could be associated with DCM in the patient. It has also been reported that Emery-Dreifuss muscular dystrophy, one of the laminopathies, is caused by *LMNA* p.Glu358Lys mutation (20).

The proband had combined AT deficiency and DCM, and the monozygotic twin brother as well as the maternal cousin also suffered from AT deficiency and cardiac conduction disturbance, which is commonly seen in DCM due to *LMNA* mutations. These findings suggested that the AT deficiency co-segregated with the *LMNA* p.Asp357His mutation, which could be associated with DCM, in this family.

Congenital AT deficiency by the *SERPINC1* (1q23-25) mutation and DCM by the *LMNA* (1q21.2-21.3) mutation are rare diseases by independent inheritance, although both genes are located in the nearby regions on the same Chromosome 1. This is the first report of a compound case of two gene mutations associated with AT deficiency and DCM. Since both AT deficiency and DCM are risks of thromboembolization (3, 4), patients with both diseases in combination could be highly thrombophilic. As expected, the proband showed the thrombus formation in the left auricular appendix on the transesophageal echocardiography, although he had been taking warfarin.

Interstitial myocardial fibrosis is an important pathologic finding in DCM, although it is not specific, and problems with its quantification and prognostic value remains unresolved (21). In terms of myocardial fibrosis and AT deficiency, it is noteworthy that myocardial fibrosis in low tissue factor mice improved after plasma levels of AT were decreased in the mouse model, although the mechanism was not clear (22). Also, histological examination revealed only focal fibrosis in the myocardial biopsy samples of the proband (data not shown). The genetic and clinical heterogeneity of familial DCM suggests that it might be caused by a single gene, with multiple other genetic and environmental factors altering its expressivity (23). Taken together, it might also be possible that AT deficiency would show some effects against interstitial fibrosis of the heart in DCM patients.

In conclusion, we investigated the molecular basis of AT deficiency and DCM in a patient with both diseases, and identified missense mutations in the *SERPINC1* and *LMNA* genes, which may cosegregate in the family. It remains to be possible that DCM in the proband is caused by another molecule mutation, but it is more likely that the *LMNA* gene mutation is associated with DCM. It is because his monozygotic twin brother, who should have the same *LMNA* mutation, showed cardiac conduction disturbance, which is common in DCM due to *LMNA* mutations. To prove that the *LMNA* p.Asp357His mutation can cause DCM, and that the *SERPINC1* p.Pro439Thr mutation can affect the pathophysiology of DCM, knock-in experiments of the mouse model will be necessary in future.

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ORIGINAL ARTICLE *Laboratory investigation*

Skewed X chromosome inactivation in fraternal female twins results in moderately severe and mild haemophilia B

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Summary. Female carriers of haemophilia B are usually asymptomatic; however, the disease resulting from different pathophysiological mechanisms has rarely been documented in females. In this study, we investigated the mechanisms responsible for haemophilia B in fraternal female twins. We sequenced the factor IX gene (*F9*) of the propositus, her father, a severe haemophilia B patient and the other family members. X chromosome inactivation was assessed by the methylation-sensitive *Hpa*II-PCR assay using X-linked polymorphisms in human phosphoglycerate kinase 1 gene (*PGK1*) and glutamate receptor ionotropic AMPA 3 gene (*GRIA3*). The twins were found to be heterozygotes with a nonsense mutation (p.Arg384X) inherited from their father. The propositus, more severely affected twin, exhibited a significantly higher percentage of inactivation in the maternally derived X chromosome carrying a normal *F9*. The other twin also showed a skewed maternal X inactivation, resulting in a patient with mild haemophilia B. Thus, the degree of skewing of maternal X inactivation is closely correlated with the coagulation parameters and the clinical phenotypes of the twins. Furthermore, we identified a crossing-over in the Xq25–26 region of the maternal X chromosome of the more severely affected twin. This crossing-over was absent in the other twin, consistent with their fraternal state. Differently skewed X inactivation in the fraternal female twins might cause moderately severe and mild haemophilia B phenotypes, respectively.

Keywords: crossing-over, female, haemophilia B, skewed inactivation, twin, X chromosome

Introduction

Haemophilia B is an X-linked recessive bleeding disorder caused by a deficiency or functional defect in the coagulation factor IX (FIX) with an incidence of 1:25 000–30 000 male births [1,2]. It is a clinically heterogeneous disorder in which bleeding severity is related to the quantity of coagulant FIX activity (FIX:C) and is classified as severe (<1%), moderately severe (≥1 to <5%) or mild (≥5 to <30%) based on the FIX:C. FIX is a vitamin K-dependent plasma protein and is important in the early phase of blood clotting.

The human FIX is encoded by the FIX gene (*F9*), which is 34-kb in length comprising eight exons and seven introns, and is located on the X chromosome (Xq27.1–27.2). It is synthesized by hepatocytes as a 461 amino acid precursor with a 46 amino acid signal peptide, which is cleaved off prior to its secretion into plasma as a mature peptide (415 amino acids). Plasma FIX is a single chain glycoprotein with a molecular weight of approximately 57 kDa, with five important functional domains; the N-terminal Gla domain, the aromatic amino acid stack domain, two epidermal growth factor (EGF)-like domains, the activation peptide domain and the C-terminal catalytic domain [2–4].

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Female carriers of haemophilia B are usually asymptomatic because the X chromosome is randomly inactivated with an approximately equal proportion of the two populations of somatic cells [5,6]. However, in rare cases, a skewed X chromosome inactivation could result in a symptomatic female in whom the normal X chromosome is predominantly inactivated [7,8]. In rare instances, structural X chromosome abnormalities such as translocations, iso-X chromosomes or others also cause haemophilia B in females [9–13].

Here, we have described fraternal twin girls carrying the FIX nonsense mutation inherited from their father, associated with differently skewed inactivation of the maternally derived normal X chromosome, causing moderately severe and mild haemophilia B phenotypes, respectively.

Patients, materials and methods

Patients and samples

The propositus was a 1-year-old girl suffering from a subaponeurotic cephalohaematoma following a head injury. She was diagnosed with moderately severe haemophilia B (FIX:C = 2.3%) and successfully treated with FIX concentrates (Fig. 1). Her fraternal twin sister was also diagnosed with mild haemophilia B (FIX:C = 24.6%). Their father was a severe haemophilia B patient (FIX:C <1.0%) and the mother had normal plasma FIX:C (88.9%). Cytogenetic analysis of the propositus showed a normal female karyotype (46, XX) without any structural abnormality. The study was approved by the Ethics Committee of the Nagoya University School of Medicine. After obtaining informed consents, citrated blood samples were obtained from the twins and their parents. From each sample, genomic DNA was isolated from the peripheral leukocytes by phenol extraction as described previously [14].

Identification of F9 mutation

The eight exons and exon-intron boundaries of the *F9* were amplified by polymerase chain reaction (PCR) using the gene-specific primers listed in Table 1. The PCR products were analysed by direct sequencing using the BigDye Terminator Cycle Sequencing Kit and the ABI Prism 310 Genetic Analyzer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) as described previously [15]. We also analysed the DNA samples by PCR-mediated *Taq*I restriction fragment length polymorphism (RFLP) to confirm the identified mutation. Thus, DNA fragments of the *F9* (exon 8) were amplified by PCR as described above, treated with *Taq*I, and then analysed by 2% agarose gel electrophoresis.

Southern blot analysis

DNA of the family members was subjected to *Apa*LI Southern blot and *Nco*I Southern blot analyses for *F9* as described previously [14].

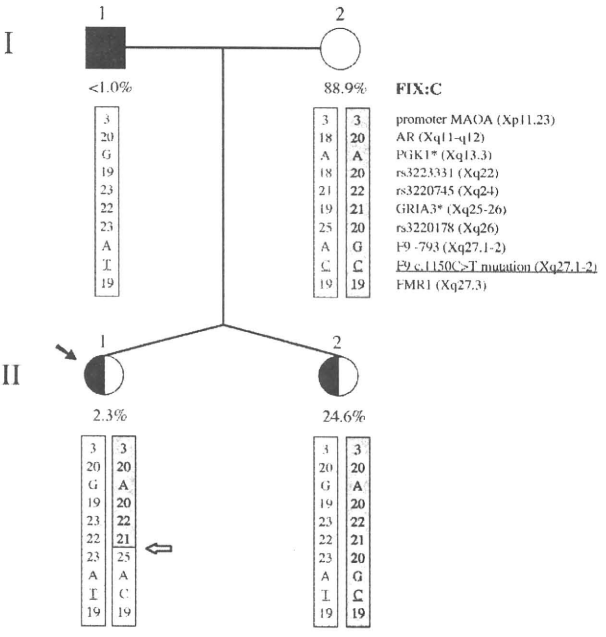

Fig. 1. Pedigree and haplotype analysis of the family. The square (□) and circle (○) symbols denote male and female, respectively. Solid symbols indicate the presence of *F9* nonsense mutation (p.Arg338X: c.1151C > T). Solid arrow (↘) indicates the propositus. Values (%) below the symbols represent individual FIX:Cs. Haplotypes of the X chromosomes are shown in the boxes, which have number of the repeat or individual nucleotide in each locus. Underline represents the *F9* mutation in this family. Asterisks (*) are loci used for X chromosome inactivation analysis. Open arrow (⇦) indicates a crossing-over in the X chromosome at Xq25-26 region of the propositus.

Assessment of X chromosome inactivation by methylation-sensitive HpaII-PCR assay

X inactivation was assessed by the methylation-sensitive *Hpa*II-PCR assay using an X-linked human phosphoglycerate kinase 1 gene (*PGK1*) polymorphism as described previously [16]. The genomic DNA samples were pretreated with or without methylation-sensitive *Hpa*II (New England BioLabs, Bervery, MA, USA) and then subjected to PCR-mediated *Bst*XI RFLP analysis for the *PGK1*. The PCR products were analysed by 1.5% agarose gel electrophoresis with ethidium bromide and the amount of each product was evaluated by the NIH image version 1.62 (http://rsb.info.nih.gov/nih-image/).

We also analysed X chromosome inactivation patterns at the X-linked glutamate receptor ionotropic AMPA 3 gene (*GRIA3*) as described previously [17]. Genomic DNA samples were treated with or without methylation-sensitive *Hpa*II and subjected to PCR amplification with specific fluorescent primers (Table 1). The PCR products were then analysed by GeneScan software on ABI Prism 310 Genetic Analyzer.

X chromosome haplotype analysis

To analyse the X chromosome haplotype of the family members, we used polymorphic markers, such as monoamine oxidase A gene (*MAOA*, Xp11.23) promoter [18], androgen receptor gene (*AR*, Xq11–12) [19], phosphoglycerate kinase 1 gene (*PGK1*, Xq13.3) [16], glutamate receptor ionotropic AMPA 3 gene (*GRIA3*, Xq25–26) [17], FIX gene (*F9* -793, Xq27.1–2) [20] and Fragile X mental retardation gene 1 (*FMR1*, Xq27.3) [16]. We also analysed short tandem repeat [13] polymorphisms, such as reference SNP IDs: rs3223331 (Xq22), rs3220745 (Xq24) and rs3220178 (Xq26).

Results and discussion

Haemophilia B occurs primarily in males. Normally, female carriers of haemophilia B do not exhibit a phenotypic manifestation of the bleeding disorder. However, there are several different mechanisms that could lead to the phenotypic expression of very low FIX:C levels in females [7]. In this study, we investigated the molecular basis of moderately severe and mild haemophilia B phenotypes in fraternal twin girls to elucidate the genetic mechanisms responsible for female haemophilia B.

First, we analysed *F9* genes of the family members by direct sequencing and found a C-T transition at nucleotide position 1150 of the coding sequence (c.1150C>T). This nonsense mutation (p.Arg384X), previously reported as FIX New York [21], was

Table 1. Primers used in this study.

	Primers 5′–3′	Annealing temperature, °C	Product size, bp
FIX sequence primers			
FIX Exon1U	AATCAGACTAACTGGACCAC		
FIX Exon1L	TATCTAAAAGGCAAGCATAC	50	536
FIX Exon2 & 3U	ATGATGTTTTCTTTTTTGCT		
FIX Exon2 & 3L	GGTTGGACTGATCTTTCTG	50	553
FIX Exon4U	TTCTAAGCAGTTTACGTGCC		
FIX Exon4L	GTAGCTTCTTGAACTCATATCC	52	331
FIX Exon5U	CCCCCAATGTATATTTGACC		
FIX Exon5L	CCGTCCTTATACTAGAAGCC	50	606
FIX Exon6U	AATACTGATGGGCCTGCT		
FIX Exon6L	AACTTGCCTAAATACTTCTCAC	50	445
FIX Exon7U	CCAATATTTTGCCTATTCCT		
FIX Exon7L	CTTCTGGTATGGAAATGGCT	50	457
FIX Exon8U-1	TGTGTATGTGAAATACTGTTTG		
FIX Exon8L-1	TTATAGATGGTGAACTTTGTAGA	60	388
FIX Exon8U-2	TTGGATCTGGCTATGTAAGT		
FIX Exon8L-2	AGTTAGTGAGAGGCCCTG	60	407
PGK 1 sequence primers			
PGK1 Up	CTGTGCTCTGTCGCAAACCT		
PGK1 Lw	CTCCTGAAGTTAAATCAACATCC	52	468
GRIA 3 sequence primers			
GRIA 3 Up	GGAGTAACCACTAATGGCCCTC		562
GRIA 3 Up 6-FAM	GCGGAGAGAGTCTCAGATTGTC		182
GRIA 3 Lw	TTTGCCAAAGCGATTTCTGT	68	

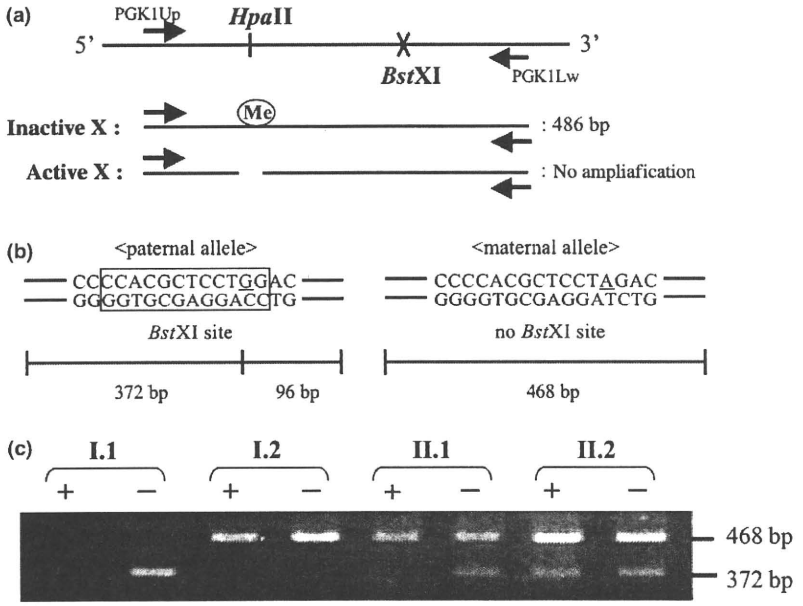

Fig. 2. X chromosome inactivation analysis at the *PGK* locus. (a) Scheme of PCR amplification after methylation sensitive *Hpa*II digestion in the *PGK1* locus. The symbol (Ⓜe) indicates a methylation site on an *Hpa*II cleavage site. (b) *Bst*XI-RFLP at *PGK1* locus. The paternal derived G allele is detected as 372-bp and 96-bp fragments, whereas the maternal derived A allele appears as a 468-bp fragment. (c) +, *Hpa*II digested DNA; —, undigested DNA; I.1, father (severe haemophilia B); I.2, mother (not informative); II.1, propositus (extremely skewed X inactivation); II.2, twin sister (mild skewed X inactivation). After *Hpa*II digestion of the DNA, PCR-mediated *Bst*XI-RFLP analysis for the propositus (II.1) showed an extremely unbalanced amplification towards the maternal A allele (99:1) indicating a severely skewed inactivation of the maternal X chromosome. Contrastingly, the other twin (II.2) showed only a moderately skewed amplification towards the maternal A allele (65:35).

responsible for the immature termination of the FIX protein synthesis in their father, a severe haemophilia B patient. This mutation was also detected in both the twins in heterozygous state (Fig. 1), which was confirmed by the *Taq*I PCR-RFLP analysis (data not shown). The variant FIX molecule did not have normal activity, consistent with the undetectable FIX:C level of their father. However, the heterozygous mutation in the twins indicated inconsistency in their moderately severe and mild haemophilia B phenotypes. *Apa*LI or *Nco*I Southern blot analysis performed for *F9* in the twin girls demonstrated no altered migration pattern suggesting the absence of gross gene abnormality in their *F9* (data not shown). Furthermore, the heterozygous state of the *F9* c.1150C>T mutation in the propositus indicated that she would not have a large gene deletion of the *F9* in her maternal allele, which could be missed in PCR analysis.

We analysed a possibility of skewed inactivation of the maternally derived normal X chromosome in the twins. To assess the heterozygosity for a G/A polymorphism in intron 1 of the *PGK1* [16], we performed PCR-mediated *Bst*XI-RFLP analysis for the family and found that both the twins were positive. Thus, the *Bst*XI-RFLP analysis for the twins showed two distinct bands, which indicated the G allele from the father (372-bp) and the A allele from the mother (468-bp) (Fig. 2). After *Hpa*II digestion of the template DNA from the peripheral blood, the PCR-mediated *Bst*XI-RFLP analysis for the propositus twin girl showed an extremely unbalanced amplification towards the maternal allele (99:1) indicating a severely skewed inactivation of the maternally derived normal X chromosome. Contrastingly, the other twin showed only a moderately skewed amplification towards the maternal allele (65:35), which was consistent with her mild haemophilia B phenotype. We also analysed X chromosome inactivation patterns at the X-linked *GRIA3* [17] and observed similar skewed inactivation results of the *PGK1* methylation analysis (data not shown). Therefore, the difference in the severity of the haemophilia B phenotype closely correlated with the degree of skewing of X inactivation.

During the survey of informative markers for X inactivation assay along the X chromosome, we found a crossing-over in the maternal X chromosome

at Xq25–26 region of the more affected twin (Fig. 1). This was absent in the other twin. This difference between the twins was consistent with their fraternal state. In the case of monozygotic (MZ) twins, Côté and Gyftodimou described that a review of twin discordance and an attempt at explaining some of the data leads one to hypothesize the impossibility for crossed X chromosomes to undergo efficient inactivation [22]. In fraternal twins, however, extreme skewed inactivation of crossed X chromosomes can result from single gene mutations that affect cell survival or growth [23]. Interestingly, it has been reported that X chromosome haplotype analysis suggests the presence of a locus for the familial skewed X inactivation in chromosome Xq25 most likely controlling the X chromosome choice in X inactivation or cell proliferation [24]. Therefore, although clarification of a precise break point of the X chromosome is necessary to confirm it, it might be possible that the crossing-over in the propositus X chromosome would disturb a gene function affecting cell proliferation leading to a selection towards the skewed X inactivation.

In conclusion, differently skewed inactivation of the maternal X chromosome in the fraternal female twins, with an inherited *F9* nonsense mutation in the paternally derived X chromosome, might cause moderately severe and mild haemophilia B phenotypes, respectively.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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REGULAR ARTICLE

Impaired binding of thrombin to thrombomodulin is associated with risk of deep vein thrombosis

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KEYWORDS
Thrombin;
Thrombomodulin;
Deep vein thrombosis

Abstract

The complex of thrombin and thrombomodulin (TM) activates protein C, and impaired binding of thrombin to TM may be a risk factor for thrombosis. In this study, we evaluated the reactivity of thrombin to TM by determining the TM-bound thrombin (TMBTh) to total thrombin generation (t-Th) ratio (TMBT ratio). We also examined whether a decreased TMBT ratio is associated with increased risk of thrombosis. TMBTh was measured on TM-coated plates. Thrombin was generated by addition of prothrombin time reagent to plasma. Levels of t-Th and TMBTh were expressed as percentages of the levels in pooled normal plasma. The study included 124 patients with deep vein thrombosis and 150 age- and sex-matched healthy subjects. The TMBT ratio (TMBTh/t-Th) was significantly lower in patients than in control subjects ($p<0.05$). Among the 124 patients, 43 (34.7%) showed TMBT ratios below the 5th percentile value of control subjects, and the odds ratio (OR) for development of deep vein thrombosis was 9.4 (95% confidence interval [CI], 4.6–19.1). When patients with a deficiency of natural anticoagulant (antithrombin III, protein C, or protein S) were excluded from analysis, the TMBT ratio in 37 (42.5%) of the remaining 87 patients was below this cutoff point, and the OR (13.1; 95% CI, 6.4–26.9) was increased compared to that in the total patient group. These results suggest that it is possible to evaluate the reactivity of thrombin to TM by determining the TMBT ratio, and this ratio may be a predictor of deep vein thrombosis.

Abbreviations: TM, thrombomodulin; t-Th, total thrombin generation; TMBTh, TM-bound thrombin; TMBT ratio, TMBTh/t-Th; rhs-TM, recombinant human soluble thrombomodulin; TM-coated plates, thrombomodulin coated plates; DSS, disuccinimidyl suberate; DMSO, dimethlsulfoxide; S-2238, D-phe-pipecolyl-Arg-*p*-nitroanilide; FI-TBS, Tris-buffed saline including fibrin inhibitor; DVT, deep vein thrombosis.

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Introduction

Thrombin is a central enzyme in the coagulation process. It activates factor V and factor VIII, which provide positive feedback for production of thrombin, and it directly activates platelets and cleaves fibrinogen to form fibrin, resulting in thrombus formation [1]. In the process of coagulation, the protein C anticoagulant pathway, which includes protein C, protein S, thrombomodulin (TM), and endothelial cell protein C receptor, plays an important role in the natural inhibitor system of thrombin regulation [2–4]. TM is an integral membrane protein that binds thrombin with high affinity. In the TM-thrombin complex, thrombin activates protein C approximately 1000-fold faster than thrombin alone, and it loses its procoagulant activities including fibrin formation and platelet aggregation [3,4]. Aided by protein S, a non-enzymatic cofactor with high phospholipid affinity, activated protein C proteolytically inhibits coagulation factors Va and VIIIa [5]. Thus, TM converts procoagulant thrombin to an anticoagulant and plays a role as a physiologically important anticoagulant on the endothelial cell surface. The normal protein C pathway depends on the precise assemblage of these factors on the surface of endothelial cells. Therefore, impairment of this assemblage may increase the risk of thrombosis.

The importance of the protein C pathway is underscored by the observation that protein C deficiency, protein S deficiency, and factor V Leiden mutation are associated with an increased risk of thromboembolic events, events that are either spontaneous or triggered by circumstantial risk factors [6–9]. In an abnormal protein C pathway, genetic abnormalities affecting TM should theoretically be associated with thrombosis. Screening for TM gene mutations in patients with thrombotic disease has shown the occurrence of several mutations, some of which are associated with venous and arterial thrombosis [10–12], but establishing the significance of these findings requires larger studies. Another possibility is that thrombosis derives from abnormal thrombin generation in individuals. Indeed, because TM-bound thrombin complex can activate protein C, a decrease in the TM-bound thrombin level may be prothrombotic. However, there has been no reported study pertaining to the reactivity to TM of thrombin generated in individuals.

The aim of this study was to determine whether a low level of TM-bound thrombin is associated with thrombosis. We developed an assay method for total thrombin generation and the binding of thrombin generated in plasma to TM to investigate whether low level TM-bound thrombin is a risk factor for deep vein thrombosis (DVT).

Materials and methods

Materials

Thromboplastin C Plus as tissue factor and factor II-depleted plasma were purchased from Dade Behring (Marburg, Germany). Human protein C-depleted plasma and reptilase were purchased from Diagnostica Stago (Asnières, France). Human protein S-depleted plasma was purchased from Biopool (Burlington, ON, Canada). Chromogenic substrate, S-2238 (D-Phe-pipecolyl-Arg-*p*-nitroanilide) was purchased from Kabi Vitrum (Stockholm, Sweden). Casein and affinity-purified horseradish peroxidase-conjugated goat anti-human IgG F(ab')2 fragment were purchased from Sigma-Aldrich (St. Louis, MO, USA). Disuccinimidyl suberate (DSS) was purchased from Research Organics (Cleveland, OH, USA). Dimethylsulfoxide (DMSO) was purchased from Pierce (Rockford, IL, USA). A soluble recombinant human thrombomodulin (rhs-TM), composed of all of the extracellular domains of thrombomodulin without chondroitin sulfate chains [13,14], was supplied by Asahi Kasei, Pharma (Tokyo, Japan). Fibrin inhibitor (of polymerization) was purchased from Biogenesis (Poole, UK). All reagents used were of the highest commercially available grade.

Preparation of rhs-TM bound plates

rhs-TM was coupled to CovaLink NH microplates (Nunc, Roskilde, Denmark) with the use of DSS as a bifunctional N-hydroxysuccinimide linker. CovaLink NH microplates were activated with 100 μl of 12.5 mg DSS/50 ml DMSO in 150 ml carbonate buffer, pH 9.6, and incubated for 1 hour at room temperature. After the plates were washed with distilled water, they were coated with 100 μl of 2.5 μg/ml rhs-TM in carbonate buffer, pH 9.6, and incubated overnight at room temperature. Blocking was performed with 0.5% (w/v) casein in phosphate-buffered saline (PBS), pH 7.2, (200 μl/well) and incubation for 15 minutes at room temperature. The microplates were washed 3 times with 0.2 M NaCl in PBS and stored in Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) at 4 °C until use.

Assay of total-thrombin generation and TM-bound thrombin

Thrombin was generated in the plasma of patients or control subjects by addition of prothrombin time reagent to plasma. In Brief, 50 μl of plasma sample, diluted 1:100 in 0.1 mg/ml fibrin inhibitor in TBS (FI-TBS), was added to each well of a microplate (FASTEC Micro Plate U; Fujirebio, Tokyo, Japan); 100 μl Thromboplastin C Plus as prothrombin time reagent was then added to each well. The plate was incubated for 30 minutes at 37 °C; 50 μl of S-2238 substrate solution (1.0 μmol/ml S-2238 in TBS) was then added to each well. Absorbance of the solution in each well was then determined at 405 nm with a Biomek Plate Reader (Beckman Instruments Inc, Miami, FL, USA), with 630 nm used as reference wave length.

TM-bound thrombin (TMBTh) levels were measured in rhs-TM-bound 96-well microplates. Plasma sample, 50 μl diluted 1:100 in FI-TBS, was added to each well of the rhs-TM binding microplate; 100 μl Thromboplastin C Plus was then added to each well. After the plate was incubated for 30 minutes at 37 °C, it was washed 3 times with TBS, and 100 μl of chromogenic substrate S-2238 solution (0.5 μmol/ml S-2238 in TBS) was added. The plate was then incubated for 1 hour at room temperature, and absorbance of the solution in each well was determined at 405 nm with a Biomek Plate Reader with 630 nm used as reference wave length. The total thrombin generation (t-Th) and TMBTh assays were

Table 1 Specificity of TMBTh assay

	rhs-TM coated plate (n=5)	Casein coated plate (n=5)
α-thrombin		
2.5 U/ml	0.845 (0.050)	0.027 (0.004)*
1.25 U/ml	0.434 (0.015)	ND
Normal plasma		
x100 diluted	0.410 (0.034)	0.026 (0.002)*
x200 diluted	0.209 (0.021)	ND
Prothrombin deficient plasma		
x100 diluted	0.030 (0.001)*	0.028 (0.001)*
FI-TBS	0.029 (0.001)	ND

Values are given as mean (SD) of OD at 405 nm absorbance.
* No significant difference from FI-TBS using Mann-Whitney U test. ND: not done.

calibrated with dilutions of normal pooled plasma, and standard curves were prepared by duplicate measurements for each plate. Levels of t-Th and TMBTh were expressed as percentages of the levels in pooled normal plasma shown by the standard curves. The TM-bound thrombin/total thrombin ratio (TMBT ratio) was calculated by dividing the level of TMBTh by the level of t-Th.

Preparation of defibrinated plasma

Diluted plasma (400 μl diluted 5 times in TBS) and reptilase (4 μl) were mixed at room temperature. After 30 minutes, the mixture was incubated for 30 minutes at 4 °C to promote the polymerization of fibrin monomer, and the mixture was defibrinated by centrifugation at 4 °C (2800 g, 10 minutes). When t-Th or TMBTh was measured, the supernatant was further diluted 20 times in TBS (final dilution, x100).

Patients and control subjects

The study included 124 patients (53 men and 71 women between 19 and 88 years of age (mean, 53 years) with DVT. Patients were referred to the Department of Cardiovascular Surgery at Osaka University Medical Hospital during an 88-month period (August 1994 through December 2001). Blood samples were obtained from these patients at least 3 months after the thrombotic episode and at least 4 weeks after discontinuation of oral anticoagulants unless this treatment could not be stopped. Patients taking oral anticoagulants were excluded from the study because of low concentrations of vitamin K-dependent proteins. None of the patients received heparin during the investigation period. DVT was diagnosed on the basis of clinical manifestations and findings of duplex scanning, radioisotope venography, and contrast venography. There was no indication of factors triggering DVT among the patients, i.e. there was no surgery, cancer, pregnancy or oral contraceptive use that could have contributed to the DVT. Among these 123 patients, 3 had had two thrombotic episode, and the remaining 120 had had one episode. Patients were also asked about the occurrence of venous thromboembolism in their families. Three of them had a family history of venous thromboembolism, and three patients had family history of myocardial infarction. Healthy Japanese subjects (150 control subjects) without any history of venous thrombosis were randomly selected from among volunteers. Patients and volunteers were informed that blood samples were being obtained for research purposes and that their privacy would be protected.

Blood samples

Venous blood samples were obtained by venipuncture with the use of a tourniquet. Blood was drawn into Vacutainer tubes containing 3.2% (w/v) trisodium citrate (0.129 M; 9:1, v/v). Platelet-poor plasma was prepared by centrifugation at 2800 *g* for 10 minutes. Aliquoted plasma was kept at −80 °C until use. Pooled normal plasma used in the study was obtained from 20 of the healthy volunteers who were not receiving medication. These individuals were not screened for abnormalities associated with an increased risk of venous thrombosis.

Statistical analysis

Between-group differences in distributions of patients and control subjects for t-Th, TMBTh, or TMBT ratio were analyzed by Student's *t*-test or Mann-Whitney U test. Between-group differences in age were examined by Student's *t*-test, and between-group differences in sex ratio were examined by chi-square test. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated as estimates of relative risk for development of DVT. All analyses were performed with StatFlex ver. 5.0 software (Artech Inc., Osaka, Japan). A p values of <0.05 were considered statistically significant.

Results

Specificity of TMBTh assay

To check the specificity of the TMBTh assay, we measured 4 samples (thrombin, normal plasma, prothrombin-deficient plasma, and FI-TBS) with TM-coated plates and casein-coated plates. With TM-coated plates, amidolitic activities, both of thrombin and normal plasma, were high, and no significant difference between prothrombin-deficient plasma and FI-TBS was observed. With casein-coated plates, no significant differences were observed between any of the samples (Table 1).

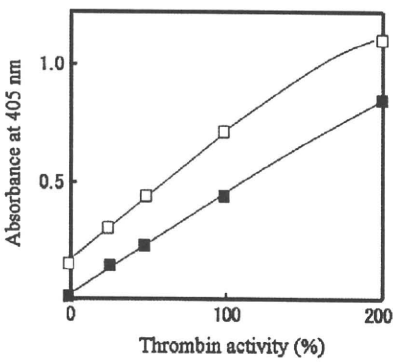

Figure 1 Standard curves for total thrombin generation (□) and thrombomodulin-bound thrombin (■). Reactions were performed as described in the Materials and methods.

Table 2 Characteristic of patients with venous thrombosis and control subjects

	Patients	Controls
	n=124	n=150
Age (y)		
Mean (y)	53	53
Range (y)	19–88	18–88
Sex		
Male (n)	53	67
Female (n)	71	83
Anti-thrombin III deficiency (n)	2	0
Protein C deficiency (n)	8	0
Protein S deficiency (n)	26	0
Protein C+Protein S deficiency (n)	1	0

Standard t-Th and TMBTh curves

For standard curves, prothrombin levels of 200% and 100% were prepared by dilution of pooled normal plasma (x50 and x100 in FI-TBS, respectively). Prothrombin levels of 50%, 25%, and 0% were prepared with factor II-depleted plasma containing each amount of normal plasma and diluted x100 in FI-TBS (50%, 25%, and 0% normal prothrombin levels). Standard t-Th and TMBT curves are shown in Fig. 1. When these standard curves were used, the coefficients of variation (n=8) were 2.38 (t-Th: mean, 85.7%), 10.11 (TMBT: mean, 88.2%), and 9.69 (TMBT ratio: mean, 1.029), respectively.

Influence of natural anticoagulant (antithrombin III, protein C, protein S) concentrations on assay of t-Th and TMBTh

To investigate influence of the protein C or protein S concentration, we used protein C- or protein S-depleted plasma. The concentration of protein C or protein S in plasma was varied by mixing protein C- or S-depleted plasma with normal plasma (0%, 25%, 50%, 75%, and 100% normal plasma), and both t-Th and TMBTh were analyzed. There was no significant difference between each concentration in the values of t-Th or TMBTh. To investigate influence of the antithrombin III concentration, we measured values in plasma with added antithrombin III (final concentrations, 90%, 190%, and 290% of normal). Both t-Th and TMBTh decreased slightly (85–76% and 89–82%, respectively) according to the increase in the concentration of antithrombin III, but the TMBTh ratio did not change (1.060–1.078).

Characteristics of the study subjects

One hundred twenty-four patients (53 men and 71 women) and 150 healthy subjects (67 men and 83 women) were recruited. Mean age±SD (range) of patients and healthy subjects was 53.2±18.8 (19–88) years and 53.5±15.5 (18–88) years, respectively. There was no significant difference in the mean age or sex ratio between patients and healthy subjects ($p=0.895$ and $p=0.807$, respectively). Mean age of patients at the first thrombotic event was 51.8±16.4 (18–

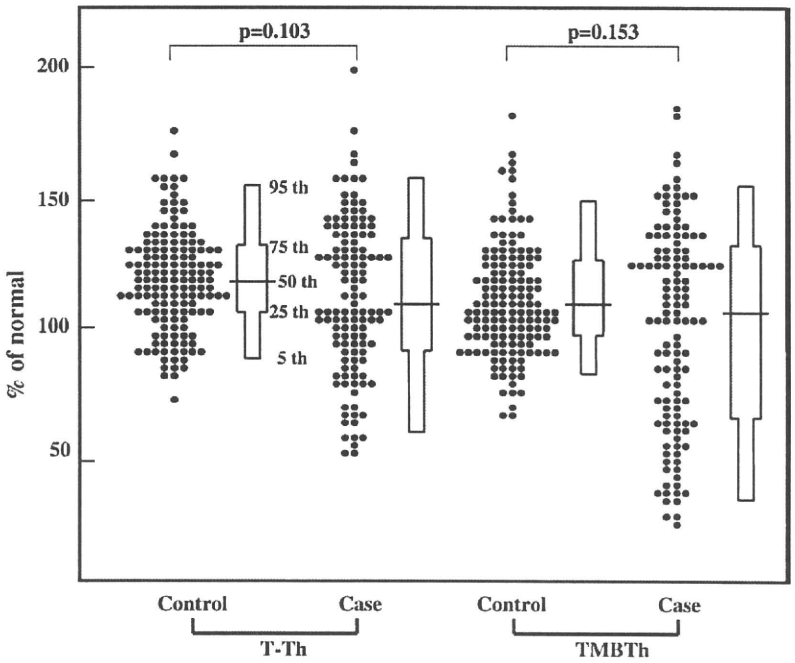

Figure 2 Distribution of total thrombin and thrombomodulin-bound thrombin activities in DVT patients (n=124) and control subjects (n=150). Values are shown as percentages of pooled normal plasma values. The 5th, 25th, 50th, 75th, and 95th percentiles are indicated. Significance was determined by Mann-Whitney U test.

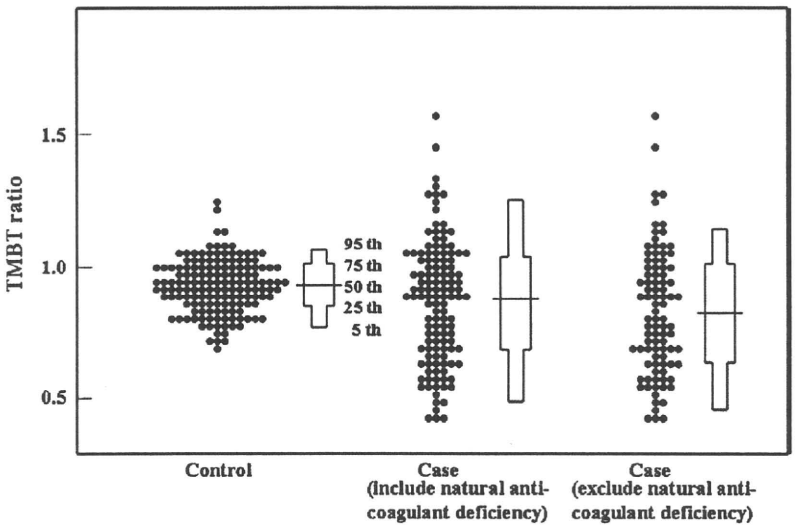

Figure 3 Binding capability to thrombomodulin in control subjects and patients with deep vein thrombosis. The results were plotted as thrombomodulin-bound thrombin ratios (TMBT ratio: TMBTh/T-Th). The 5th, 25th, 50th, 75th, and 95th percentiles are indicated. Differences in TMBT ratio between the total DVT patients and those without natural anticoagulant (antithrombin III, protein C, and protein S) deficiencies and control subjects were significant ($p<0.05$ and $p<0.001$, respectively).

88) years. A thrombophilic cause was found in 37 patients (29.8%), i.e., deficiency of antithrombin III (n=2), protein C (n=8), protein S (n=26), or deficiency of both protein C and protein S (n=1) (Table 2). Factor V Leiden and prothrombin G20210A mutation were not examined because of the extremely low prevalence of these mutations in the Japanese population.

t-Th, TMBTh, and TMBT ratios

Gaussion distribution was noted for the t-Th and TMBTh levels in the control group and the t-Th level in the patient group. The t-Th level did not differ significantly between patients (median, 91.6; IQR, 44.0; range, 50–200) and control subjects (median, 104.7; IQR, 25.9; range, 71–176) ($p=0.103$). Nor did the TMBTh level differ significantly between patients (median, 66.0; IQR, 63.8; range, 23–186) and control subjects (median, 94.63; IQR, 30.0; range, 66–186) ($p=0.153$) (Fig. 2). TMBT ratios were significantly lower in patients than in control subjects ($p<0.05$). Few patients had low ratios, and the distribution of values among patients was distinctly bimodal: 35% fell below the 5th percentile of control values, and 58% fell below the 50th percentile of control values. When patients with natural anticoagulant deficiencies were examined separately, there was no significant difference in TMBT ratio between this group and the total patient group (Fig. 3).

Table 3 Crude odds ratios (ORs) for DVT, by TMBT ratio

	Patients (n=124)	Control subjects (n=150)	OR	95%CI
1st percentile (0.700)			28.0	10.0–78.0
at or below	34	2		
above	90	148		
2.5th percentile (0.732)			16.7	7.2–39.1
at or below	39	4		
above	85	146		
5th percentile (0.760)			9.4	4.6–19.1
at or below	43	8		
above	81	142		
10th percentile (0.800)			4.0	3.2–10.8
at or below	49	15		
above	75	135		

CI=confidence interval.

TMBT ratio as a risk factor for DVT

We examined whether a low TMBT ratio is a risk factor for development of DVT. Forty percent of patients had a TMBT ratio that fell below the 10th percentile of the control

Table 4 TMBT ratio (TMBTh/t-Th) and risk of DVT

	Positive/total	OR	95%CI
Control	8/150		
Case			
Including deficiencies*	43/124 (34.7%)	9.4	4.6–19.1
Excluding deficiencies*	37/87 (42.5%)	13.1	6.4–26.9

Odds ratios (ORs) are given as determinants of relative risk. The risk was evaluated with 5th percentile of the distribution of TMBT ratios amongst controls.
* Deficiencies of antithrombin III, protein C and protein S.

value. Thus, individuals with a TMBT ratio below the 10th percentile had a 3.4-fold increased risk of developing DVT compared to that of individuals with a TMBT ratio above this cutoff value. Further analyses revealed that lower TMBT ratios, (e.g., below the 5th, 2.5th, and 1st percentiles) were associated with higher ORs for DVT. This dose-response relation of (higher risk associated with low TMBT ratio) suggested a threshold level for development of DVT (Table 3).

Of the 124 patients, 43 (34.7%) showed TMBT ratios below the 5th percentile of control values, and the OR was 9.4 (95% CI, 4.6–19.1). Six of the 43 patients with a low TMBT ratio were deficient in protein S; other deficiencies (antithrombin III or protein C) were not found. When patients deficient in natural anticoagulant were excluded from the analysis, 37 (42.5%) of the remaining 87 patients had TMBT ratios below this cutoff point, and the OR (13.1; 95% CI, 6.4–26.9) increased compared to that of the total patient group (Table 4). This result suggested that the TMBT ratio, as a risk factor for DVT, is not dependent on these deficiencies.

Influence of fibrinogen or fibrin monomer on the assay

The concentration of fibrinogen did not correlate with the TMBT ratio in the patient group ($p=0.496$). Defibrination was performed for 25 patients with a TMBT ratio below the 5th percentile of the control group to identify any influence of fibrinogen or fibrin monomer on this assay method, and the TMBT ratio was determined. The median TMBT ratio obtained in non defibrinated plasma was 0.557 (mean, 0.584; SD, 0.079; range, 0.385 – 0.705); that for defibrinated plasma was 0.542 (mean, 0.605; SD, 0.109; range, 0.429 – 0.828). The ratio did not differ significantly between non defibrinated plasma and defibrinated plasma ($p=0.43$).

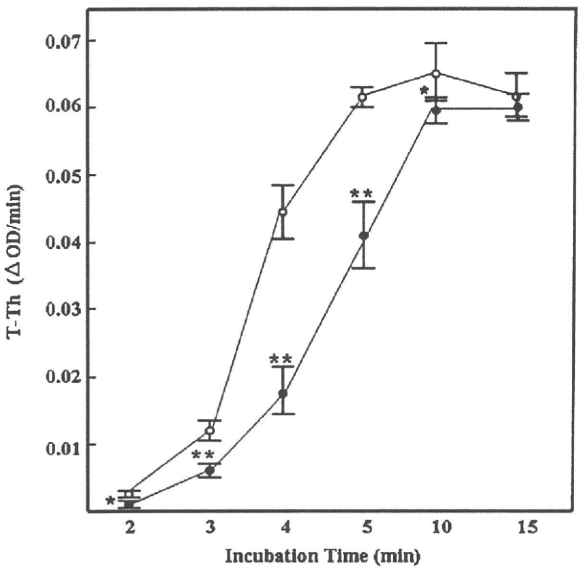

Figure 4 Comparison of thrombin generation in samples with normal and low TMBT ratios. Time courses of thrombin generation in normal (○) and low (●) TMBT ratios are shown. Thrombin generation is represented by ΔOD/minutes at 405 nm absorbance. Symbols and bars represent mean ± SE of 8 samples for normal and 11 samples for low TMBT ratio. Significance was determined by Mann-Whitney U test. *$p<0.05$, **$p<0.01$ compared to corresponding data at each time point.

Time course of thrombin generation

Because the cause of the low TMBT ratio may be abnormality of thrombin generation, we examined the time course of thrombin generation with chromogenic substrate in 2 groups: 8 subjects with normal TMBT ratios (2 control subjects and 6 DVT patients; mean, 1.063; SD, 0.152) and 11 DVT patients with low TMBT ratios (mean, 0.581; SD, 0.041). There was no significant difference in t-Th values between the normal TMBT ratio group (mean: 110.1, SD: 12.6, range: 98.1 to 134.3) and low TMBT ratio group (mean, 101.9; SD, 12.4; range, 91.0 – 126.1). This assay for the generation of thrombin over time used the same conditions as the t-Th assay (see Materials and methods), and the results are expressed as ΔOD/minute at 405 nm. In both groups, thrombin generation was first detected at 2 minutes. However, by 2, 3, 4, 5, and 10 minutes, thrombin generation decreased in the low TMBT ratio group compared to that in the normal TMBT ratio group (Fig. 4).

Discussion

We describe a new test for screening the ability of thrombin generated in plasma of individuals to bind TM. The assay we developed is based on the extrinsic pathway, with tissue factor used on rhs-TM coated plates. Thus, the concentrations of natural anticoagulants (antithrombin, protein C, and protein S) and the presence of fibrinogen (fibrin monomer) and high molecular polycations may influence this assay. However, we showed that the concentrations of protein C and protein S did not influence the total thrombin generation or TM-bound thrombin. These results indicate that this assay is not influenced by protein C pathway activation. Dargaud et al. [14] studied thrombin generation by the addition of thrombomodulin to plasma and reported that the measurement of thrombin generation by calibrated thrombinography in the presence of TM can detect the prothrombotic phenotype (factor V Leiden mutation, prothrombin G20210A mutation, protein C deficiency, and protein S deficiency). We believe that our assay is not affected by the protein C pathway because thrombin generation was performed on plates coated with a very low amount of rhs-TM. Furthermore, with respect to the antithrombin III concentration, the amount of thrombin generated and the amount of thrombin bound to TM decreased according to

increases in the antithrombin III concentration, but there was no influence on the TMBT ratio. It has been reported that the inactivation of thrombin by antithrombin is stimulated in the presence of TM [15–20] and that the chondroitin sulfate moiety on TM is responsible for this stimulation [17,19]. There was no chondroitin sulfate moiety in the rhs-TM we used. Thus, the decrease in total thrombin generation or in thrombin bound to rhs-TM was not due to inactivation of thrombin by antithrombin.

We used fibrin inhibitor to prevent the polymerization of fibrin monomer in our assay. However, thrombin binds to fibrinogen (fibrin monomer) in addition to TM [21]. To confirm that the low TMBT ratio was not related to fibrinogen, we examined defibrinated plasma from patients with low TMBT ratios. There was no significant difference in the TMBT ratio between non defibrinated plasma and defibrinated plasma.

Preissner et al. [22] reported that direct interference of TM function affects high molecular polycations such as those in platelet lysates, which are known to contain several glycosaminoglycan-binding proteins such as platelet factor 4, histidine-rich glycoprotein, fibronectin, and thrombospondin. The presence of these polycations in plasma due to platelet activation following thrombosis development cannot be denied in either of our study groups. We measured thrombin generation and the amount of thrombin binding to TM in freeze-thaw preparations of platelet-rich plasma ($20\times10^4/\mu l$ platelets). Neither assay result was affected. Thus, it is possible to evaluate the reactivity of thrombin to TM by dividing the amount of thrombin bound to TM by the total amount of thrombin generated.

To determine whether the decrease in TM-bound thrombin is associated with an increased risk of thrombosis, we studied 124 patients with DVT and 150 age- and sex-matched healthy subjects. Mean t-Th and TMBTh did not differ significantly between the two groups. However, we found that a low TMBT ratio, defined as a value below the 10th percentile of the value in control subjects, is a risk factor for DVT. Lower cutoff values for the TMBT ratio (i.e., below the 5th or 2.5th percentile) were associated with a markedly higher risk of DVT; moreover, this association was not due to the effect of antithrombin III deficiency or protein C or S deficiency. Indeed, when the cutoff was below the 5th percentile, the OR was 9.4 (95% CI, 4.6–19.1) for all patients and 13.1 (95% CI, 6.4–26.9) for patients without deficiencies in natural anticoagulants.

The generation of activated protein C is reduced by decreasing the binding of thrombin to TM on endothelial cells and vessel segments (3), and residual free thrombin activates its natural substrates, platelets, fibrinogen, and factors V, VIII, and XIII. Thrombin dysfunction, i.e., decreased binding to TM, may lead to an increase in free thrombin and decreased protein C activation; impaired TM function has been shown to be a risk factor for venous thromboembolism [10]. The mechanism linking the low TMBT ratio to venous thrombosis, as either an inherited or acquired thrombin abnormality, is unknown.

The amount of thrombin that binds to TM is influenced by the amount of thrombin generated and the amount of time that thrombin is exposed to TM. We showed that thrombin generation in the low TMBT ratio group was markedly decreased compared to that in the normal TMBT ratio group during the reaction period (2–10 minutes) although there was no significant difference in t-Th values between the normal TMBT ratio group and the low TMBT ratio group. This suggests that the delay in thrombin generation in cases of DVT is attributable to the time of thrombin generation, and this delay is considered to lead to decreased binding of thrombin to TM in this measurement system. However, 21 (17%) of the DVT patients showed TMBT ratios higher than the 95th percentile in the control group. Previous studies have shown that hyperlipidemia is an etiologic factor in DVT and that most Japanese idiopathic DVT patients are hyperlipidemic [23,24]. In addition, in hyperlipidemic patients, the prothrombic state is reported to be characterized by increases in factor VII, fibrinogen, and plasminogen activator inhibitor 1 levels [25]. Therefore, this phenomenon of a high TMBT ratio may depend on early completion of thrombin generation with and increased factor VII level.

Our patient group was similar to a cohort we previously described [26]: deficiencies of natural anticoagulants (protein C, protein S, and antithrombin III) were found in approximately 30% of patients. Deficiencies of these inhibitor proteins are established causes of thrombosis. The overall prevalence of deficiencies of these inhibitor proteins is much higher in thrombophilic Japanese patients than in thrombophilic Caucasian patients [26,27]. Moreover, the factor V Leiden mutation and the prothrombin G20210A mutation that are found in Caucasians [28,29] are not found in Japanese [30,31]. In Japanese patients with thrombosis, the prevalence of cases of unknown cause remains greater than 60%. In 35% of Japanese patients in the present study, the TMBT ratio was below the 5th percentile of control values.

Results of our study may suggest that abnormalities resulting in decreased binding of thrombin to TM in anticoagulation are highly prevalent in Japanese patients with venous thrombosis. It will be important to confirm the finding in a larger study.

Acknowledgements

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Frequencies of mild factor V, VII and X deficiencies in a Japanese population

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We investigated the frequencies of coagulation factor deficiencies in a Japanese population. We measured factor II, V, VII and X activity in 100 healthy individuals. A specific factor deficiency was determined according to the factor activity and the ratio of the factor activity to that of other coagulation factors. Seven samples showed factor activity less than the mean −2SD of standardized factor activity (factor II: three; factor V: one; factor VII: one; factor X: one and factor V+factor VII: one). The samples with low factor II and factor VII activity were determined not to be due to deficiency because the ratios of these factor activities to other factor activities were within the range of the mean ± 2SD. We measured activity ratios in the remaining 97 samples and identified one sample with factor V deficiency and two with factor VII deficiency. Thus, six samples with coagulation factor deficiency were identified (factor X: one; factor V: two; factor VII: two and factor V + factor VII: one). These results suggest that the Japanese population has relatively high frequencies of mild factor V, factor VII and factor X deficiencies, in which activity is reduced to approximately 50% (36–64%) of normal plasma.
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Introduction

Coagulation factors II, VII, IX and X are vitamin K-dependent serine proteases and coagulation factor V is a pivotal nonenzymatic cofactor of the prothrombinase complex [1,2]. The related factors of the extrinsic coagulation pathway are factors II, V, VII and X, and the extrinsic coagulation pathway is activated by tissue factor (TF) and factor VII in the presence of Ca^{2+} [3]. Prothrombin time (PT) is used as a screening test of members of the coagulant activity of the extrinsic coagulation pathway, including fibrinogen, factors II, V, VII and X. A prolonged PT indicates the possibility of one or more deficiency of these factors. Deficiency of factors II, V, VII or X is very rare, and there is a low prevalence of these deficiencies in the general population. However, we often observe prolonged PT in Japanese individuals without a history of bleeding. Prolonged PT may be due to a coagulation factor deficiency or differences in TF reagent sensitivity. We investigated the prevalence of coagulation factor deficiency in the extrinsic coagulation pathway and determined whether the prolonged PT was due to TF reagent sensitivity.

Participants and methods

Participants

Participants were 100 healthy volunteers (37 men and 63 women; age, 21–63 years; mean age, 32 years) from our hospital staff and students at our university without a bleeding tendency. Samples were handled with full anonymity; all identifying information was eliminated.

Blood samples

Venous blood samples were collected into a one-tenth volume of 3.13% trisodium citrate. Platelet-poor plasma was prepared by centrifugation at 2800*g* for 10 min and kept at −80°C until use.

Coagulation factor assays

Factor II, V, VII and X activities were assayed by the one-stage clotting method with either rabbit TF (Thromboplastin C Plus; Dade Behring, Marburg, Germany) or recombinant human TF (Recombiplastin; Instrumentation Laboratory, Lexington, Massachusetts, USA) and coagulation factor deficient-plasma (HemosIL; Instrumentation Laboratory) with Coagrex-800 (Sysmex, Kobe, Japan). Pooled plasma was collected to create standard curves for each factor activity and PT. Factor VII antigen was measured by enzyme-linked immunosorbent assay (ELISA) (Asserachrom FVII:Ag kit; Diagnostica Stago, Asnieres-sur-seine, France). Factor V and X antigens were measured by ELISA, using an affinity-purified sheep antihuman factor V and factor X immunoglobulin G (IgG) as a coating antibody with a peroxidase-conjugated sheep anti-factor V antibody and

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a peroxidase-conjugated rabbit anti-factor X antibody as a second antibody, respectively, according to the manufacturer's protocol (Cedarlane, Hornby, Ontario, Canada). The value of each test was expressed as a percentage of the normal pooled plasma. A specific factor deficiency was determined according to the factor activity and the ratio of the factor activity to that of other coagulation factors.

Liver function tests

Aspartic aminotransferase, alanine aminotransferase, γ-glutamyltranspeptidase, cholinesterase, albumin, alkaline phosphatase, total protein, total bilirubin and total cholesterol were measured with a JCA-BM6010 (Japan Electron Optical Laboratory, Tokyo, Japan) as evaluation of a liver function. PIVKA (protein induced by vitamin K absence or antagonist) II was measured using Picolumi PIVKA-II (Sanko Junyaku co., Ltd., Tokyo, Japan) as evaluation of vitamin K absence.

Statistical analysis

StatFlex version 5.0 (Artech, Osaka, Japan) was used for statistical analysis and for determination of mean, standard deviation (SD) and skewness of distribution values.

Results

Distribution of prothrombin time and factors II, V, VII and X

Figure 1 shows the distribution of PT and factors II, V, VII and X. The normal range (mean ± 2SD) for each of the factors and PT studied in the 100 samples was as follows: factor II, 82–118%; factor V, 64–136%; factor VII, 59–143%; factor X, 69–137%; PT, 10–12.5 s.

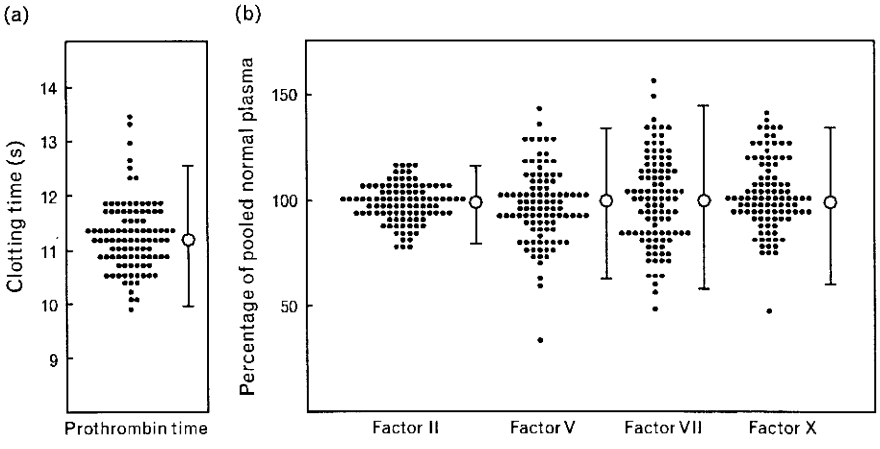
Determination of coagulation factor deficiency from factor activity

Table 1 shows the normal range of ratios used to determine the deficiency of a coagulation factor. Deficiency was suspected when the activity level was less than the mean −2SD of the normal range. Of the 100 sample activities, 3, 1, 1, 1 and 1 were less than the mean −2SD of normal range for factor II, V, VII, X and V + VII, respectively (Fig. 1b). The samples, which had less than mean −2SD of normal for factors V, VII and X, corresponded with the prolonged PT samples. The low values for factor II and VII activity were determined not to indicate deficiency because the ratios of these factor activities to other factor activities (factor II/VII, II/X, VII/II and VII/X) were within the range of the mean ± 2SD. One sample, which had low values for factor V and VII activity, fell below the ratios of factor V/II, V/X, VII/II and VII/X, but the ratio of factor V/VII was within the range of the mean ± 2SD. Three samples with factor deficiency were identified from factor activity (factor II: 0 sample; factor V: one; factor VII: 0; factor X: one and factor V + VII; one) (Table 2A).

Determination of coagulation factor deficiency from ratios of factor activity

We examined the ratios of coagulation factor activity in the 97 remaining samples and identified three samples with ratios less than the mean −2SD. One of these samples was less than the mean −2SD of the ratios of factor V/II, V/VII and V/X, and one of the other two samples was less than the mean −2SD of the ratios of factor VII/II and VII/X. In addition, one sample was less than the mean −2SD of the ratio of factor VII/II, and the factor VII/X ratio was 0.59 (Table 2B). Thus, six samples showed a coagulation factor deficiency according to this

Fig. 1



Distribution of prothrombin time and factors II, V, VII and X in normal individuals. (a) The distribution of prothrombin time; the results were plotted as clotting time. (b) The distribution of factors II, V, VII and X. The results were plotted as percentage of normal pooled plasma. Open circles and bars represent mean ± 2SD of 100 samples for normal individuals.

Table 1 Normal range of ratios used to determine the deficiency of a coagulation factor

	FII/FVII[a]	FII/FX[a]	FVII/FII[a]	FVII/FX[a]	FX/FII[a]	FX/FVII[a]	FV/FII[a]	FV/FVII[a]	FV/FX[a]
Upper limit	1.49	1.30	1.41	1.38	1.32	1.54	1.35	1.49	1.43
Lower limit	0.59	0.66	0.61	0.58	0.76	0.58	0.67	0.59	0.57

Ranges are mean ± 2 SD. F, factor. [a]Activity of factors

method (factor X: one sample; factor V: two; factor VII: two and factor V+VII: one).

Influence of sensitivity of tissue factor reagent

To examine whether low levels of factor activity were associated with differences in TF reagent sensitivity, we measured factor activity in the samples identified with factor deficiencies with the use of recombinant human TF. Five of the six samples showed no difference in factor activity or ratios with rabbit TF and recombinant human TF (data not shown). However, one sample (sample no. 94; factor VII deficiency) showed discrepancies in factor VII activity with rabbit TF, recombinant human TF and factor VII antigen (factor VII activity with rabbit TF: 61%; with recombinant human TF: 97%; factor VII antigen: 140%).

Discussion

In the present study, we found a relatively high prevalence of factor V, VII and X deficiency. The prevalence of factor deficiency was as follows: factor II, 0% (0/100); factor V, 2% (2/100); factor VII, 2% (2/100); factor X, 1% (1/100); and factor V+VII, 1% (1/100). Factors II, VII, IX and X are synthesized by hepatocytes, and the complete synthesis of these factors requires vitamin K as a cofactor for hepatic vitamin K-dependent carboxylase. In the absence of vitamin K or the presence of vitamin K antagonists, these factors are synthesized as the protein induced by vitamin K absence or antagonist (PIVKA) [4]. We measured PIVKA-II to determine whether the factor deficiencies were due to an absence of vitamin K and obtained negative results (data not shown). The liver function of our factor deficient case, which we decided in the present study, showed healthy liver function. Therefore, the deficiencies of factor VII and X are not considered to be due to acquired conditions. Factor V is a cofactor that plays an important role in the coagulation cascade. Approximately 80% of blood factor V circulates in the plasma and the remaining 20% is stored in platelet α-granules. Plasma-derived factor V is synthesized by hepatocytes, and platelet factor V is synthesized by megakaryocytes and absorbed from plasma factor V [2]. We compared factor V activity with those of other factors (factors II, VII and X) to confirm factor V deficiency.

Factor V, VII and X deficiencies are rare autosomal recessive bleeding disorders with an incidence of 1/1 000 000, 1/500 000 and 1/1 000 000, respectively, in the general population. The classification of factor deficiency is based on the results of both immunologic and functional assays. Our participants showed levels of factors V, VII and X that were reduced to approximately 50% (36–64%) of normal plasma. The activities of factor V deficiencies were 41, 61 and 67% and their antigen were 36, 63 and 64%, respectively. The activity and antigen of factor X deficiency was 48 and 89%, respectively. Therefore, the type of factor V and X deficiencies in our study was mild type 1 and type 2, respectively. We examined the effect of different TF reagents on factor activity in the six samples with identified deficiencies. Only one sample with factor VII deficiency showed discrepant values with rabbit TF reagent and recombinant human TF reagent. This factor VII deficiency is considered to be due to abnormal factor VII protein. We also identified a combined factor V and factor VII deficiency. This deficiency is a rare bleeding disorder, with only four cases reported in the literature [5–8].

Only three samples with factor deficiency were identified from a screen of factor activities and PT in the present study. The diagnosis of factor deficiency and an estimate of the prevalence of factor deficiency are complicated by factor activities that are borderline to the normal range.

Table 2 Coagulation factor deficiencies identified in this study

Sample no.	PT (s)	Activity (%)		Ratio		State (deficiency)
A. Determination from factor activity						
6	13.3	FV:36	FV/FII:0.39	FV/FVII:0.43	FV/FX:0.37	FV
36	13.0	FV:63	FV/FII:0.63	FV/FVII:1.31	FV/FX:0.65	FV + FVII
		FVII:48	FVII/FII:0.48	FVII/FX:0.49		
53	13.5	FX: 48	FX/FII:0.50	FX/FVII:0.56		FX
B. Determination from ratios of factor activity						
67	12.3	FV:64	FV/FII:0.65	FV/FVII:0.52	FV/FX:0.57	FV
26	12.5	FVII:64	FVII/FII:0.60	FVII/FX:0.48		FVII
94	12.3	FVII:61	FVII/FII:0.58	FVII/FX:0.59		FVII

Normal range (mean ± 2SD) of PT is from 12.5 to 10.0. F, factor; PT, prothrombin time.

Borderline activity levels may represent a problem for the diagnosis of coagulation factor deficiency.

Results of the present study suggest that the Japanese population may have a relatively high prevalence of mild factor V, VII and X deficiencies. It is necessary to confirm these findings in larger studies of the Japanese population.

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