Table 4 Spearman's correlation coefficients between patient characteristics and thrombogenetic factors in patients taking dual antiplatelet therapy

	Platelet aggregation*	PAI-1	E-selectin	D-dimer	Soluble fibrin
Patient characteristics					
Age (years)	0.038 ($P = 0.729$)	−0.051 ($P = 0.645$)	−0.241 ($P = 0.026$)*	0.167 ($P = 0.127$)	0.079 ($P = 0.472$)
BMI (kg/m^2)	−0.057 ($P = 0.804$)	0.234 ($P = 0.032$)*	0.310 ($P = 0.004$)**	−0.254 ($P = 0.020$)*	−0.163 ($P = 0.139$)
Hypertension	0.042 ($P = 0.452$)	0.181 ($P = 0.132$)	−0.166 ($P = 0.128$)	−0.016 ($P = 0.396$)	0.029 ($P = 0.794$)
Diabetes mellitus	0.063 ($P = 0.570$)	−0.150 ($P = 0.172$)	0.253 ($P = 0.019$)*	−0.009 ($P = 0.943$)	0.145 ($P = 0.186$)
Prior myocardial infarction	−0.106 ($P = 0.334$)	0.058 ($P = 0.598$)	0.131 ($P = 0.131$)	−0.273 ($P = 0.011$)*	−0.046 ($P = 0.579$)
Number of vessel diseases	0.089 ($P = 0.420$)	−0.006 ($P = 0.953$)	0.011 ($P = 0.918$)	−0.185 ($P = 0.073$)	0.248 ($P = 0.022$)*
Biochemical markers					
Fasting glucose	0.243 ($P = 0.025$)*	−0.119 ($P = 0.277$)	0.205 ($P = 0.059$)	0.090 ($P = 0.411$)	−0.097 ($P = 0.378$)
Total cholesterol	−0.010 ($P = 0.928$)	−0.125 ($P = 0.256$)	−0.154 ($P = 0.158$)	0.095 ($P = 0.387$)	0.426 ($P < 0.001$)***
BNP	0.146 ($P = 0.191$)	−0.071 ($P = 0.527$)	−0.177 ($P = 0.111$)	0.411 ($P < 0.001$)***	0.296 ($P = 0.005$)**
Adrenalin	0.099 ($P = 0.367$)	0.169 ($P = 0.122$)	0.022 ($P = 0.843$)	0.002 ($P = 0.969$)	0.148 ($P = 0.177$)
hsCRP	0.009 ($P = 0.932$)	0.115 ($P = 0.295$)	0.132 ($P = 0.230$)	0.064 ($P = 0.581$)	0.049 ($P = 0.655$)
Physiological markers					
AHI	0.048 ($P = 0.671$)	0.304 ($P = 0.005$)**	0.269 ($P = 0.015$)*	0.082 ($P = 0.465$)	0.111 ($P = 0.320$)
ABI	−0.078 ($P = 0.480$)	−0.010 ($P = 0.920$)	−0.111 ($P = 0.836$)	−0.009 ($P = 0.933$)	−0.452 ($P < 0.001$)***
24-h SBP	−0.128 ($P = 0.494$)	0.109 ($P = 0.333$)	0.135 ($P = 0.231$)	−0.056 ($P = 0.620$)	0.032 ($P = 0.779$)
24-h DBP	−0.188 ($P = 0.255$)	0.148 ($P = 0.186$)	0.292 ($P = 0.008$)**	−0.042 ($P = 0.707$)	−0.120 ($P = 0.285$)

*Light transmission assessed by 1 μg/mL of collagen.
BMI, body mass index; hsCRP, high-sensitivity C-reactive protein; BNP, brain natriuretic peptide; PAI-1, plasminogen activator inhibitor-1; SBP, systolic blood pressure; DBP, diastolic blood pressure; AHI, apnea–hypopnea index; ABI, ankle-brachial index.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

Table 5 Multivariate analyses for determination of thrombogenetic factors in patients taking dual antiplatelet therapy

	(R^2; P)	Variables	β*	β (95%CI)	P
PAI-I	(0.09, 0.006)	BMI	0.167	–	0.147
		AHI	0.300	0.402 (0.116–0.687)	0.006
E-selectin	(0.203, <0.001)	Age	−0.127	–	0.236
		BMI	0.323	0.983 (0.365–1.601)	0.002
		Diabetes mellitus	0.279	5.736 (1.566–9.906)	0.008
		AHI	0.126	–	0.253
		24-h DBP	0.169	–	0.136
D-dimer	(0.126, 0.001)	BMI	−0.064	–	0.564
		Prior MI	−0.075	–	0.484
		BNP	0.356	1.928 (0.793–3.063)	0.001
Soluble fibrin	(0.366, <0.001)	Number of VD	0.085	–	0.372
		Total cholesterol	0.349	0.075 (0.035–0.113)	<0.001
		BNP	0.222	3.681 (0.651–6.711)	0.018
		ABI	−0.330	−17.953 (−28.203–7.704)	0.001

β*, standardized coefficient; CI, confidence interval; PAI-1, plasminogen activator inhibitor-1; AHI, apnea–hypopnea index; BMI, body mass index; BNP, brain natriuretic peptide; DBP, diastolic blood pressure; ABI, ankle-brachial index; VD, vessel diseases.

surface that provokes the coagulation cascade, thereby amplifying thrombin generation.[38,39] However, residual platelet activation was not correlated with systemic thrombin generation assessed by plasma SF and resultant fibrinolytic activation assessed by the D-dimer level. The major determinant of thrombin generation was found to be independently associated with total cholesterol, BNP, and ABI, suggesting that thrombin generation in PCI subjects under dual antiplatelet therapy is mainly determined by the degree of impaired cardiac function and/or arteriosclerosis. Plasma PAI-1 was also associated with the presence of sleep apnea syndrome. Although circulating platelets account for increases in plasma PAI-1 and release it following activation,[40] platelet aggregability was not associated with PAI-1. Taken together, these data suggested that many factors may be involved in systemic thrombogenicity, independent of platelet aggregability.

Our data suggested that some patients may benefit from the addition of anticoagulant treatment after PCI. The American College of Cardiology/American Heart Association guidelines recommend anticoagulant therapy in patients with an acute ST-elevation myocardial infarction with extensive regional wall motion abnormalities. However, the routine use of anticoagulant drugs without thienopyridine should be avoided in patients who have undergone PCI because treatment with aspirin and ticlopidine results in a lower rate of stent thrombosis as compared with a combination of aspirin plus warfarin.[41] No trial has closely evaluated the safety and efficacy of anticoagulant therapy in combination with dual antiplatelet therapy in patients undergoing PCI. Large-scale trials are thus needed to confirm any recommendations. Our study should be interpreted in light of its limitations; for ethical reasons we could not obtain proper control patients who had not taken any antiplatelet drug after PCI. This was because dual antiplatelet therapy is the gold standard to reduce clinical events in patients who have undergone PCI.

In conclusion, the current study has demonstrated that dual antiplatelet therapy effectively inhibited its pharmacological targets, although we found inter-individual variability in platelet aggregation, which was at least partly explained by hyperglycemia. On the other hand, thrombin generation, inhibition of fibrinolytic activity, and endothelial dysfunction were not determined by platelet aggregability, but by other aspects of the patients' backgrounds, such as obesity, sleep apnea, diabetes mellitus, cardiac dysfunction, and/or atherosclerotic burden. Our findings indicated that some patients remain at risk of subsequent thrombotic complications after PCI despite adequate dual antiplatelet therapy. Large-scale prospective studies are required to determine which markers are associated with the risk of further cardiovascular events after PCI and to examine interventions such as tight plasma glucose control, anticoagulation, and continuous positive air way pressure therapy.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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REGULAR ARTICLE

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

Etsuji Suehisa [a,*], Tomio Kawasaki [b], Masayuki Toku [a], Yoh Hidaka [a]

[a] *Laboratory for Clinical Investigation, Osaka University Hospital, Osaka, Japan*
[b] *Cardiovascular Surgery, Osaka University Graduate School of Medicine, Osaka, Japan*

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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.

* Corresponding author. Laboratory for Clinical Investigation, Osaka University Hospital, 2-15 Yamada-oka, Suita-City, Osaka, 565-0871, Japan. Tel.: +81 6 6879 6655; fax: +81 6 6879 6657.
E-mail address: esuehisa@hp-lab.med.osaka-u.ac.jp (E. Suehisa).

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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).

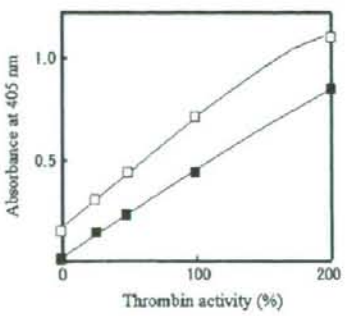

Figure 1 Standard curves for total thrombin generation (□) and thrombomodulin-bound thrombin (■). Reactions were performed as described in the Materials and methods.

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–

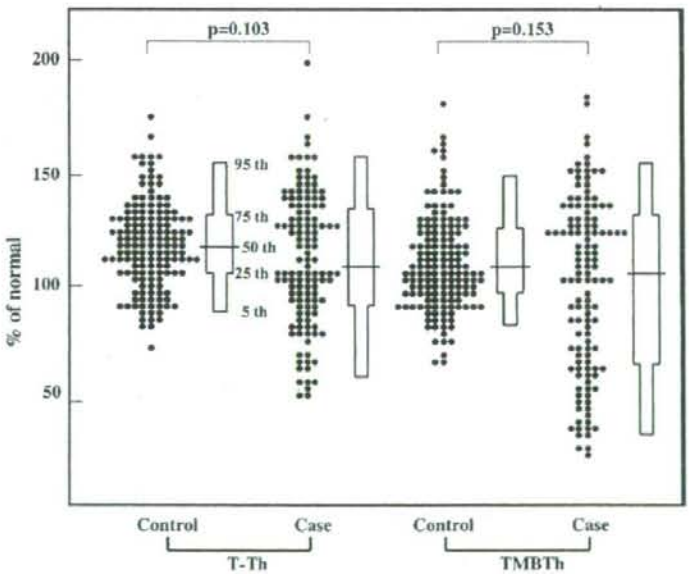

Figure 2 Distribution of total thrombin and thrombomodulin-bound thrombin activities in DVT patients (n=124) and control subjects (n=150). Values are shown as percentages of pooled normal plasma values. The 5th, 25th, 50th, 75th, and 95th percentiles are indicated. Significance was determined by Mann-Whitney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

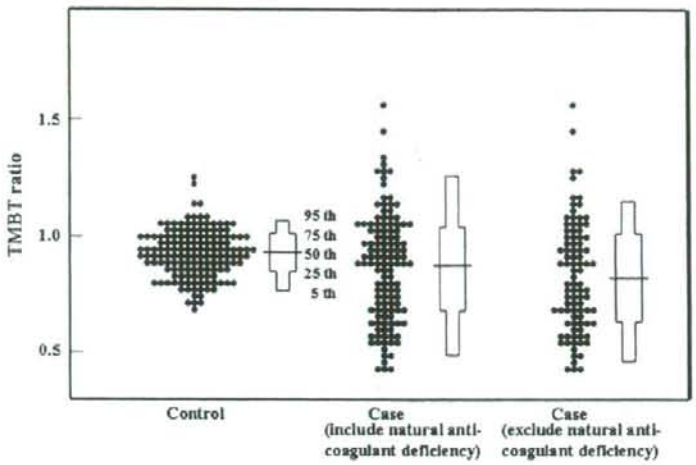

Figure 3 Binding capability to thrombomodulin in control subjects and patients with deep vein thrombosis. The results were plotted as thrombomodulin-bound thrombin ratios (TMBT ratio: TMBTh/T-Th). The 5th, 25th, 50th, 75th, and 95th percentiles are indicated. Differences in TMBT ratio between the total 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 ($p=0.43$).

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).

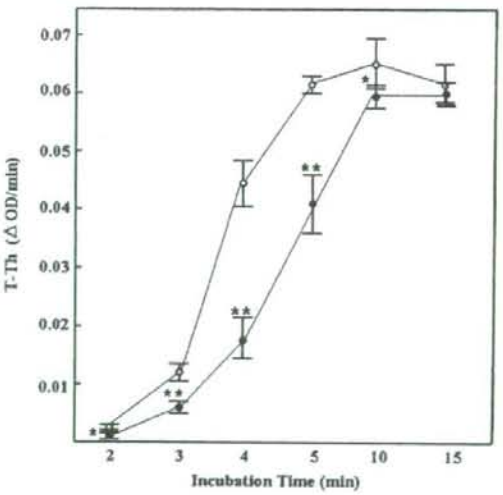

Figure 4 Comparison of thrombin generation in samples with normal and low TMBT ratios. Time courses of thrombin generation in normal (○) and low (●) TMBT ratios are shown. Thrombin generation is represented by ΔOD/minutes at 405 nm absorbance. Symbols and bars represent mean ± SE of 8 samples for normal 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.

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 ($20 \times 10^4/\mu l$ 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

Chiduko Yasunaga[a], Etsuji Suehisa[a], Masayuki Toku[a], Tomio Kawasaki[b] and Yoh Hidaka[a]

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. *Blood Coagul Fibrinolysis* 19:597–600 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: coagulation factor deficiency, frequency, Japanese, factor V, factor VII, factor X

[a]Laboratory for Clinical Investigation, Osaka University Hospital and [b]Department of Cardiovascular Surgery, Osaka University Graduate School of Medicine, Suita, Japan

Correspondence to Etsuji Suehisa, Laboratory for Clinical Investigation, Osaka University Hospital, 2-15 Yamada-oka, Suita 565-0871, Japan
Tel: +81 6 6879 6655; fax: +81 6 6879 6655;
e-mail: esuehisa@hp-lab.med.osaka-u.ac.jp

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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

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.

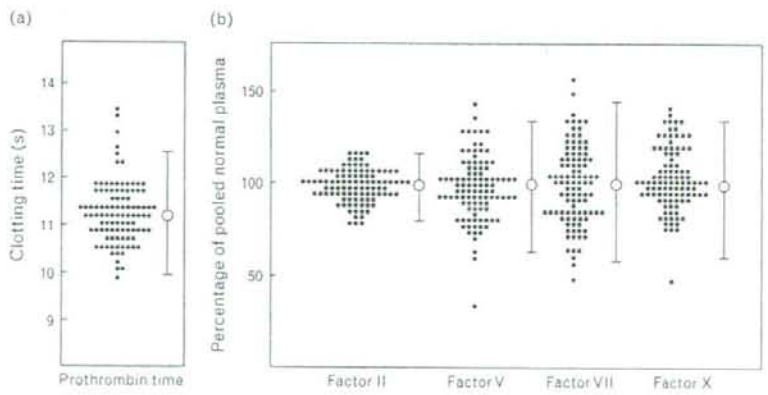
Determination of coagulation factor deficiency from factor activity

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						
87	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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Safety and efficacy of lower-dose unfractionated heparin for prophylaxis of deep vein thrombosis and pulmonary embolism in an Asian population

Hiromi Ugaki[a], Takayuki Enomoto[a], Kazuko Fujiwara[a], Tadashi Kimura[a] and Tomio Kawasaki[b]

The objective of this study is to analyze the tolerance and efficacy of the subcutaneous administration of a reduced 2500-unit low-dose unfractionated heparin given for an efficacious, yet Asian population-sensitive, prophylaxis for deep vein thrombosis and fatal pulmonary embolism. Eighty-seven Japanese patients were operated on either for abdominal or pelvic complications or both, as well as for gynecologic conditions including ovarian, cervical, and corpus cancers. Thirty-two of the patients were administered the experimental low dose of unfractionated calcium heparin for prophylaxis. The 2500 units of low-dose unfractionated heparin were given subcutaneously 2 h preoperatively and again 12 h postoperatively. Other standard methods of mechanical prophylaxis, including graduated compression stockings and intermittent pneumatic compression, were performed. Fifty-five of the patients were not administered heparin, but did receive the same standard mechanical graduated compression stockings and intermittent pneumatic compression prophylaxis. We compared the surgical and postsurgical complications noted for low-dose unfractionated heparin patients with the results of those who received no heparin prophylaxis and analyzed this data using the Mann–Whitney *U*-test. There was no significant difference in the mean of the blood loss volumes. There were also no significant differences found in the perioperative bleeding complications between the two groups. However, three (3/55; 6%) of the patients in the no-heparin group suffered a symptomatic pulmonary embolism, although none were fatal. There were no pulmonary embolism onsets in the heparin prophylaxis group. We feel that we have provided evidence that several serious complications, such as perisurgical hemorrhage, deep vein thrombosis, fatal pulmonary embolism, and increased postoperative recovery times, can be prevented by prophylaxis with 2500-unit low-dose unfractionated heparin. *Blood Coagul Fibrinolysis* 19:585–589 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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[a]Department of Obstetrics and Gynecology and [b]Department of Surgery, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

Correspondence to Hiromi Ugaki, MD, Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 567-0871, Japan
Tel: +81 6 6879 3351; fax: +81 6 6879 3359; e-mail: hirohiromimi@hotmail.com

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Introduction

Deep venous thrombosis (DVT) and thrombotic pulmonary embolism are significant complications in postoperative patients. The risk of perioperative development of DVT among patients with malignant gynecologic disease undergoing major operations ranges from 20 to 38% if no preventive measures are taken [1–3]. Approximately, 50% of these perioperative DVT form during the actual operation, an additional 25% occur within 72 h of surgery [4,5]. Only 5% of all perioperative DVT form later than 1 week postoperatively.

Forty percent of all deaths directly resulting from gynecologic surgery are estimated to be attributable to a thrombotic pulmonary embolism [6]. One study shows the risk factors for DVT to be the following: age more than 40 years, obesity, perioperative immobility, trauma, malignancy, previous radiation therapy, and medical conditions, including diabetes mellitus, cardiac disease such as heart failure, severe varicose veins, previous DVT (with or without embolization), chronic pulmonary disease, and antithrombin, protein C, or protein S deficiency [7]. Another study discusses the risk factors occurring during abdominal and pelvic surgery [2].

Intraoperative factors associated with postoperative DVT include an increased duration of anesthesia, increased blood loss, and the need for a transfusion in the operating room. Recognizing that these risk factors exist, it becomes imperative that we consider and implement more effective prophylactic measures for perisurgical DVT.

Prophylaxis of DVT is generally recommended for patients undergoing general surgical procedures [8–10]. There is good evidence that prophylaxis with low-dose unfractionated heparin (LDUH), with 5000 units being the dose, significantly reduces the incidences of both DVT

and fatal pulmonary embolism. In addition to its proven efficacy, LDUH is inexpensive and easily administered.

Several reports regarding native Asian populations have critically noted that they have a uniquely lower prevalence of DVT and pulmonary embolism [11–19]. One report suggests that prevalence of DVT and pulmonary embolism among Hong Kong Chinese was only 10% of that in Western countries, and that when pulmonary embolism occurred it was clinically milder [16]. Kobayashi *et al.* [20] suggest that this may be in part due to their diet and note that the incidence of pulmonary embolism is on the rise as diets change to a more Western style.

Perioperative and postoperative DVT and pulmonary embolism in Asian populations are, however, still problematic. Although levels of fatal pulmonary embolism dropped significantly when Japan instituted its insurance-reimbursed venous thromboembolism prophylactic measures in 2004, the rate has now become stabilized in the last 3 years, and is not improving further [21]. The question arises as to whether Asian surgical teams need to use as intensive a DVT prophylaxis dose as is performed in Western countries, since the appearance of DVT and pulmonary embolism in Asians is still currently significantly lower than it is in Westerners.

The objective of this study is to analyze the safety and efficacy of the subcutaneous administration of a reduced 2500-unit LDUH given for an efficacious, yet Asian population-sensitive, prophylaxis for DVT and fatal pulmonary embolism.

Materials and methods

This is a retrospective study conducted at the Osaka University Hospital of Japan. From November 2005 to February 2007, 87 Japanese patients were operated on either for abdominal or pelvic complications or both, as well as for gynecologic conditions including ovarian, cervical, and corpus cancers. The procedures were performed under general anesthesia. Patients were excluded from the study if they required presurgical anticoagulant or antiplatelet therapy. They were also excluded if they had hepatic or renal failure, or a history of systemic bleeding diathesis.

All patients who were scheduled to have an operation were required to have a preoperative DVT screening based upon comparative measurements of the diameter of their thighs and legs, as well as a clinical examination by palpation for Homan's sign. DVT was suspected if the difference in diameter measurement between the left and right thighs or legs was 2 cm or above [22]. A diagnosis of DVT was then confirmed using ultrasonography. Patients were excluded from the study if they were diagnosed with DVT prior to the operation.

All patients provided a written informed consent. The patients were operated on by the same two well respected surgeons to ensure the most accurate results for this study. The majority of patients who received the experimental 2500-unit LDUH for DVT prophylaxis did so after March 2006. They were compared with a similar cohort of patients, who had received no heparin prophylaxis, most of whose surgeries occurred prior to March 2006.

Thirty-two of the patients were administered the experimental low-dose of unfractionated calcium heparin for prophylaxis. The 2500 units of LDUH were given subcutaneously 2 h preoperatively and again 12 h postoperatively. Other standard methods of mechanical prophylaxis, including graduated compression stockings (GCS) and intermittent pneumatic compression, (IPC) were performed. Fifty-five of the patients were not administered heparin, but did receive the same standard mechanical GCS and IPC prophylaxis.

Perioperative blood loss was recorded from sponge weights and suction aspirates. We compared the surgical and postsurgical complications noted for LDUH patients with the results of those who received no heparin prophylaxis and analyzed the data using the Mann–Whitney *U*-test.

Results

Table 1 shows several important characteristics of the patients, including the patient's age, BMI, activated partial thromboplastin time (APTT), hemoglobin content, and platelet counts. The characteristics of the two groups had no significant differences. The mean of the operational duration was not significantly different between the two groups.

Table 1 Patient characteristics

	LDUH ($n=30$)	No heparin prophylaxis ($n=57$)	*P*
Mean age (years)	55.3 ± 11.6	54.8 ± 11.8	0.8509
BMI	22.9 ± 5.0	21.7 ± 3.9	0.2463
APTT (s)	30.2 ± 4.3	30.0 ± 3.5	0.857
Hemoglobin (g/dl)	12.7 ± 1.6	12.7 ± 1.3	0.594
Platelets ($\times 10^4$/dl)	26.9 ± 9.7	28.7 ± 12.7	0.5123
Diagnosis			
Ovarian cancer	11	18	
Corpus cancer	16	26	
Cervical cancer	3	13	
Surgical procedure			
TAH BSO	3	7	
TAH BSO PLN	8	5	
TAH BSO PLN PAN	9	26	
RH BSO PLN	10	19	
Surgical data			
Mean operative time (min)	317.8 ± 144.4	363.0 ± 138.1	0.0969

APTT, activated partial thromboplastin time; BMI, body mass index; BSO, bilateral salpingo-oophorectomy; LDUH, low-dose unfractionated heparin; PAN, para aortic lymphadenectomy; PLN, pelvic lymphadenectomy; RH, radical hysterectomy; TAH, total abdominal hysterectomy.

Table 2 Bleeding during surgical procedure

	LDUH ($n=30$)	No heparin prophylaxis ($n=57$)	P
Mean operative blood loss (ml)	1054.0 ± 1221.0	912.0 ± 814.0	0.9602
Diagnosis			
Ovarian cancer	1773.0 ± 1864.2	1457.6 ± 1153.3	0.7958
Corpus cancer	704.3 ± 554.5	524.6 ± 373.6	0.4293
Cervical cancer	598.8 ± 123.9	975.9 ± 474.4	0.1464
Surgical procedure			
TAH BSO	693.3 ± 828.6	504.3 ± 864.6	0.6667
TAH BSO PLN	396.3 ± 343.3	286.0 ± 101.9	0.9433
TAH BSO PLN PAN	1538.0 ± 1835.8	1072.4 ± 972.4	0.7885
RH BSO PLN	1204.5 ± 799.6	1004.2 ± 554.9	0.5818

APTT, activated partial thromboplastin time; BMI, body mass index; BSO, bilateral salpingo-oophorectomy; LDUH, low-dose unfractionated heparin; PAN, para aortic lymphadenectomy; PLN, pelvic lymphadenectomy; RH, radical hysterectomy; TAH, total abdominal hysterectomy.

Table 2 shows the bleeding volume during the surgical procedure, comparing the blood loss volume in the surgical procedures where a prophylactic 2500 units of LDUH was administered with the group that received no prophylaxis. There was no significant difference in the means of the blood loss volumes. There were also no significant differences found in the perioperative bleeding complications between the two groups. However, three (3/55; 6%) of the patients in the no-heparin group suffered a symptomatic pulmonary embolism, although none were fatal. There were no deaths in this study attributable to thromboembolism. We did not observe that resurgery was required for postsurgical abdominal hematomas during this study.

Discussion

Gynecologic surgery patients undergoing pelvic surgical procedures are at a higher-than-average risk than for other surgical patients of developing perioperative and postoperative DVT and pulmonary embolism. According to the American College of Chest Physician's guidelines [23], patients undergoing extensive surgery for malignancy, and for patients with additional DVT/pulmonary embolism risk factors, are recommended to have routine prophylaxis with LDHU [5000 units three times daily (t.i.d.), grade 1A], or a higher dose of low-molecular-weight heparins (LMWH) (i.e., >3400 units/day, grade 1A). Alternative considerations include IPC alone continued until hospital discharge (grade 1A), or a combination of LDUH or LMWH plus mechanical prophylaxis with graduated compression stockings (GCS) or intermittent pneumatic compression (IPC) (all grade 1C).

Most prophylaxis trials of subcutaneous LDUH administered a dose of 5000 units 1–2 h before surgery, followed by administration of 5000 units b.i.d. (twice daily) or t.i.d. until patients were either ambulating or were discharged from the hospital. A meta-analysis of 46 randomized clinical trials in general surgery compared therapy with LDUH with no prophylaxis or a placebo [8]. The rate of DVT was significantly reduced [from 22 to 9%; odds ratio (OR) 0.3; needed to be treated (NNT) 7], as were the rates of symptomatic pulmonary embolism (from 0.8 to 0.3%; OR 0.4; NNT 182), and the all cause mortality (from 4.2 to 3.2%; OR 0.8; NNT 97). Prophylaxis with LDUH was associated with a small increase in the rate of bleeding events (from 3.8 to 5.9%; OR 1.6; NNT 47). These findings were verified in another meta-analysis in which the rate of wound hematomas was increased with LDUH use (6.3 vs. 4.1% in control subjects; OR 1.6; NNT 45), although the rate of major bleeding was not [24]. Although both meta-analyses concluded that the administration of heparin, 5000-units t.i.d., was more efficacious than that of 5000-units b.i.d., without increasing bleeding, this was based on indirect comparisons, and we are not aware of any studies that directly compared these two regimens.

LMWH are fragments of unfractionated heparin produced by either chemical or enzymatic depolymerization. LMWH are as effective as LDUH in the prevention of perioperative DVT [25]. For general surgery patients, LDUH and LMWH have similar efficacy and bleeding rates. In high-risk general surgery patients, higher doses of LMWH provide greater protection of perioperative DVT than lower doses. Bergqvist *et al.* [26] reported that in cancer patients, prophylaxis with dalteparin, 5000 unit daily was significantly more efficacious than with 2500 unit daily, without an increased risk of bleeding in northern Europeans. However, according to our results, it could be sufficiently efficacious at a 2500 unit dose of LMWH for prophylaxis of DVT and pulmonary embolism in an Asian population. Some studies have reported significantly fewer wound hematomas and other bleeding complications with LMWH than with LDUH, whereas, other trials have shown the opposite effect [27–33]. Two meta-analyses that found similar efficacy for LDUH and LMWH, described differences in bleeding rates that were dependent on the dose of LMWH used. Lower doses of LMWH (i.e., ≤3400 U daily) were associated with less bleeding than LDUH (3.8 vs. 5.4%, respectively; OR 0.7), whereas, higher doses resulted in more bleeding events (7.9 vs. 5.3%, respectively; OR 1.5) [34].

Furthermore, the clinical advantage of LMWH over LDUH include its once-daily administration and the lower risk of heparin-induced thrombocytopenia (HIT) [35]. Therefore, LMWHs are becoming more commonly used.

The reasoning for using LDUH administered as a reduced 2500 units in this study, as opposed to the more traditional 5000 units, is that in Japan no other form of chemoprophylaxis against venous thromboembolism except for LDUH is permitted. Additionally, there are significantly lowered risk factors for venous thrombosis when comparing Asian with Western patient populations,

so by lowering the dosage we might lower some of the undesired side effects of LDUH.

To minimize risk, we have developed criteria to screen out patients who have had a DVT history before surgery. Criado and Burnham [22] evaluated the clinical presentation of DVT and found that a difference in calf circumference of less than 2 cm demonstrated a negative predictive value of 85% among outpatients, and of 93% in inpatients. Therefore, we were able to eliminate patients with a high risk of DVT and pulmonary embolism before surgery. We can find no other reports of 2500-unit LDUH administration preoperatively and postoperatively, such as in our current study.

In conclusion, we feel that we have provided good evidence that complications such as perisurgical hemorrhage, DVT, fatal pulmonary embolism and increased postoperative recovery times can be prevented by prophylaxis with 2500-unit LDUH. We expect that the tolerance and efficacy of prophylaxis with 2500-unit LUDH is not significantly different from 5000-unit LDUH or LMWH. However, because this study is small in patient number, we hope to receive future funding to extend this study by conducting an initial randomized control study for corroborative evidence.

A low-dose of LMWH for prophylaxis of DVT and pulmonary embolism could be just as efficacious as 2500 unit of LDUH in our Asian populations; however, we can find no reports concerning such a low dose of LMWH for prophylaxis for Asian populations, so further study would be necessary to show that efficacy and unit.

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