidolytic assay and Western blotting analysis, respectively, and expressed as the concentrations of AT where purified plasma AT was used as a standard.

As shown in Fig. 3, both AT mutants of E381del and P16H were secreted normally from the transfected HEK293 cells to media as in the case of wild-type AT. The specific activities of wild-type and P16H mutant were 0.99±0.22 units/ng (mean±S.D., n=5) and 0.90±0.40 units/ng (mean±S.D., n=5), respectively, while the activity of E381del mutant was below a detectable level (Table 2).

Discussion

AT circulates in blood in an inactive form and becomes active upon association with glycosaminoglycans such as heparin and heparan sulphate which interact with the helix D region of AT [27,28]. The unusual long N-terminus region and the carbo-

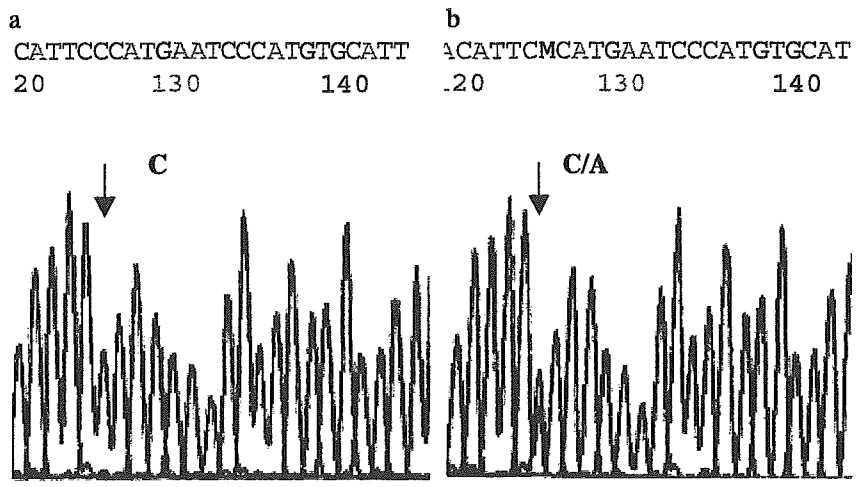

Figure 2 Nucleotide sequences of antithrombin exon 2 from patient 2. A substitution of C to A was observed in the patient's exon 2. (a) Normal control; (b) patient.

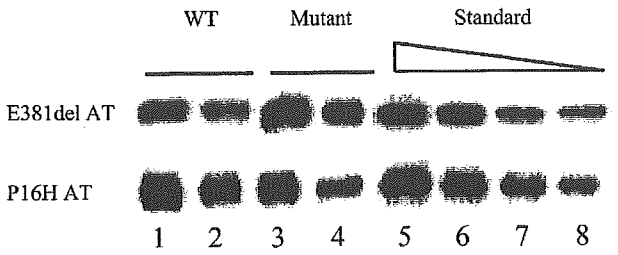

Figure 3 Immunoblot analysis of antithrombin in culture supernatant from E381del mutant (patient 1) under Reducing Conditions. Five microliters of culture supernatant (1:0—1:2 dilution) and standard plasma AT (1.8—14.3 ng of AT) was separated by SDS-PAGE (10% acrylamide) and the gels were electroblotted onto PVDF membranes. Antithrombin was stained with goat anti-human-antithrombin antibody (see Materials and methods). 1: wild; 2: wild (1:2 dilution); 3: mutant, 4: mutant (1:2 dilution); 5—8: standard plasma AT (14.3, 7.3, 3.7, 1.8 ng of AT, respectively).

hydrate attached at Asn135 are specific in AT among the serpin superfamily and affect the heparin binding to AT [8]. The effect of heparin binding to the helix D region is to expel the sheet-inserted residue P15 (Gly379) and P14 (Ser380) from β-sheet A, so that the whole reactive center loop is exposed [29,30]. The allosteric effect between the hinge region (Gly379—Glu381) and the heparin binding region is essential for the heparin activation [31].

The AT deficiency is roughly divided into type I and type II deficiencies. Type I deficiency ("Classical deficiency") has reduced levels of immunologically and functionally determined AT. Type II deficiency has been applied to the cases in which the functionally determined AT is reduced. Type II deficiency is further divided into the heparin binding site (HBS)-type in which heparin binding is abnormal; reactive site (RS)-type in which the reactive center loop is abnormal; and pleiotropic effect (PE)-type in which the influence is pleiotropic [32]. Patient 1 in this study was type II deficiency showing a normal immunological (protein) level and a reduced AT activity (Table 1), and the E381del mutation was detected by base sequencing (Fig. 1). Patient 2 was type I with reduced AT level and reduced activity in plasma (), and base sequencing detected a mutation at P16H on the N-terminal (Fig. 2).

Expression experiments of these AT mutants transfected into HEK293 cells indicated that both of the mutant molecules were secreted normally into culture media (Fig. 3). However, the E381 del mutation had no AT activity, while the P16H mutant had the same specific activity as that of wild type (Table 2). Glutamic acid at 381 (P13) is in the hinge region (P15—P8; Gly379—Thr386) of the reactive center loop and is highly conserved to the same extent as P12 (Ala382) in the serpin superfamily [33,34]. The hinge region and the reactive center loop play pivotal roles for the structural/function relationship of AT [6,8,29,30] and A382T at P12 mutation is known as AT Hamilton or Glasgow II [35—37]. A glutamic acid (Glu381) in the reactive center loop at position P13 has a central role for the allosteric activation of AT by heparin binding [38]. The crystal structure shows that Glu381 contacts stabilize the activated conformation [38]. The loop in almost all the serpins is formed by 17 residues [33] and the tight conservation of the length of serpin reactive center loops is striking when compared with the wide variance in the length of surface loops for other protein families. The inhibitory activity of AT to factor Xa depended upon the length of the N-terminal portion of the reactive center loop, and the deletion of one or two residues lowered the inhibitory activity of AT as well as PAI-1 and PAI-2 [39]. The deletion of residues in the reactive center loop converts the serpin into a substrate [39]. Considering these evidence, we would safely conclude that E381 del mutation had lost the AT activity because the reactive center loop was shortened by deleting glutamic acid at 381.

In the case of P16H mutant of Patient 2, the AT level secreted into the culture media and the specific activity were similar to those of the wild-type (Fig. 3 and Table 2), suggesting that both the protein processing for secretion and the inhibitory activity were normal. Since conservation of proline at position 16 is low in the serpin superfamily [33], its involvement in the retention of the stereostructure of AT and proteinase-inhibitory activity may be low. Incubation of P16H mutant at 40°C did not demonstrate the thermal instability compared to the wild type (data not shown). How do we then explain the fact that Patient 2 exhibited the phenotype of type I deficiency (see Table 2)? In this study, we sequenced all seven exons and the exon—intron boundaries of AT gene by PCR (see Materials and methods). Our method applied in this study, how-

Table 2 AT specific activities in normal pooled plasma and in media secreted from HEK293 cells transfected with wild-type and mutants AT genes

Type of AT genes	Specific activity [arbitrary unit] (units/ng)
Wild-type	0.99±0.22 (n=5)
E381-del mutant	undectectable (n=3)
P16H mutant	0.90±0.40 (n=5)
Normal pooled plasma	1.35±0.33 (n=3)

ever, is not a valid method for detecting partial gene deletion or rearrangement, indicating that we could not exclude a possibility of gene deletion or rearrangement. Patient 2 was a 64-year-old female with deep vein thrombosis in the right leg induced by infectious arthritis. She has been suffering from myeloma for 10 years and also suffering from liver dysfunction due to hepatitis B. Her deep vein thrombosis in the right leg was induced by infectious arthritis in the same leg. She responded to heparin/warfarin treatment during the course of a month, indicating that the deep vein thrombosis was relatively mild. The P16H missense mutation was not due to polymorphism (see Results). Kondo et al. analyzed the molecular deficiency mechanism of heparin cofactor II, and reported a patient whose heparin cofactor II mutant was secreted normally into the culture media of transfected HEK293 cells, although the heparin cofactor II level was decreased in the pateint's plasma [40]. Although the P16H mutant did not show the thermal instability at 40°C, it might be possible that the half-life is shortened due to mutation in some reasons, thereby increasing elimination from the circulation. Regarding the genesis of the decreased plasma AT level in Patient 2, either the P16H mutant may have been secreted into the circulation, but was rapidly degraded, or the mutation may not have been directly involved in the reduction of AT in Patient 2. At the present time, however, we are unable to offer a reasonable explanation that she exhibited the phenotype of type I AT deficiency. It could be due to a complication of myeloma, liver dysfunction and arthritis.

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Protein S and protein C gene mutations in Japanese deep vein thrombosis patients

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Abstract

Objectives: Coagulation factor V Leiden has not been detected in Japanese patients suffering from thrombosis. Hitherto, the constitutional background of Japanese thrombotic patients has never been systematically examined. We have performed a systematic investigation to determine pathogenesis for deep vein thrombosis in a Japanese population.

Design and methods: Routine coagulation and fibrinolysis tests were performed to determine the activities of protein S, protein C, antithrombin, plasminogen and fibrinogen. Gene analysis was performed in thrombotic patients having low activities of these factors.

Results: Our study indicates that the frequency (19/85 = 0.22) of mutations of protein S gene in the Japanese patients was 5–10 times higher than that of mutations of protein S gene in Caucasian patients, and the frequency (8/85 = 0.09) of mutations of protein C gene was almost three times higher than that of Caucasian patients. The frequency of antithrombin gene mutation was similar in both populations.

Conclusion: Our study reinforces that the genetic anomaly in the protein S/protein C anticoagulation system is an important risk factor for thrombophilia in the Japanese population.

Keywords: Deep vein thrombosis; Protein S gene mutation; Protein C gene mutation; Factor V Leiden; Japanese thrombophilia

Introduction

Thrombophilia is defined as an increased tendency to thrombosis, and can be either inherited or acquired. Thrombus formation is regulated by the anticoagulatory and fibrinolytic systems on the vascular endothelial cells. Importance of thrombotic factors as the cause of thrombosis has been clarified [1] and studies have indicated that abnormalities of the anticoagulation system and fibrinolytic factors contribute to thrombosis [2,3]. Venous thrombosis is the most common clinical manifestation of such thrombophilia. The prevalence of inherited anomalies of anticoagulation factors such as antithrombin, protein S (PS) and protein C (PC) in patients with venous thrombosis has been reported to be approximately 5–10% [4–6]. In European and American Caucasian patients, factor V Leiden (R506Q), a polymorphism of coagulation factor V, is a major risk factor for venous thrombosis [7,8]. Although the constitutional background of Japanese thrombotic patients has not been well examined, factor V Leiden (R506Q) is not detected in Japanese patients suffering from thromboses [9]. Since 1994, at Kyushu University Hospital, we have been investigating constitutional predispositions of patients suffering from not only venous thrombosis but also arterial and small vessel thromboses in the Japanese population [10], indicating the important role of PS in the pathogenesis of thromboses in Japanese population. Recently, two studies from Taiwan have reported that reduced activities of the PS/PC anticoagulation system are the most important risk factors for thrombophilia in the Taiwanese–Chinese population [11,12].

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