発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
田中一郎、天野景裕、瀧正志、岡敏明、酒井道生、鳴緑倫、白幡　聡、竹谷英之、花房秀次、日笠聡、福武勝幸、藤井輝久、堀越泰雄、松下正、松本剛史、三室淳、吉岡章	東日本大震災で被災された血友病患者への支援	日本血栓止血学会誌	22(5)	259-265	2011
Saito T, Ohashi K, Utoh R, Shimizu H, Ise K, Suzuki H, Yamato M, Okano T, Gotoh M.	Reversal of diabetes by the creation of neo-islet tissues into a subcutaneous site using islet cell sheets.	Transplantation.	92	1231-1236	2011
Ohashi K, Mukobata S, Utoh R, Yamashita S, Masuda T, Sakai H, Okano T.	Production of islet cell sheet using cryopreserved islet cells.	Transplant Proc.	43	3188-3191	2011

研究成果の刊行物・別刷

Thrombosis Research xxx (2011) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Lack of association between serum paraoxonase-1 activity and residual platelet aggregation during dual anti-platelet therapy

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ARTICLE INFO

Article history:
Received 3 August 2011
Received in revised form 18 October 2011
Accepted 30 October 2011
Available online xxxx

Keywords:
antiplatelet agent
platelet pharmacology
coronary syndrome

ABSTRACT

High residual platelet aggregability during thienopyridine treatment occurs because of low levels of the active drug metabolite, and is associated with an increased rate of major adverse cardiovascular events. Recent findings suggest that paraoxonase-1 (PON1) is a major determinant for clopidogrel efficacy. The aim of this study was to assess the impact of serum PON1 activity on platelet aggregability in thienopyridine-treated patients. In 72 patients receiving treatment with aspirin and ticlopidine after acute coronary syndrome, various laboratory data including the formation of platelet aggregations induced by agonists were compared with serum PON1 activities, measured as paraoxonase and homocysteine thiolactone hydrolase (HTLase). Serum paraoxonase activity was significantly associated with HTLase activity ($R=0.4487$, $P<0.0001$). These PON1 activities were not correlated with any parameters for platelet aggregation, hypertension, sleep apnea, and diabetes mellitus. In contrast, serum PON1 activities seemed to be involved in cardiac function, with brain natriuretic peptide and ejection fraction being significantly correlated with serum HTLase activity ($R=-0.2767$, $P=0.0214$) and paraoxonase activity ($R=0.2558$, $P=0.0339$), respectively. Paraoxonase activity also demonstrated a significant association with increased levels of ankle-brachial index ($R=0.267$, $P=0.0255$). Serum PON1 activities did not influence platelet aggregability during treatment with thienopyridine. However, they might modulate cardiac function after acute coronary syndrome and progression of atherosclerosis.

Introduction

The concept of antiplatelet resistance, particularly poor responsiveness to thienopyridine, has received increasing attention in recent years because of its reported involvement in cardiovascular events after percutaneous coronary artery intervention (PCI) [1-3]. Thienopyridines such as clopidogrel and ticlopidine are rapidly absorbed prodrugs, and must therefore be converted to an active metabolite to exert their inhibitory actions at the target $P2Y_{12}$ ADP nucleotide receptor on platelets. This conversion is *via* a two-step process involving the hepatic cytochrome P450 (CYP) enzyme pathway [4]. Resistance to clopidogrel was thought to result mainly from decreased CYP function leading to reduced active metabolite production [4]. Indeed, individuals carrying the loss-of-function polymorphism of the *CYP2C19* allele had significantly lower levels of the active metabolite of clopidogrel, and a higher rate of major adverse cardiovascular events [5,6]. Drug interaction with the CYP2C19 inhibitor, omeprazole, might also reduce the production of active metabolites [7,8].

Very recently, it was reported that paraoxonase-1 (PON1) is a major and essential factor in the production of active metabolites from clopidogrel [9]. PON1 hydrolyses 2-oxoclopidogrel (an oxidative metabolite of clopidogrel) to form the final active metabolite, a thiol derivative of clopidogrel (Supplemental Fig. 1) [9]. PON1 is a high-density lipoprotein-associated enzyme that prevents oxidative modification of low-density lipoprotein [10]. The *PON1* genotype (Q192 allele) has significant dose-dependent associations with decreased levels of serum PON1 activity and with increased levels of oxidative stress [11]. PON1 has multiple enzyme activities including paraoxonase, arylesterase, and thiolactonase (Supplemental Fig. 1). Although the full range of endogenous substrates hydrolysed by PON1 remains to be elucidated, PON1 has been shown to produce homocysteine from homocysteine thiolactone *via* its homocysteine thiolactone hydrolase (HTLase) activity [12].

Abbreviations: PON1, paraoxonase-1; HTLase, homocysteine thiolactone hydrolase; PCI, percutaneous coronary artery intervention; CYP, cytochrome P450; BNP, brain natriuretic peptide.

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doi:10.1016/j.thromres.2011.10.033

We have previously investigated the mechanisms and clinical backgrounds that determine residual platelet aggregability, and attempted to ascertain whether platelet aggregability is involved in systemic thrombogenicity during dual antiplatelet therapy [13]. Using this previous population, we have retrospectively measured actual serum PON1 activities, measured as paraoxonase and HTLase, in 72 patients treated with ticlopidine and aspirin, and assessed the correlation between PON1 and platelet aggregability.

Methods

Patients

The institutional review board approved all study protocols, and informed consent was obtained from all participants. The design and protocol of this study has been described previously [13]. Briefly, we enrolled consecutive hospitalized patients from July 2006 to April 2007 who were treated by PCI because of symptomatic coronary artery disease. After normalization of cardiac enzymes, patients underwent blood sampling, ankle-brachial index monitoring and cardiorespiratory monitoring.

Blood collection and platelet aggregation

Platelet aggregation was assessed as described previously [14]. A fasting venous sample was carefully collected, and platelet-rich plasma was obtained by centrifugation. The aggregation response was measured based on the light scattering intensities obtained with a PA-200 platelet aggregation analyzer (Kowa Co. Ltd., Tokyo, Japan). This device is particularly sensitive for detecting and classifying the size of platelet aggregates (small, medium, and large) [14]. Platelet aggregation was stimulated with collagen (Hormon-Chemie, Munich, Germany), ADP (MC Medical Co., Tokyo, Japan) and thrombin receptor-activating peptide (TRAP; Invitrogen Co., Carlsbad, CA), a specific agonist for protease activating receptor-1. Blood samples (serum and plasma) were stored at −80 °C until analysis.

Laboratory testing

Plasma levels of plasminogen activator inhibitor-1 antigen, D-dimer, E-selectin and soluble fibrin were assayed using an automated latex agglutination assay (LPIA-S500; Mitsubishi Chemical Medience Co., Tokyo, Japan) based on conjugated monoclonal antibodies. The concentrations of brain natriuretic peptide (BNP) (ShionoRIA BNP kit; Shionogi USA, Inc. Florham Park, NJ) were measured by SRL Inc. (Tokyo, Japan).

Measurement of serum PON1 activities

We quantified paraoxonase and HTLase activities as a measure of serum PON1 activity (Supplemental Fig. 1). Serum paraoxonase activity was measured by using paraoxon as a substrate (Fully Automated Paraoxonase Activity Measurement Kit, Rel Assay Diagnostics, Gaziantep, Turkey). HTLase activity was measured by a hydrolysis of γ-thiobutyrolactone (Alfresa Auto HTLase, Alfresa Pharma Corp., Osaka, Japan). HTLase hydrolyzes the lactone ring of the substrate γ-thiobutyrolactone, producing free thiols that are detected using Ellman's reagent (DTNB; 5,5'-dithiobis (2-nitrobenzoic acid)). Assay reproducibility was high (coefficient of variation was less than 6%).

Statistical analysis

Statistical analyses were performed using Prism v5 (GraphPad software, Inc, La Jolla, CA). The associations between the individual parameters were calculated using Spearman's correlation method. All reported P values are two-sided; a P value of less than 0.05 was considered to indicate statistical significance.

Results

Patients

Of the 85 patients from our previous study, we selected 72 patients taking 100 mg / day of aspirin and 200 mg / day of ticlopidine after acute coronary syndrome. Base line characteristics of the study population are summarized in Table 1.

Lack of correlation of serum PON1 activities with platelet aggregation

We initially examined serum PON1 activities (measured by paraoxonase and HTLase activity). As show in Fig. 1, serum HTLase activity, but not paraoxonase activity, appeared to be normally distributed across the study population (HTLase: 130.3 ± 36.7 U/L; paraoxonase: 62.65 ± 25.27 U/L). These PON1 activities were significantly correlated ($R = 0.4487$, $P < 0.0001$). To examine whether serum PON1 activities determine platelet aggregability during dual antiplatelet therapy, serum PON1 activities were compared with several parameters of platelet aggregation. However, none of these parameters was significantly associated with PON1 activities (Fig. 2 and Table 2).

Correlation between serum PON-1 activities and cardiac function

We next compared serum PON1 activities with parameters for hypertension, sleep apnea, diabetes mellitus, hyperlipidemia, blood coagulation, arteriosclerosis, and cardiac dysfunction. Using linear regression analysis, we determined that only HDL cholesterol and BNP were correlated with HTLase activity (Table 3). Paraoxonase activity was associated with triglyceride, D-dimer, ankle-brachial index, and ejection fraction (Table 3). The medication including use of diuretics, angiotensin II receptor blocker, angiotensin converting enzyme inhibitor, beta blocker, calcium channel blocker, or statin did not demonstrate a significant association with serum PON1 activities (Supplemental Table 1). These data suggest that decreased levels of PON1 activity might lead to the acceleration of atherosclerosis and cardiac dysfunction after acute coronary syndrome.

Table 1
Characteristics of the study population.

Variables	Total subjects (n = 72)
Age, years	62.15 ± 11.62
Men, n (%)	57 (80)
BMI, kg/m^2	25.11 ± 3.514
Systolic blood pressure (mmHg)	125.3 ± 21.08
Diastolic blood pressure (mmHg)	76.6 ± 11.28
Pulse rate (/min)	72.39 ± 14.59
Blood sugar (mg/dl)	118.3 ± 50.43
HbA1c (%)	6.76 ± 1.891
Triglyceride (mg/dl)	130.2 ± 53.01
Total cholesterol (mg/dl)	167.7 ± 36.77
LDL cholesterol (mg/dl)	100.4 ± 30.35
HDL cholesterol (mg/dl)	41.3 ± 12.72
CPK max (U/L)	2,194 ± 2,211
BNP (pg/ml)	151.4 ± 183.6
Concomitant medications	
Antiplatelet agents, n (%)	
Aspirin + Ticlopidine	72 (100)
Antihypertensive medication, n (%)	66 (91.7)
Statin, n (%)	55 (76.4)
NSAIDs, n (%)	0 (0)

Data for continuous variables are expressed as the mean ± SD. BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BNP, brain natriuretic peptide; NSAID, non-steroidal anti-inflammatory drug.

Please cite this article as: Ohmori T, et al, Lack of association between serum paraoxonase-1 activity and residual platelet aggregation during dual anti-platelet therapy, Thromb Res (2011), doi:10.1016/j.thromres.2011.10.033

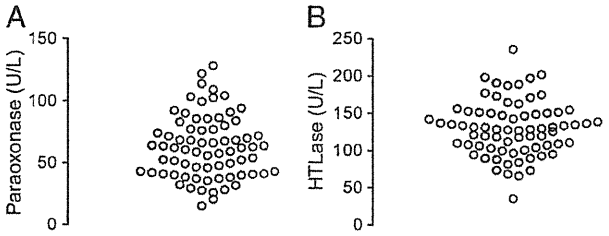

Fig. 1. Serum paraoxonase and HTLase activities in the study population.

Discussion

Inhibition of the $P2Y_{12}$ nucleotide receptor, an ADP receptor on platelets, is currently the gold-standard therapy for the prevention of ischemic events in patients undergoing PCI [15,16]. Although the second-generation thienopyridine, clopidogrel, is recommended by a number of current clinical guidelines, the inter-individual variability of its efficacy is a major drawback in its clinical use [17]. Better understanding of this variability in the efficacy of clopidogrel and other thienopyridines is vital at a time when the number of PCIs is increasingly rapidly. The loss-of-function polymorphism of the CYP2C19 allele has attracted attention as a potential factor in clopidogrel efficacy [4-6], while an elegant recent study suggested that PON1 is a major determinant in the production of the final active metabolite of clopidogrel [9]. In this study, we measured serum two PON1 activities in acute coronary syndrome and compared them with platelet aggregability in patients receiving dual antiplatelet therapy. We could identify no correlation between PON1 activities and any parameter for platelet aggregation in our population.

Several explanations may exist for the discrepancy between our result and the previous report. First, genetic divergence between Caucasian and Japanese patients might affect the result. The Japanese population is reported to express predominantly the 192R allele of *PON1* (192QQ: 18.2%; 192QR: 40.9%; 192RR: 40.9%) [18], whereas the Caucasian population in a large cohort study tended to express the 192Q variant (192QQ: 46.3%; 192QR: 43.9%; 192RR: 9.8%) [11]. The Q allele of *PON1* genotype was significantly and dose-dependently associated with decreased serum PON1 activity, whereby 192QQ, 192QR and 192RR had comparatively low, intermediate and high PON1 activity, respectively [11]. In contrast, the frequency of polymorphism for *CYP2C19*, a key enzyme in clopidogrel oxidation, varies among races, with loss-of-function polymorphisms reportedly being more common in Asian patients [19,20]. However, even in a genetically homogenous population, the *CYP2C19* allele was reported to account for only 12% of the variability in clopidogrel efficacy, whereas the *PON1*

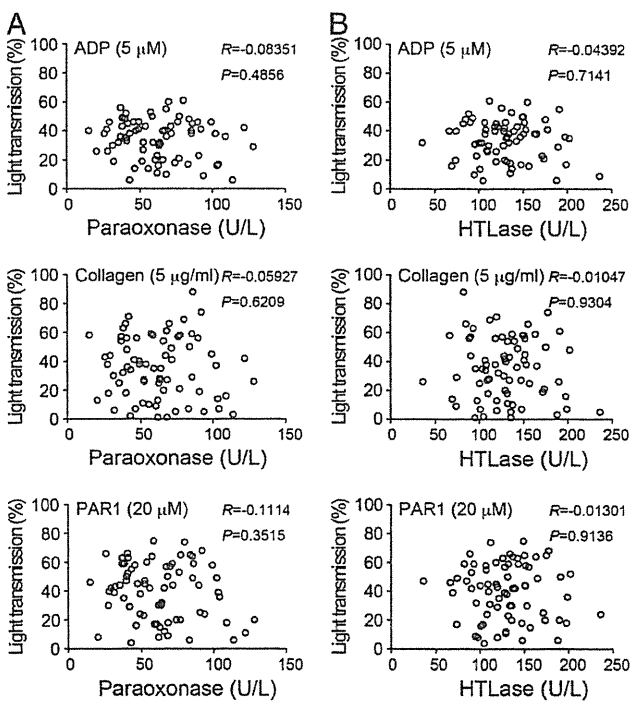

Fig. 2. Association between PON1 activities and platelet aggregation. Platelet aggregation induced by 5 μM ADP, or 5 μg / ml of collagen, or 20 μM TRAP was assessed by aggregometry, and was expressed as light transmission (%). Serum paraoxonase activities (U / L) (A) and HTLase activities (U / L) (B) were compared with platelet aggregation using Spearman's rank correlation coefficient.

Table 2
Correlation between serum paraoxonase activities and platelet aggregation.

	Paraoxonase		HTLase	
	R	*P* value	*R*	*P* value
ADP 2 μM-LT	−0.1252	0.2945	−0.03844	0.7486
ADP 2 μM-Small	−0.2083	0.0791	−0.07811	0.5143
ADP 2 μM- Med	−0.1335	0.2636	−0.01776	0.8823
ADP 2 μM-Large	−0.03798	0.7514	0.085	0.4777
ADP 5 μM-LT	−0.08351	0.4856	−0.04392	0.7141
ADP 5 μM-Small	−0.2212	0.0619	−0.09755	0.415
ADP 5 μM- Med	−0.2317	0.0501	−0.1055	0.3776
ADP 5 μM-Large	−0.1406	0.2389	−0.06589	0.5824
Coll 1 μg/ml-LT	−0.1072	0.37	0.02695	0.8222
Coll 1 μg/ml-Small	−0.1524	0.2012	0.06594	0.5821
Coll 1 μg/ml- Med	−0.1174	0.3262	0.04772	0.6906
Coll 1 μg/ml-Large	−0.00214	0.9857	0.04327	0.7182
Coll 5 μg/ml-LT	−0.05927	0.6209	−0.01047	0.9304
Coll 5 μg/ml-Small	−0.1489	0.212	−0.1001	0.4029
Coll 5 μg/ml- Med	−0.1269	0.2881	−0.03555	0.7669
Coll 5 μg/ml-Large	−0.1113	0.352	0.007927	0.9473
TRAP 20 μM-LT	−0.1114	0.3515	0.01301	0.9136
TRAP 20 μM -Small	−0.1585	0.1835	−0.1742	0.1434
TRAP 20 μM - Med	−0.09187	0.4427	−0.04854	0.6855
TRAP 20 μM -Large	−0.05235	0.6623	0.03127	0.7943

LT, light transmission; Small, small aggregates; Med, medium aggregates; Large, Large aggregates; Coll, collagen; TRAP, thrombin receptor-activating peptide (SFLLRN). *$P<0.05$.

Table 3
Correlation between paraoxonase activities and other laboratory data.

	Paraoxonase		HTLase	
	R	*P* value	*R*	*P* value
SBP	−0.0953	0.443	−0.118	0.3417
DBP	−0.121	0.3294	−0.00797	0.949
HR	−0.1096	0.3774	0.02895	0.8161
AHI	0.06843	0.5735	0.05563	0.6474
Blood suger	−0.06266	0.609	0.06714	0.5836
HbAlc	−0.08087	0.5121	−0.09806	0.4263
Triglyceride	0.2958	0.0129*	0.00273	0.9821
Total cholesterol	0.01199	0.9215	0.1166	0.3365
LDL cholesterol	−0.0158	0.8975	0.05602	0.6475
HDL cholesterol	−0.01299	0.915	0.2646	0.0269*
PAI-1	−0.08214	0.4928	0.01523	0.899
E-selectin	−0.0007075	0.9953	−0.1618	0.1746
Soluble Fibrin	−0.03224	0.788	−0.08483	0.4787
D-dimer	−0.2348	0.0471*	−0.2229	0.0598
max CPK	−0.1691	0.1616	−0.02521	0.8359
BNP	−0.1306	0.2849	−0.2767	0.0214*
Pulse wave velocity	−0.1665	0.1682	−0.04456	0.7141
ABI	0.267	0.0255*	−0.01957	0.8722
Ejection fraction	0.2558	0.0339*	0.1632	0.1803

SBP, systolic blood pressure; DBP, diastolic blood pressure; AHI, apnea-hypopnea index; LDL, low-density lipoprotein; HDL, high-density lipopurotein; PAI-1, plasminogen activator inhibitor-1; BNP, brain natriuretic peptide; ABI, ankle-brachial index. *$P<0.05$.

Please cite this article as: Ohmori T, et al, Lack of association between serum paraoxonase-1 activity and residual platelet aggregation during dual anti-platelet therapy, Thromb Res (2011), doi:10.1016/j.thromres.2011.10.033

Q192R polymorphism was estimated to be responsible for 72.5% of the variability in ADP-stimulated platelet aggregation after clopidogrel administration [9]. It is therefore important that we clarify which polymorphism combinations (of *PON1* and *CYP2C19*) are the most relevant in the metabolism of thienopyridines in our population.

The first-generation thienopyridine, ticlopidine, was used instead of clopidogrel in our study because ticlopidine was the only approved drug for acute coronary syndrome in Japan during our study period. We acknowledge the possibility that the rate-limiting enzyme for ticlopidine metabolism to its active metabolite may differ from that of clopidogrel. All thienopyridines including ticlopidine, clopidogrel, and prasugrel are prodrugs that need to be converted into active metabolite through the formation of thiolactone metabolites (2-oxo-ticlopidine, 2-oxo-cloipdogrel, and prasugrel thiolactone, respectively (see Supplemental Fig. 1)) [4]. The free active thiol of these active metabolites forms disulfide bonds with, and therefore binds irreversibly to, cysteine residues Cys17 and Cys270 of $P2Y_{12}$ [21]. It is of great importance, therefore, to understand whether thiolactone metabolites of all thienopyridines are hydrolyzed mainly by PON1, or are instead oxidized by CYP.

We found correlations between PON1 activities and cardiac function in our study population. PON1 has a protective effect against oxidation of lipoproteins, and a *PON1* polymorphism (the 192Q allele) that produces decreased levels of PON1 activity was associated with systemic oxidative stress and higher rates of major cardiovascular events [11]. It is possible that decreased levels of PON1 activities enhance the progression of atherosclerosis in the coronary artery, resulting in decreased cardiac function after acute coronary syndromes. Indeed, reduced paraoxonase activity was significantly associated with a decreased ankle-brachial index in our study. Further studies are needed to assess the possible mechanisms and biological effect of PON1, particularly the severity of its effects on cardiac function after coronary artery disease.

Some limitations in this study merit discussion. First, we could assess platelet function testing in the patients treated with ticlopidine, but not clopidogrel. We cannot exclude the possibility that results may differ with other thienopyridines, as described above. In addition, we assessed only the correlation between serum PON1 activities and platelet response to ticlopidine, and we did not assess gene polymorphisms. Although it is accepted that serum PON1 activities are determined by *PON1* polymorphism, more data regarding genetic variation in *CYP* and *PON1* may have extended our findings relating to the mechanism(s) of the platelet response during dual antiplatelet therapy. Finally, the analysis reported here is *post hoc* analysis of a previously reported population and the number of participants is limited. We previously estimated that at least 62–85 participants would be required for the study ($\alpha = 0.05$, $\beta = 0.20$, and expected correlation coefficient, $R = 0.30$–0.35) [13]. Weak association due to β-error may affect the strength of any conclusions based on these data.

Conclusions

The current study has demonstrated that serum PON1 activities did not influence platelet aggregation in patients receiving thienopyridine treatment, but was involved in cardiac function. Our data suggest the need for a re-evaluation of the importance of PON1 (and/or CYP) in the production of active metabolites from thienopyridines. We may also need to consider how expression of the rate-limiting enzymes for thienopyridine metabolism differs between individual drugs and racial populations. During the preparation of this article, it was reported that no association exists between *PON1* genotype and platelet response to clopidogrel and stent thrombosis in a *post hoc* analysis of prospective studies [22]. Further large-scale prospective studies are required to determine which enzyme (PON1 or CYP) is critical for the production of active metabolites from thienopyridines, and therefore for cardiovascular events during thienopyridine administration.

Supplementary materials related to this article can be found online at doi:10.1016/j.thromres.2011.10.033.

Conflict of interests statement

T.O. has received financial support from Daiichi Sankyo. The other authors declare that they have no competing interest.

Acknowledgements

We would like to acknowledge Dr. Y. Yatomi (The University of Tokyo) for very helpful discussions. The authors are grateful for the hard work of the Coronary Care Unit staff in patient recruitment and management. We also thank N. Matsumoto and M. Ito for their excellent technical assistance. This study was supported by grants from the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

[1] Alexopoulos D. Clopidogrel pretreatment in PCI: absolute requirement or obsolete myth? Int J Cardiol 2011;146:305–10.
[2] Farre AJ, Tamargo J, Mateos-Caceres PJ, Azcona L, Macaya C. Old and new molecular mechanisms associated with platelet resistance to antithrombotics. Pharm Res 2010;27:2365–73.
[3] Maree AO, Fitzgerald DJ. Variable platelet response to aspirin and clopidogrel in atherothrombotic disease. Circulation 2007;115:2196–207.
[4] Cattaneo M. The platelet P2Y receptor for adenosine diphosphate: congenital and drug-induced defects. Blood 2011;117:2102–12.
[5] Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med 2009;360:354–62.
[6] Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Meneveau N, et al. Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 2009;360:363–75.
[7] Sibbing D, Morath T, Stegherr J, Braun S, Vogt W, Hadamitzky M, et al. Impact of proton pump inhibitors on the antiplatelet effects of clopidogrel. Thromb Haemost 2009;101:714–9.
[8] O'Donoghue ML, Braunwald E, Antman EM, Murphy SA, Bates ER, Rozenman Y, et al. Pharmacodynamic effect and clinical efficacy of clopidogrel and prasugrel with or without a proton-pump inhibitor: an analysis of two randomised trials. Lancet 2009;374:989–97.
[9] Bouman HJ, Schomig E, van Werkum JW, Velder J, Hackeng CM, Hirschhauser C, et al. Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 2011;17:110–6.
[10] Goswami B, Tayal D, Gupta N, Mallika V. Paraoxonase: a multifaceted biomolecule. Clin Chim Acta 2009;410:1–12.
[11] Bhattacharyya T, Nicholls SJ, Topol EJ, Zhang R, Yang X, Schmitt D, et al. Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. JAMA 2008;299:1265–76.
[12] Ferretti G, Bacchetti T, Marotti E, Curatola G. Effect of homocysteinylation on human high-density lipoproteins: a correlation with paraoxonase activity. Metabolism 2003;52:146–51.
[13] Yano Y, Ohmori T, Hoshide S, Madoiwa S, Yamamoto K, Katsuki T, et al. Determinants of thrombin generation, fibrinolytic activity, and endothelial dysfunction in patients on dual antiplatelet therapy: involvement of factors other than platelet aggregability in Virchow's triad. Eur Heart J 2008;29:1729–38.
[14] Ohmori T, Yatomi Y, Nonaka T, Kobayashi Y, Madoiwa S, Mimuro J, et al. Aspirin resistance detected with aggregometry cannot be explained by cyclooxygenase activity: involvement of other signaling pathway(s) in cardiovascular events of aspirin-treated patients. J Thromb Haemost 2006;4:1271–8.
[15] Becker RC, Meade TW, Berger PB, Ezekowitz M, O'Connor CM, Vorchheimer DA, et al. The primary and secondary prevention of coronary artery disease: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest 2008;133:776S–814S.
[16] Wright RS, Anderson JL, Adams CD, Bridges CR, Casey Jr DE, Ettinger SM, et al. 2011 ACCF/AHA Focused Update of the Guidelines for the Management of Patients With Unstable Angina/Non-ST-Elevation Myocardial Infarction (Updating the 2007 Guideline) A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines Developed in Collaboration With the American College of Emergency Physicians, Society for Cardiovascular Angiography and Interventions, and Society of Thoracic Surgeons. J Am Coll Cardiol 2011;57:1920–59.
[17] Gurbel PA, Becker RC, Mann KG, Steinhubl SR, Michelson AD. Platelet function monitoring in patients with coronary artery disease. J Am Coll Cardiol 2007;50:1822–34.
[18] Togawa T, Mukai Y, Ohata K, Suzuki T, Tanabe S. Measurement of homocysteine thiolactone hydrolase activity using high-performance liquid chromatography with fluorescence detection and polymorphisms of paraoxonase in normal human serum. J Chromatogr B Analyt Technol Biomed Life Sci 2005;819:67–72.

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[19] Kim KA, Song WK, Kim KR, Park JY. Assessment of CYP2C19 genetic polymorphisms in a Korean population using a simultaneous multiplex pyrosequencing method to simultaneously detect the CYP2C19*2, CYP2C19*3, and CYP2C19*17 alleles. J Clin Pharm Ther 2010;35:697–703.
[20] Jinnai T, Horiuchi H, Makiyama T, Tazaki J, Tada T, Akao M, et al. Impact of CYP2C19 polymorphisms on the antiplatelet effect of clopidogrel in an actual clinical setting in Japan. Circ J 2009;73:1498–503.
[21] Ding Z, Kim S, Dorsam RT, Jin J, Kunapuli SP. Inactivation of the human P2Y12 receptor by thiol reagents requires interaction with both extracellular cysteine residues, Cys17 and Cys270. Blood 2003;101:3908–14.
[22] Sibbing D, Koch W, Massberg S, Byrne RA, Mehilli J, Schulz S, et al. No association of paraoxonase-1 Q192R genotypes with platelet response to clopidogrel and risk of stent thrombosis after coronary stenting. Eur Heart J 2011;32:1605–13.

Please cite this article as: Ohmori T, et al, Lack of association between serum paraoxonase-1 activity and residual platelet aggregation during dual anti-platelet therapy, Thromb Res (2011), doi:10.1016/j.thromres.2011.10.033

Haemophilia

Haemophilia (2011), 1–8

DOI: 10.1111/j.1365-2516.2011.02686.x

ORIGINAL ARTICLE

Immune response against serial infusion of factor VIII antigen through an implantable venous-access device system in haemophilia A mice

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Summary. Haemophilia A is a life long bleeding disorder caused by an inherited deficiency of factor VIII (FVIII). About 30% of haemophilia A patients develop neutralizing antibodies as a consequence of treatment with FVIII concentrates. Immune tolerance protocols for the eradication of inhibitors require daily delivery of intravenous FVIII. We evaluated the immune responses to serial intravenous administration of FVIII in preimmunized haemophilia A mice. We introduced an implantable venous-access device (iVAD) system into haemophilia A mice to facilitate sequential infusion of FVIII. After preimmunization with FVIII, the haemophilia A mice were subjected to serial intravenous administration of FVIII through the iVAD system. In all mice with serial infusion of FVIII, high titers of anti-FVIII inhibitory antibodies developed at 10 exposure days (EDs). However, the anti-FVIII IgG titers were decreased after 150 EDs of sequential low-dose infusion of FVIII [0.05 U g^{-1} body weight (BW) five times per week]. Proliferative response to *ex vivo* FVIII stimulation was significantly suppressed in splenic $CD4^+$ T cells from mice with serial low-dose FVIII infusion compared with those from mice with high-dose FVIII infusion (0.5 U g^{-1} BW five times per week) or preimmunized mice. Moreover, splenic $CD4^+$ T cells from mice with serial low-dose infusion of FVIII failed to produce interleukin-2 and interferon-γ. These data suggest that serial infusion of FVIII could induce T-cell anergy in haemophilia A mice with inhibitor antibodies.

Keywords: anergy, factor VIII, haemophilia A mice, inhibitor, venous-access device

Introduction

Haemophilia A is a life-long bleeding disorder caused by an inherited deficiency of factor VIII (FVIII) because of mutations in the FVIII gene [1]. About 30% of severe haemophilia A patients who received replacement therapy with intravenous FVIII products develop neutralizing antibodies that inhibit the function of substituted FVIII [2,3]. Once an inhibitor develops, treatment of bleeding episodes is quite difficult due to partial or complete lack of efficacy of replacement therapy. Immune tolerance induction (ITI) therapy using regular applications of FVIII is the only strategy that has been proven successfully to combine eradication of FVIII inhibitors and induction of FVIII-specific immune tolerance [2,4].

Central venous-access devices (VADs) are often used in haemophiliacs undergoing ITI to overcome difficulties of regular venous puncture [5,6]. The fully implantable devices offer many advantages compared with external catheters, because they generally have longer useful duration with lower rate of infectious complication and cannot be accidentally displaced [7]. Although ITI approach was introduced several decades ago, little is known about the immunological mechanisms that cause down-modulation of FVIII-specific immune responses and the induction of long-lasting immune tolerance against FVIII.

In this study we introduced an implantable VAD (iVAD) system into haemophilia A mice to facilitate serial intravenous infusion of FVIII and evaluated immune responses against FVIII in preimmunized haemophilia A mice. We demonstrated that sequential administration of FVIII through the iVAD system could induce T-cell anergy in adult haemophilia A mice with inhibitors.

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Accepted after revision 29 September 2011

Methods

Animal models

Haemophilia A mice (B6; $_{129}S_4$-F8^{tm1Kaz}/J) with targeted destruction of exon 16 of the FVIII gene were kindly provided by Dr H.H. Kazazian Jr (University of Pennsylvania, Philadelphia, PA, USA) [8]. All mice were housed and used in a pathogen-free facility at Jichi Medical University, Shimotsuke, Tochigi, Japan. All animal experiments were performed in accordance with regulations of the Japanese Council for Animal Care; Jichi Medical University Animal Care Committee approval all animal protocols.

iVAD system and intravenous injection of FVIII

Haemophilia A mice aged 12 weeks were anaesthetized by inhalation with 2.5% isoflurane in the anaesthesia unit (Univentor, ZTN 08, Malta). An iVAD system (SoloPort; Instech Laboratories, Plymouth Meeting, PA, USA) was placed into a pocket of back skin in the chest wall of each animal (Fig. 1). The catheter was then tunneled under the skin and introduced into the superior vena cava through a cut-down site of jugular vein under a zoom stereomicroscope (Nikon, Tokyo, Japan). The entire system was flushed with saline solution after insertion. Mice were infused with intravenous recombinant human FVIII formulated with sucrose (Kogenate FS; Bayer Healthcare, Leverkusen, Germany) through the iVAD system.

Assay for FVIII inhibitors

Inhibitory FVIII antibodies were measured according to the Bethesda assay [9]. In brief, mouse plasma was serially diluted in Owren's Veronal Buffer (Dade Behring, Deerfield, IL, USA), such that the remaining FVIII activity for each sample was between 25% and 75%, and mixed 1:1 with normal pooled human plasma at 37°C for 2 h. Residual human FVIII activity was measured by one-stage assay using 50 μL of FVIII-deficient human plasma (Kokusai-Shiyaku, Kobe, Japan) and a 50-μL sample from the previous incubation on a automated coagulometer (CA-500; Sysmex, Kobe, Japan). One BU mL^{-1} was defined as the dilution of plasma containing FVIII inhibitory activity that results in 50% inhibition of FVIII activity.

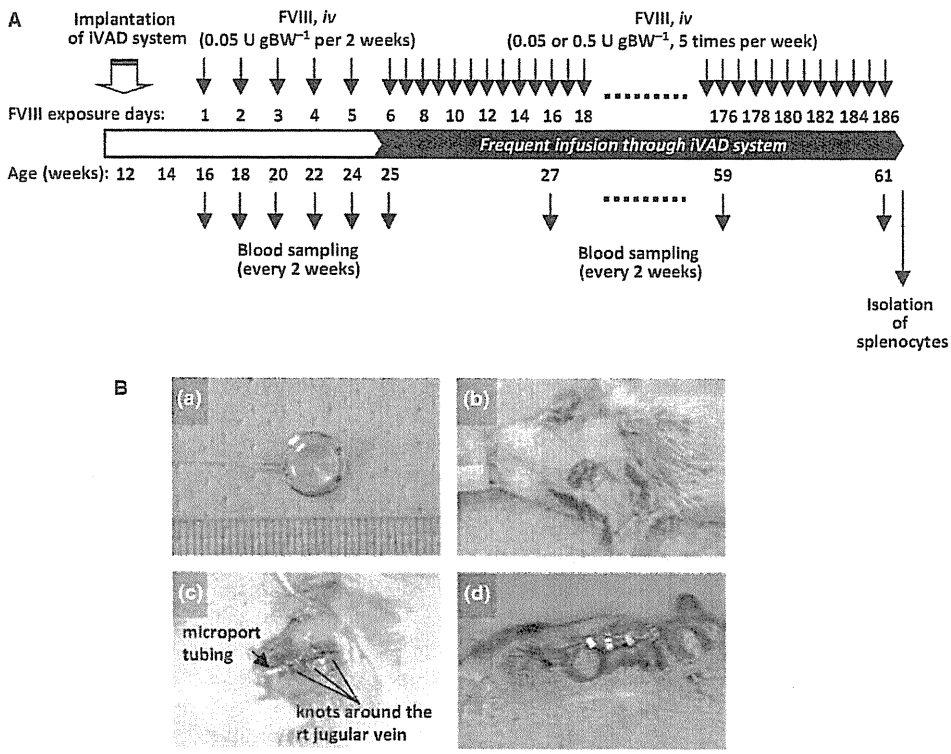

Fig. 1. Protocol for serial intravenous administration of FVIII through an implantable venous access device (iVAD) system in haemophilia A mice. A. Haemophilia A mice were implanted an iVAD system at age 12 weeks, then they were preimmunized with intravenous injection of 0.05 U g^{-1} BW recombinant human FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently administered with FVIII (0.05 or 0.5 U g^{-1} BW, five times per week) through the iVAD system. B. (a) The iVAD system consists of a stainless steel port with a molded silicon rubber and an 1.2 Fr catheter. After incision of the cervical skin in a haemophilia A mouse, the jugular vein was exposed (b), then the catheter was inserted into superior vena cava by cut-down procedure (c). The other side of the catheter was tunneled to the port that was set beneath the pocket of back skin (d).

Anti-FVIII measurement

Anti-FVIII IgG concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in microtiter wells (Nunc, Roskilde, Denmark) coated with 1 μg mL^{-1} recombinant human FVIII. After blocking with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), serial dilutions of murine plasma were added at 4°C for 16 h. Each well was washed with 0.5% BSA in PBS containing 0.05% Tween-20. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA) was added at 37°C for 1 h. ABTS Microwell substrate (KPL, Gaithersburg, MD, USA) was added, and the absorbance at 405 nm was read. Anti-FVIII antibody concentrations were calculated from the linear portion of a standard curve obtained using antihuman FVIII monoclonal antibodies (kindly provided by Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan).

Determination of anti-FVIII IgG subclasses

Microtiter wells were coated with 1 μg mL^{-1} recombinant human FVIII in PBS for 16 h at 4°C. After blocking with 5% BSA in PBS, serial dilutions of murine plasma were added for 2 h at 37°C. The wells were washed with 0.5% BSA in PBS containing 0.05% Tween-20. The IgG1, IgG2a, IgG2b and IgG3 subtypes of anti-FVIII antibodies bound to immobilized human FVIII were determined by incubation with isotype-specific rabbit anti-mouse IgGs (Mouse Typer; BioRad, Hercules, CA, USA) for 1 h at 37°C. After washing with 0.5% BSA in PBS containing 0.05% Tween-20, the wells were incubated with goat anti-rabbit HRP conjugate for 1 h at 37°C. Substrate development was performed for 15 min at 25°C, using ABTS Microwell substrate as described above.

Proliferation assay with [^{3}H]-thymidine incorporation

Mice splenic $CD4^+$ T cells were prepared by depletion of non-$CD4^+$ T cells with the autoMACS cell sorting system (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Antigen-presenting cells were prepared from mice splenocytes by depletion of T cells using the magnetic sorting system with anti-CD90 (Thy1.2)-conjugated microbeads (Miltenyi Biotech) followed by irradiation with a single dose of 20 Gy (Gamma Cell; Norton International, ON, Canada), to prevent non-specific proliferative responses during the *in vitro* FVIII stimulation assay. To measure T-cell proliferation, 3×10^5 $CD4^+$ T cells per well were cultured with 0–3 nM human recombinant FVIII in the presence of antigen-presenting cells derived from FVIII-immunized mice (five times injection of 0.05 U g^{-1} BW FVIII, every 2 weeks) at 37°C for 72 h in complete RPMI-1640 (Gibco BRL, Rockville, MD, USA). [^{3}H]-Thymidine (Amersham Bioscience, Uppsala, Sweden) was added (0.037 MBq per well) at 37°C for 18 h. Then, cells were harvested, and [^{3}H]-thymidine incorporation was determined by scintillation counting (Top count; Packard, Meriden, CT, USA).

Cytokine assays

Splenocytes were incubated in 24-well plates at 1.0×10^6 cells per well in the absence or presence of 3 nM human recombinant FVIII at 37°C in 5% CO_2. Production of interleukin (IL)-2, IL-4, IL-12 and interferon (IFN)-γ by $CD4^+$ T cells derived from each mouse was analyzed at 72 h by ELISA kits (Biotrak ELISA System; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. In addition, levels of IL-10 were measured at 96 h by ELISA system (Biotrak ELISA System).

Statistical analysis

Data are expressed as mean ± SE. Normally distributed variables were compared by Student's *t*-test. Variables not normally distributed were analyzed by two-sided Mann–Whitney U test. The data were considered statistically significant at P values < 0.05.

Results

Serial intravenous administration of FVIII through an iVAD system in preimmunized haemophilia A mice

We securely implanted venous access devices into haemophilia A mice at 12 weeks using a zoom microscopy, therefore, we could avoid using FVIII concentrates for haemostatic control during the procedure (Fig. 1B). After the operation-related wounds had healed, we developed immunized mice against FVIII by intravenous injection of FVIII (0.05 U g^{-1} BW) at 2-week intervals. Titers of anti-FVIII inhibitory antibodies of the mice were elevated to 100–400 BU mL^{-1} after the fifth exposure of FVIII. Thereafter, we performed serial infusion of FVIII into the preimmunized haemophilia A mice through the venous access device system. High titers (>2000 BU mL^{-1}) were developed after 10 exposure days (EDs) in mice with administration of FVIII (0.05 U g^{-1} BW five times per week) and were continued over 100–120 EDs. However, after 130–150 EDs their titers were gradually decreased despite continuing sequential stimulation of FVIII (Fig. 2a). One of the five mice was discontinued at 140 EDs because of bleeding from the site of catheter insertion (Fig. 2a; LD#3). In contrast, > 2000 BU mL^{-1} of anti-FVIII inhibitory antibodies were sustained over

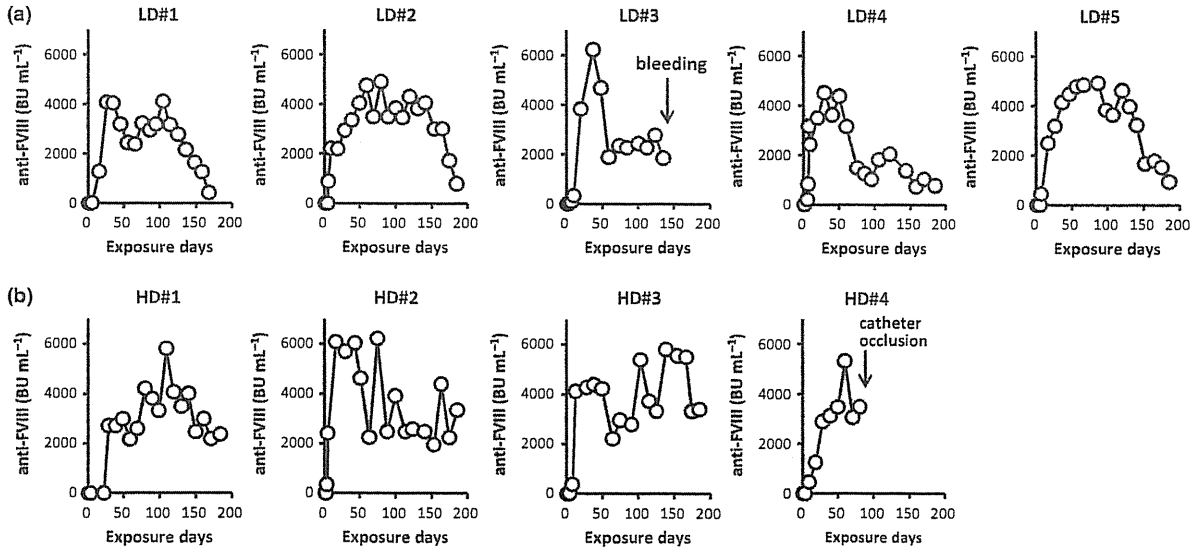

Fig. 2. Effect of serial intravenous administration of FVIII on anti-FVIII inhibitory antibody formation in preimmunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently administered with FVIII [(a) 0.05 U g^{-1} BW five times per week; (b) 0.5 U g^{-1} BW, five times per week] through the iVAD system. The mice were bled at every 2 weeks, and their anti-FVIII inhibitor titers were determined by Bethesda assay.

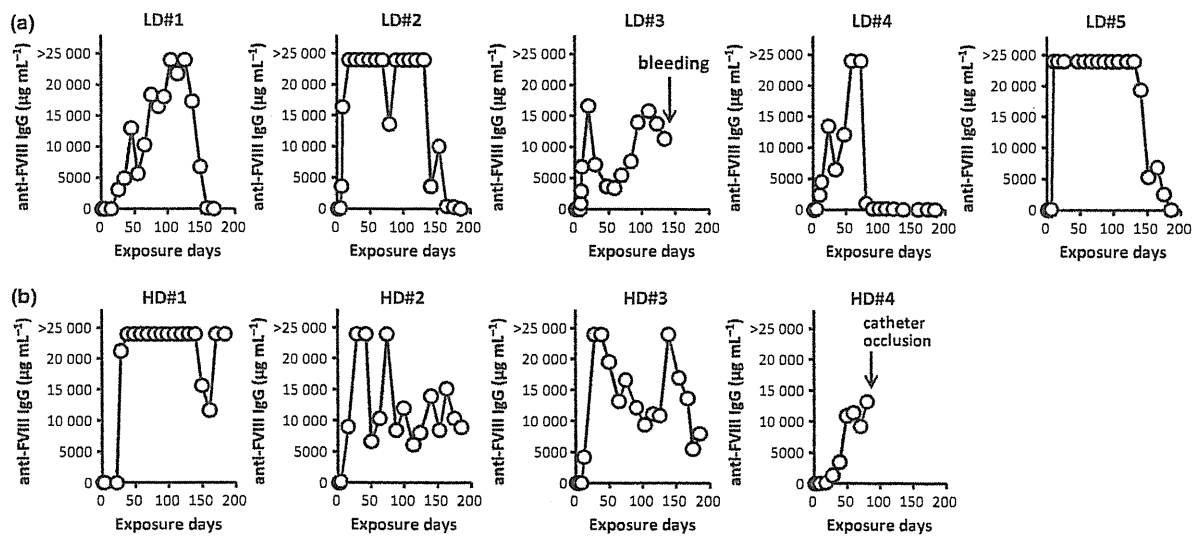

Fig. 3. Effect of repeated intravenous infusion of FVIII on FVIII-specific IgG formation in haemophilia A mice with inhibitors. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were repeatedly infused with FVIII [(a), 0.05 U g^{-1} BW, five times per week; (b) 0.5 U g^{-1} BW, 5 times per week. The mice were bled at every two weeks just before each infusion. Plasma levels of FVIII-specific IgG were measured by ELISA as described in Methods.

180 EDs in mice with serial infusion of high-dose FVIII (0.5 U g^{-1} BW five times per week) (Fig. 2b).

Effect of serial intravenous infusion of FVIII on FVIII-specific IgG and subclasses formation

Anti-FVIII IgG was detectable immediately after serial infusion of FVIII (0.05 U g^{-1} BW five times per week) in preimmunized haemophilia A mice, and were persisted for more than 80–100 EDs (Fig. 3a). Interestingly, titers against FVIII were markedly decreased after 80–150 EDs. By contrast, preimmunized mice followed by serial intravenous infusion of high-dose FVIII (0.5 U g^{-1} BW five times per week) showed high titer of anti-FVIII IgG over 150–180 EDs (Fig. 3b). One of four mice receiving sequential high-dose FVIII infusion was discontinued due to occlusion of central vein catheter at 80 EDs (Fig. 3b; HD#4). All IgG isotypes of anti-FVIII IgG

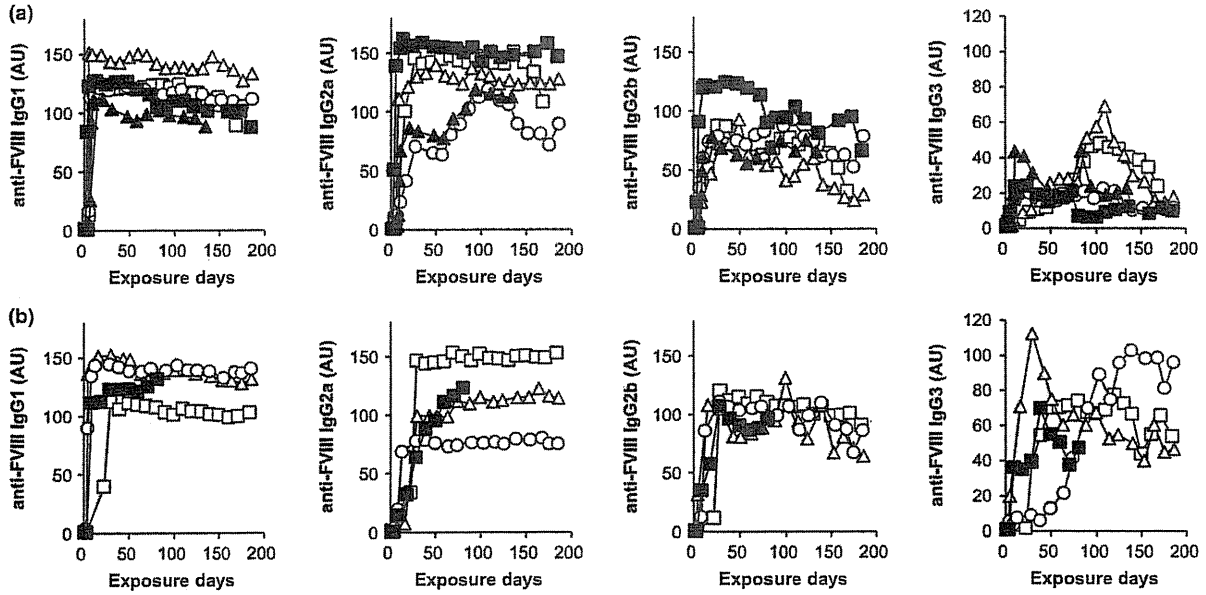

Fig. 4. Effect of serial intravenous injection on anti-FVIII IgG subclasses formation in preimmunized haemophilia A mice. (a) Haemophilia A mice (LD1, open squares; LD#2, open triangles; LD#3, closed triangles; LD#4, closed squares; LD#5, open circles) were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were repeatedly infused with FVIII (0.05 U g^{-1} BW five times per week). (b) Haemophilia A mice (HD#1, open squares; HD#2, open triangles; HD#3, open circles; HLD#4, closed squares) were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were repeatedly infused with FVIII (0.5 U g^{-1} BW five times per week). Each of the mice was bled at every two weeks just before FVIII infusion. Titers of IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) were determined by ELISA as described in Methods.

antibodies were rapidly increased after serial infusion of 0.05 U g^{-1} and 0.5 U g^{-1} BW of FVIII (Fig. 4). However, in mice with repeated administration of 0.05 U g^{-1} BW FVIII titers of IgG3 subclass antibodies were decreased after 80–100 EDs (Fig. 4b).

Effect of serial administration of FVIII on anti-factor VIII CD4⁺ T cells proliferation

Next, we evaluated whether serial infusion of FVIII exerts a suppressive effect on FVIII-specific T cells, $CD4^+$ T cells obtained after the final injection were assayed for a T-cell proliferative response to FVIII. We observed a dose-dependent $CD4^+$ T-cell proliferative response to FVIII in preimmunized mice (five times injection of FVIII every two weeks, Fig. 5). In the group with sequential infusion of 0.5 U g^{-1} BW FVIII the T cells significantly proliferated in response to FVIII stimulation. By contrast, no response was observed at any FVIII dose in $CD4^+$ T cells from the mice after serial infusion of 0.05 U g^{-1} BW FVIII.

Effect of serial infusion of FVIII on cytokine response

Mice that were immunized with FVIII every two weeks developed splenocytes, which proliferated and produced

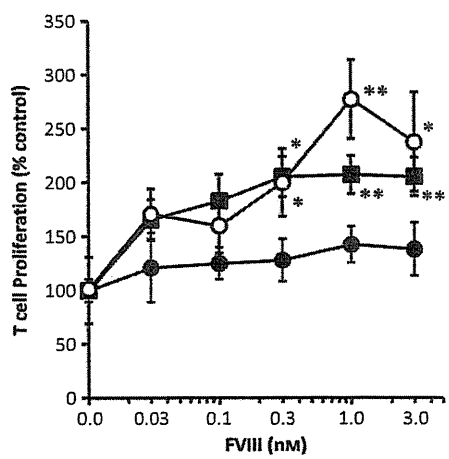

Fig. 5. Effect of repeated administration of FVIII on anti-factor VIII T-cell proliferation of haemophilia A mice. Haemophilia A mice were given intravenous injection of 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently infused with FVIII through the iVAD system. $CD4^+$ T cells of preimmunized mice (n = 5; open circles), mice with infusion of FVIII (0.05 U g^{-1} BW, five times per week; n = 4; closed circles), and mice with injection of FVIII (0.5 U g^{-1} BW, five times per week; n = 3; closed squares) were obtained three days after final immunization. The amount of ^{3}H-thymidine incorporation was measured under *in vitro* stimulation with FVIII (0–3 nM) in the presence of the FVIII-immunized mice-derived antigen-presenting cells by scintillation counting as described in the Methods. Data are means ± SD. $*P < 0.05$; $**P < 0.03$ when compared with the proliferation in the absence of FVIII.

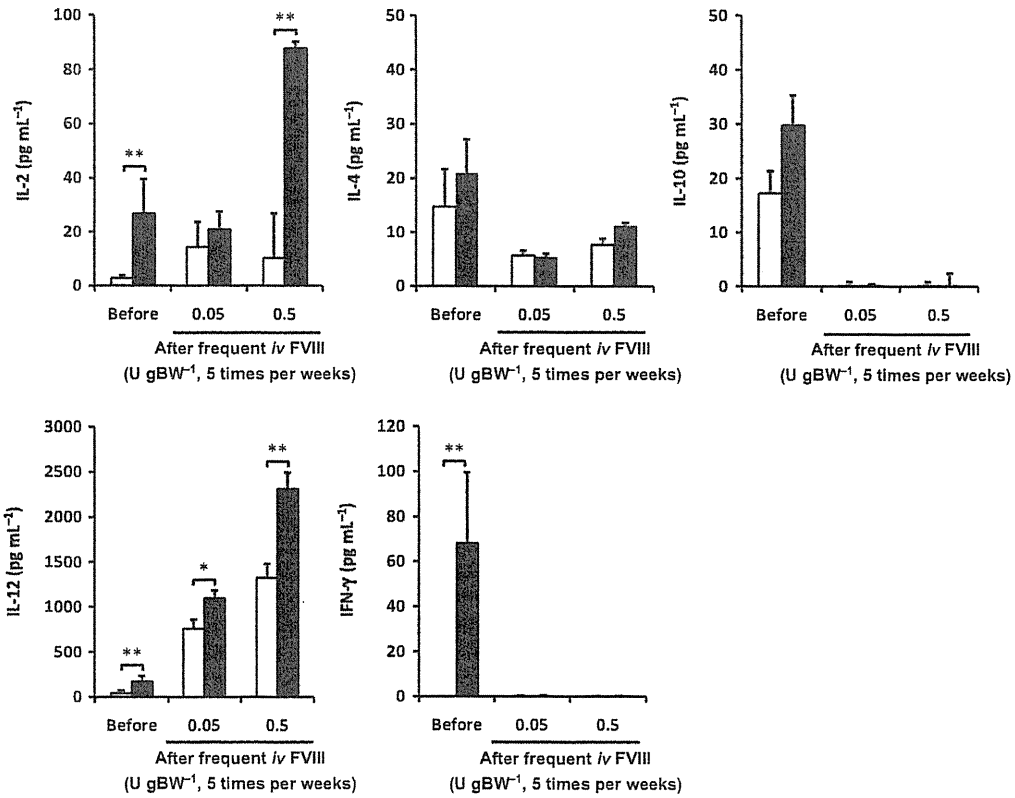

Fig. 6. Effect of serial infusion of FVIII on cytokine response of pre-immunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were frequently administered with FVIII (0.05 or 0.5 U g^{-1} BW five times per week) through iVAD system. Splenocytes from pre-immunized (before, n = 5), frequently FVIII-infused [0.05 (n = 4) or 0.5 (n = 3) U g^{-1} BW five times per week] mice were cultured in the absence (open bars) or presence (closed bars) of 3 nM FVIII, and their cytokine production (IL-2, IL-4, IL-10, IL-12 and IFN-γ) were analyzed by ELISA as described in Methods. Values (pg mL^{-1}) are means ± SD. *P < 0.05; **P < 0.03.

IL-2, IL-12 and IFN-γ in response to *in vitro* FVIII stimulation (Fig. 6). In contrast, splenocytes deriving from mice with serial infusion of 0.05 U g^{-1} BW FVIII did not increase their production of IL-2 and IFN-γ, although they could secrete IL-12 after addition of FVIII. Moreover, 0.5 U g^{-1} BW FVIII-repeated administrated mice produced significant amounts of IL-2 and IL-12, but did not change IFN-γ levels even after stimulation of FVIII.

Discussion

Haemophilia A patients with inhibitors are infused daily FVIII according to immune tolerance protocols with the aim of eradicating the antibody [10,11]. Central VADs have been used to facilitate repeated administration of clotting factor concentrates in haemophiliac children requiring ITI [12,13]. We here established a method to implant a VAD into haemophilia A mice (Fig. 1). We could prevent exposure to FVIII antigen in the mice during the surgical procedure, because it is known that the innate immune system is activated by endogenous 'danger signals' such as tissue damage that involves necrotic cell death [14]. Indeed, titers of anti-FVIII inhibitory antibodies of the mice were elevated up to 400 BU mL^{-1} after the fifth intermittent stimulation of FVIII, in good agreement with previous findings [9]. Central VADs are associated with infectious and thrombotic complications necessitating the removal [6], although recent data from the international-ITI study showed that infectious episodes during ITI may not influence treatment outcome [15]. In our animal models, one mouse that had been frequently administered 0.05 U g^{-1} BW of FVIII exhibited a catheter-related bleeding (Fig. 2; LD#3), whereas another one with 0.5 U g^{-1} BW of FVIII had occlusion of iVAD system (Fig. 2, HD#4). Nonetheless, the iVAD would be a useful tool to evaluate immune response against sequential infusion of FVIII antigen in haemophilia A mice because they could be repeatedly infused more than 180 times over 50 weeks.

Recent study showed that port systems are suitable for inhibitor-expressing children with good predictors of ITI success [16,17]. In our murine model, high titers against FVIII (>2000 BU mL^{-1} during 50–100 EDs) were decreased to <500 BU mL^{-1} after 130–150 EDs in all

mice with serial infusion of 0.05 U g^{-1} BW of FVIII, even though they were continually exposed to FVIII antigen (Fig. 2). In contrast, mice administered high-dose 0.5 U g^{-1} BW FVIII five times a week had high titers of anti-FVIII inhibitory antibodies over 180 EDs, suggesting that dose of FVIII antigen might be crucial for the immune response in haemophilia A mice. We could not induce immune tolerance in any adult haemophilia A mouse with sequential infusion of FVIII antigen, according to the international consensus in which successful immune tolerance induction in haemophilia A is currently defined as both an undetectable inhibitor titer (less than or equal to 0.6 BU mL^{-1}) and normalized FVIII pharmacokinetics [18]. However, anti-FVIII IgG titers were markedly decreased to undetectable levels after 80–180 EDs in mice with serial infusion of 0.05 U mL^{-1} FVIII (Fig. 3). The discrepancy between anti-FVIII inhibitory titers and anti-FVIII IgG titers may be dependent on assay methods in which the former was one-stage APTT measurement and the latter was ELISA using an anti-FVIII monoclonal antibody as standard.

In haemophilia A patients, several researchers reported that IgG4 is the major component of anti-FVIII antibodies, although all IgG subclasses have been found [19,20]. In murine models, we showed that kinetics of anti-FVIII IgG1, IgG2a and IgG2b titers of haemophilia A mice with serial infusion of 0.05 U g^{-1} BW FVIII were similar to those administered 0.5 U g^{-1} BW FVIII (Fig. 4). In contrast, titers of anti-FVIII IgG3 subclass were decreased after 50–100 EDs in mice with serial infusion of 0.05 U g-1 BW FVIII. The Th1 immune response is believed predominant in patients with inhibitors in the long term [21], and was also the predominant response in mice that developed antibodies after challenge in adulthood [22,23]. We demonstrated a dose-dependent $CD4^+$ T-cell proliferative response to FVIII in preimmunized mice (five times injection of FVIII every 2 weeks), which is compatible with previous studies demonstrating that human FVIII is highly immunogenic in haemophiliac mice (Fig. 5) [24]. Interestingly, we observed that haemophilia A mice with sequential infusion of 0.05 U g^{-1} BW FVIII after 180 EDs failed to develop $CD4^+$ T-cell proliferative response to *in vitro* stimulation of FVIII antigen (Fig. 5). These T cells could not produce any IL-2, IL-4, IL-10, nor IFN-γ (Fig. 6), whereas those from mice immunized with five-times infusion of 0.5 U g^{-1} BW FVIII were able to secrete significant amounts of IL-2, IL-12 and IFN-γ. It is known that Th1 cells are initiators of antibody responses, and that they participate in class switching by releasing IFN-γ, which preferentially induces IgG2a and IgG3 in mouse [25]. Consequently, the FVIII-specific Th1 cytokine response may be partially suppressed by serial administration of FVIII in haemophilia A mice with inhibitors.

Several potential mechanisms of ITI have been identified [26]. These include clonal deletion (i.e. removal of immune-response cells through programmed cell death or apoptosis), anergy (failure of immune cells to respond to the FVIII molecule), or ignorance (i.e. the immune-response cells are 'blind' to the presence of FVIII). Our data suggest that sequential exposure of FVIII antigen could partially block anti-FVIII inhibitory antibody production, inducing T-cell anergy in haemophilia A mice with inhibitor, although our murine ITI model against heteroantibodies is fundamentally different from human ITI therapy against alloantibodies. However, further evaluation using completely continuous infusion system for the exposure of FVIII antigen will be necessary to confirm its efficacy in inducing immune tolerance [27]. Furthermore, understanding of the underlying mechanisms of immune tolerance induced by serial administration of FVIII is essential for the development of this strategy for haemophilia A patients with inhibitors.

Acknowledgement

We thank D.V.M. Hisae Yamauchi, D.V.M. Akane Hirosawa and Ms. Chizuko Nakamikawa for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (#19591133, #20591155, #21790920 and #21591249) from the Ministry of Education, Culture, Sports, Science and Technology, and by a Health and Labor Sciences Research Grant for Research from the Ministry of Health, Labor and Welfare, by Support Program for Strategic Research Platform, Baxter Hemophilia Scientific Research & Education Fund, and by JKA promotion funds from KEIRIN RACE.

Author contributions

SM designed and performed the research, analyzed data and wrote the paper; EK, YK, AY and AS performed experiments; SM, TO, JM and YS analyzed data and revised the paper.

Disclosures

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

References

1 Hoyer LW. Hemophilia A. *N Engl J Med* 1994; 330: 38–47.
2 Ehrenforth S, Kreuz W, Scharrer I *et al.* Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet* 1992; 339: 594–8.
3 Hoyer LW. Why do so many haemophilia A patients develop an inhibitor? *Br J Haematol* 1995; 90: 498–501.
4 Kreuz W, Becker S, Lenz E *et al.* Factor VIII inhibitors in patients with hemophilia A: epidemiology of inhibitor development and induction of immune tolerance for factor VIII. *Semin Thromb Hemost* 1995; 21: 382–9.
5 Ewenstein BM, Valentino LA, Journeycake JM *et al.* Consensus recommendations for use of central venous access devices in haemophilia. *Haemophilia* 2004; 10: 629–48.
6 Valentino LA, Ewenstein B, Navickis RJ, Wilkes MM. Central venous access devices in haemophilia. *Haemophilia* 2004; 10: 134–46.

7 Santagostino E, Mancuso ME. Venous access in haemophilic children: choice and management. *Haemophilia* 2010; **16**(Suppl 1): 20–4.

8 Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 1995; 10: 119–21.

9 Madoiwa S, Yamauchi T, Hakamata Y *et al.* Induction of immune tolerance by neonatal intravenous injection of human factor VIII in murine hemophilia A. *J Thromb Haemost* 2004; **2**: 754–62.

10 Brackmann HH. Induced immunotolerance in factor VIII inhibitor patients. *Prog Clin Biol Res* 1984; **150**: 181–95.

11 Nilsson IM, Berntorp E, Zettervall O. Induction of immune tolerance in patients with hemophilia and antibodies to factor VIII by combined treatment with intravenous IgG, cyclophosphamide, and factor VIII. *N Engl J Med* 1988; **318**: 947–50.

12 Liesner RJ, Vora AJ, Hann IM, Lilleymann JS. Use of central venous catheters in children with severe congenital coagulopathy. *Br J Haematol* 1995; **91**: 203–7.

13 Santagostino E, Gringeri A, Muca-Perja M, Mannucci PM. A prospective clinical trial of implantable central venous access in children with haemophilia. *Br J Haematol* 1998; **102**: 1224–8.

14 Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* 2008; **8**: 279–89.

15 DiMichele DM, Goldberg I, Foulkes M, Hay RM. International prospective randomized immune tolerance (ITI) study: preliminary results of therapeutic efficacy and safety. *Haemophilia* 2010; **16**(Suppl 4): 29.

16 DiMichele DM, Hoots WK, Pipe SW, Rivard GE, Santagostino E. International workshop on immune tolerance induction: consensus recommendations. *Haemophilia* 2007; **13**(Suppl 1): 1–22.

17 Mancuso ME, Mannucci PM, Sartori A, Agliardi A, Santagostino E. Feasibility of prophylaxis and immune tolerance induction regimens in haemophilic children using fully implantable central venous catheters. *Br J Haematol* 2008; **141**: 689–95.

18 Astermark J, Morado M, Rocino A *et al.* Current European practice in immune tolerance induction therapy in patients with haemophilia and inhibitors. *Haemophilia* 2006; **12**: 363–71.

19 Fulcher CA, de Graaf Mahoney S, Zimmerman TS. FVIII inhibitor IgG subclass and FVIII polypeptide specificity determined by immunoblotting. *Blood* 1987; **69**: 1475–80.

20 Gilles JG, Arnout J, Vermylen J, Saint-Remy JM. Anti-factor VIII antibodies of hemophiliac patients are frequently directed towards nonfunctional determinants and do not exhibit isotypic restriction. *Blood* 1993; **82**: 2452–61.

21 Reding MT, Lei S, Lei H, Green D, Gill J, Conti-Fine BM. Distribution of Th1- and Th2-induced anti-factor VIII IgG subclasses in congenital and acquired hemophilia patients. *Thromb Haemost* 2002; **88**: 568–75.

22 Wu H, Reding M, Qian J *et al.* Mechanism of the immune response to human factor VIII in murine hemophilia A. *Thromb Haemost* 2001; **85**: 125–33.

23 Sasgary M, Ahmad RU, Schwarz HP, Turecek PL, Reipert BM. Single cell analysis of factor VIII-specific T cells in hemophilic mice after treatment with human factor VIII. *Thromb Haemost* 2002; **87**: 266–72.

24 Qian J, Borovok M, Bi L, Kazazian HH Jr, Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost* 1999; **81**: 240–4.

25 Stavnezer J. Immunoglobulin class switching. *Curr Opin Immunol* 1996; **8**: 199–205.

26 Behrmann M, Pasi J, Saint-Remy JM, Kotitschke R, Kloft M. Von Willebrand factor modulates factor VIII immunogenicity: comparative study of different factor VIII concentrates in a haemophilia A mouse model. *Thromb Haemost* 2002; **88**: 221–9.

27 Abe C, Tashiro T, Tanaka K, Ogihara R, Morita H. A novel type of implantable and programmable infusion pump for small laboratory animals. *J Pharmacol Toxicol Methods* 2009; **59**: 7–12.

Thrombosis Research 128 (2011) e137–e143

Contents lists available at ScienceDirect

Thrombosis Research

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Regular Article

Predictive blood coagulation markers for early diagnosis of venous thromboembolism after total knee joint replacement

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ARTICLE INFO

Article history:
Received 6 May 2011
Received in revised form 14 July 2011
Accepted 15 July 2011
Available online 11 August 2011

Keywords:
Cross-linked fibrin degradation products by leukocyte elastase
D-dimer
Soluble fibrin monomer complex
Total knee arthroplasty
Venous thromboembolism

ABSTRACT

Pulmonary embolism development may be prevented if asymptomatic venous thromboembolism (VTE) can be predicted and treated preoperatively or soon after total knee arthroplasty (TKA). The purpose of this study was to evaluate whether asymptomatic VTE can be predicted by blood coagulation markers preoperatively or early after TKA. This prospective single-centre study enrolled 68 patients (6 men, 62 women; mean age: 71 years) who underwent TKA between September 2004 and August 2009. Sixteen-row multidetector computed tomography was performed 4 days before and after surgery for diagnosis of asymptomatic VTE. Blood samples were taken to measure the plasma levels of soluble fibrin monomer complex (SFMC), D-dimer and cross-linked fibrin degradation products by leukocyte elastase (e-XDP) at 4 days preoperatively, and at 1 hour, 1 day and 4 days postoperatively. The preoperative SFMC, D-dimer and e-XDP levels did not differ significantly between the thrombus ($n=36$) and no-thrombus ($n=32$) groups. D-dimer and e-XDP levels showed the most significant increases at days 4 and 1, respectively, after surgery in the thrombus group. With cut-off points of 7.5 μg/ml for D-dimer and 8.2 U/ml for e-XDP, the sensitivities were 75% and 75%, and the specificities were 63% and 59%, respectively. By multiple logistic regression analysis, D-dimer at day 4 and e-XDP at day 1 postoperatively were independent markers for early diagnosis of VTE (odds ratio = 1.61 and 1.19, $P=0.01$ and 0.04, respectively). The postoperative occurrence of new asymptomatic VTE may be predicted by D-dimer at day 4 and e-XDP at day 1 after TKA.

Introduction

After arthroplasty, it is extremely important to prevent the development of postoperative venous thromboembolism (VTE), particularly symptomatic and fatal pulmonary embolism (PE), in orthopaedic surgery [1]. Since the 1990s, antithrombotic therapies using agents such as unfractionated and low molecular weight heparin have been administered to patients after surgery. However, despite the implementation of aggressive antithrombotic protocols, including those mandated by the American College of Chest Physicians (ACCP) [2], the incidence of fatal PE remains at 0.15% [3] and that of symptomatic PE remains at 0.41% [4] after total knee arthroplasty (TKA), with no changes since the 1990s. Pellergrini et al. [5] reported that 17% of patients with untreated deep vein thrombosis (DVT) experienced symptomatic PE after total hip arthroplasty (THA). While it is thought that prophylactic antithrombotic treatments are necessary to prevent postoperative fatal and symptomatic PE, previous reports found no differences in the incidences of fatal or symptomatic PE, regardless of whether or not prophylactic antithrombotic therapy was given [3,4,6], and that the infection rate was increased owing to haematoma caused by haemorrhage [7–9] and coagulation abnormalities [10] associated with prophylactic antithrombotic therapy early after surgery. It is also important for orthopaedic surgeons to avoid these complications, because such infections can last a lifetime or the patients can have a relapsing course if they achieve remission from the infection. The routine administration of prophylactic antithrombotic treatment is not recommended in the Japanese Guideline for Prevention of Venous Thromboembolism [11].

Abbreviations: ACCP, American College of Chest Physicians; e-XDP, Cross-linked fibrin degradation products by leukocyte elastase; DVT, deep vein thrombosis; MDCT, multidetector-row computed tomography; PE, pulmonary embolism; SFMC, Soluble fibrin monomer complex; THA, Total hip arthroplasty; TKA, total knee arthroplasty; VTE, Venous thromboembolism.

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doi:10.1016/j.thromres.2011.07.030

Based on these observations, it is clinically important to detect asymptomatic VTE that may cause fatal or symptomatic PE before or shortly after surgery without prophylactic antithrombotic treatments to reduce postoperative infections in low-risk patients, and to start antithrombotic therapy only in those patients who need it [12].

The purpose of this study was to determine whether the postoperative occurrence of new asymptomatic VTE can be predicted by preoperative and postoperative measurements of blood coagulation markers in patients undergoing TKA, and to identify independent markers that will facilitate early diagnosis of asymptomatic VTE. We performed a prospective study using multidetector-row computed tomography (MDCT), which can detect PE and DVT simultaneously, to evaluate the predictive accuracy of various blood coagulation markers as indicators for postoperative asymptomatic VTE.

Materials and methods

Patients

The study protocol was approved by the Ethics Review Board of our university. This prospective single-centre study enrolled patients who underwent TKA at our institution between September 2004 and August 2009 and gave consent to participate in the study (Fig. 1). The necessary sample size was calculated for an alpha of 0.05 and a power of 0.90 using the statistical software 'G*Power 3' [13,14], and found to be 67. For exclusion criteria, patients with a past history of symptomatic VTE, cerebral haemorrhage, cerebral infarction, cardiac infarction and drug allergy to a contrast medium were excluded from the study. In addition, patients with liver disease, renal disease and congenital clotting factor deficiencies and those undergoing antithrombotic therapy or haemodialysis were excluded from the study. Patients with asymptomatic VTE by preoperative MDCT were also excluded from the study (Fig. 1). However, patients with hypertension, diabetes mellitus and rheumatoid arthritis were included in this study.

We finally enrolled 68 patients with low risk factors who underwent TKA for osteoarthritis (45 knees) or rheumatoid arthritis (23 knees). The patients comprised 6 men and 62 women, with a mean age of 71 years (range, 49–84 years). TKA was performed under general anaesthesia in all patients and a pneumatic tourniquet was used. During and after the surgery, the patients wore elastic stockings and used an intermittent pneumatic compression device until the initiation of walking training, in accordance with the Japanese Guideline for Prevention of Venous Thromboembolism [11]. No postoperative prophylactic antithrombotic therapy was administered. If the patients developed symptomatic VTE and if VTE was detected by MDCT, the study was discontinued and aggressive antithrombotic therapy was initiated.

MDCT

For diagnosis of VTE, 16-row MDCT was performed at 4 days preoperatively (day of admission) and 4 days postoperatively (Fig. 2), the point at which the incidences of PE and VTE are reported to be high [15] and the earliest point at which the patients could comfortably undergo MDCT during the postoperative period. The slice thicknesses were 2 mm in the thoracic region and 5 mm from the abdomen to the lower limbs. The window levels were 40–60 and 40–50 and the window widths were 400–500 and 200–400, respectively. A single radiologist (M.D.) evaluated the MDCT images in a blinded manner before and after the surgery. The incidence of postoperative new asymptomatic VTE was calculated.

Patients with asymptomatic VTE ($n=7$) by preoperative MDCT were excluded from the study. Preoperative MDCT revealed asymptomatic PE of the pulmonary segmental artery in one patient, proximal asymptomatic DVT in one patient and distal asymptomatic DVT in five patients (Fig. 1). These patients did not show D-dimer abnormalities. Preoperative MDCT revealed no asymptomatic VTE in the 68 patients included in the study. For the patients with proximal asymptomatic DVT and asymptomatic PE, antithrombotic therapy was initiated because the occurrence of fatal or symptomatic PE was considered likely [16].

The thrombus group was defined as patients with new asymptomatic VTE detected by MDCT after the surgery, and the no-thrombus group was defined as patients without asymptomatic VTE by MDCT after the surgery.

Blood coagulation markers

Blood samples were taken to measure the plasma levels of soluble fibrin monomer complex (SFMC), D-dimer and cross-linked fibrin

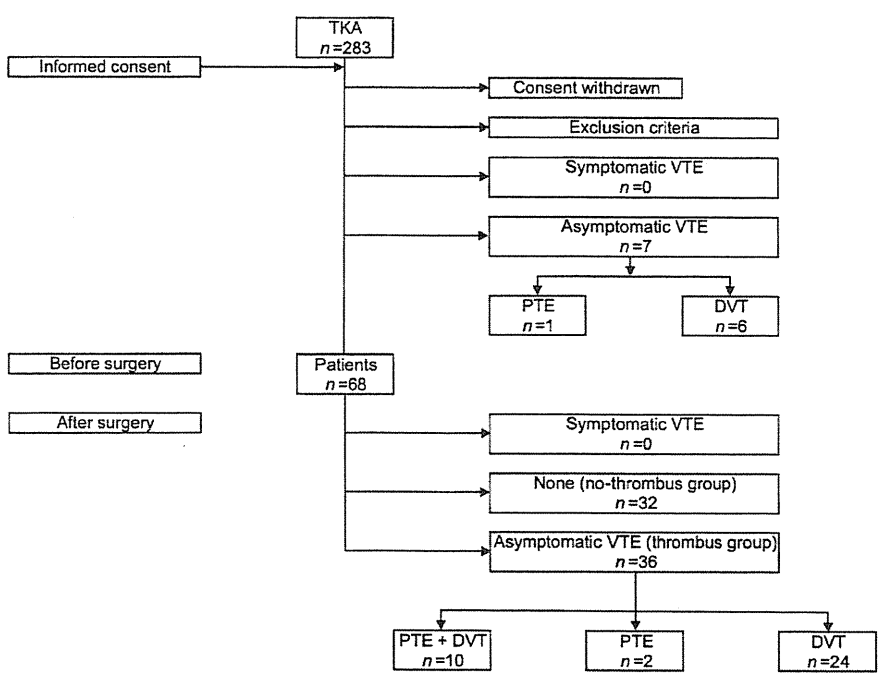

Fig. 1. Flowchart of the 283 patients undergoing TKA during the study period. *n*, number of patients.

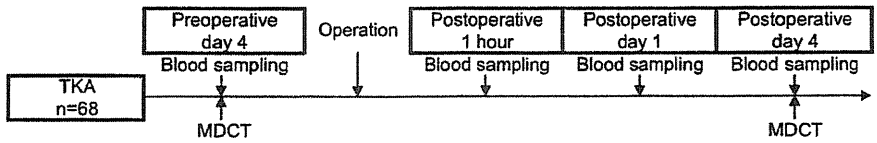

Fig. 2. Study protocol.

degradation products by leukocyte elastase (e-XDP) at 4 days preoperatively, and then at 1 hour, 1 day and 4 days postoperatively (Fig. 2). Citrated plasma samples were stored at −80 °C until analysis. The plasma SFMC levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) using the monoclonal antibody IF-43 [17]. Plasma D-dimer levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody JIF-23 [18]. Plasma e-XDP levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody IF-123 [19].

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, USA). SFMC, D-dimer and e-XDP levels were analysed by the Shapiro–Wilk test if they did not fit a normal distribution. SFMC, D-dimer and e-XDP levels were compared at 4 days preoperatively and at 1 hour, 1 day and 4 days preoperatively using the Friedman test. If a significant difference was noted, the data were compared using the Wilcoxon signed rank test and corrected using Bonferroni's inequality. SFMC, D-dimer and e-XDP levels were compared between the thrombus and no-thrombus groups using the Mann–Whitney *U* test. Sex and diabetes mellitus distributions were compared between the thrombus and no-thrombus groups using Fisher's exact test, while hypertension distributions were compared between the thrombus and no-thrombus groups using the chi-square test. Age, volume of intraoperative haemorrhage, operation time, other presurgical factors and blood markers were compared using an unpaired *t*-test. Multiple logistic regression analyses were used to determine whether blood coagulation markers were independent predictors of the postoperative occurrence of new asymptomatic VTE or were affected by other factors. The level of statistical significance was set at $P<0.05$ for all tests.

Results

No patients developed symptomatic VTE after TKA in this study (Fig. 1). Postoperative MDCT revealed asymptomatic VTE in 36 patients (thrombus group) and no VTE in 32 patients (no-thrombus group) (Fig. 1). Aggressive antithrombotic therapy was initiated in the 36 patients in whom new asymptomatic VTE was detected on postoperative MDCT.

Preoperative blood coagulation markers

There were no significant differences in preoperative SFMC, D-dimer and e-XDP levels between the thrombus and no-thrombus groups ($P=0.13$, $P=0.18$ and $P=0.15$, respectively; Fig. 3).

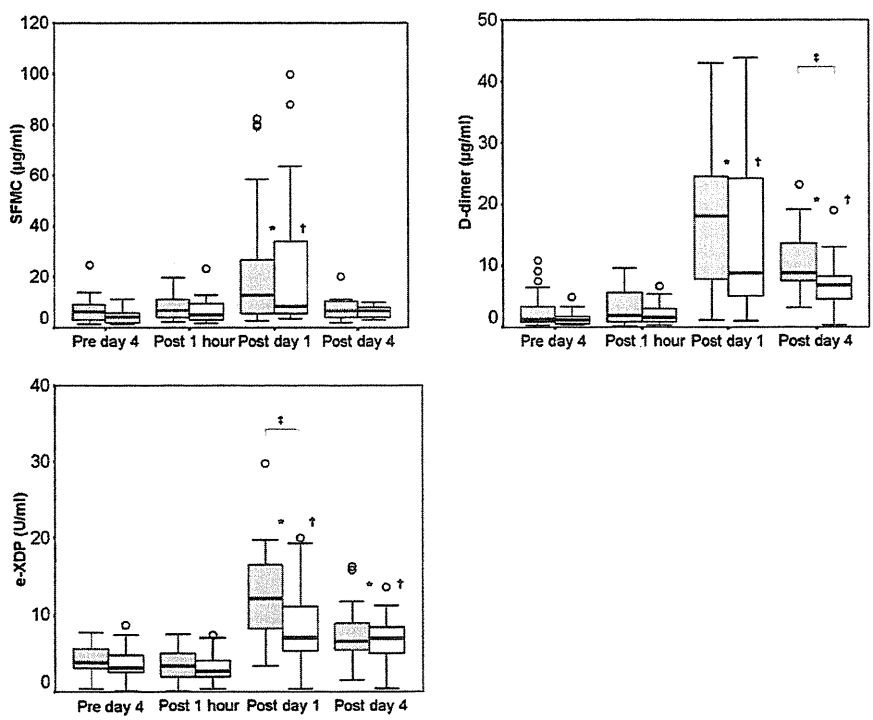

Fig. 3. Preoperative and postoperative SFMC, D-dimer and e-XDP levels. Gray boxes, thrombus group; white boxes, no-thrombus group. The circles are outliers. *$P<0.05$ versus the preoperative level in the thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. †$P<0.05$ versus the preoperative level in the no-thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. ‡$P<0.05$, thrombus group versus no-thrombus group by the Mann–Whitney *U* test.

Postoperative blood coagulation markers

SFMC

SFMC levels differed significantly at day 1 postoperatively in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, a significant increase in the SFMC level was observed at postoperative day 1 (median: 12.8 μg/ml; interquartile range: 5.3 to 27.2) compared with the preoperative value (median: 6.2 μg/ml; interquartile range: 3.1 to 9.3; $P=0.01$). Similarly, in the no-thrombus group, a significant increase in the SFMC level was observed at postoperative day 1 (median: 8.5 μg/ml; interquartile range: 5.6 to 34.9) compared with the preoperative value (median: 4.2 μg/ml; interquartile range: 2.2 to 6.4; $P=0.01$). However, postoperative changes in the SFMC level at postoperative day 1 did not differ significantly between the thrombus and no-thrombus groups ($P=0.85$; Fig. 3). SFMC levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour (median: 6.9 μg/ml; interquartile range: 4.3 to 11.4; $P=0.10$; and median: 5.3 μg/ml; interquartile range: 3.1 to 9.8; $P=0.07$, respectively) and 4 days (median: 6.8 μg/ml; interquartile range: 4.1 to 10.6; $P=0.27$; and median: 6.6 μg/ml; interquartile range: 4.2 to 8.2; $P=0.06$, respectively) postoperatively (Fig. 3).

D-dimer

D-dimer levels differed significantly at postoperative days 1 and 4 in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, significant increases in D-dimer levels were observed at postoperative day 1 (median: 18.1 μg/ml; interquartile range: 7.3 to 25.8) and day 4 (median: 8.8 μg/ml; interquartile range: 7.4 to 13.7) compared with the preoperative value (median: 1.3 μg/ml; interquartile range: 0.8 to 3.9; $P=0.01$ for both time points). In the no-thrombus group, significant increases in D-dimer levels were observed at postoperative day 1 (median: 8.8 μg/ml; interquartile range: 4.8 to 25.2) and day 4 (median: 6.8 μg/ml; interquartile range: 4.3 to 8.6) compared with the preoperative value (median: 1.2 μg/ml; interquartile range: 0.6 to 1.8; $P=0.01$ for both time points). With regard to the postoperative changes, the D-dimer level at postoperative day 4 was significantly higher in the thrombus group than that in the no-thrombus group ($P=0.01$; Fig. 3). With a cut-off D-dimer level of 7.5 μg/ml, the sensitivity was 75%, the specificity was 63% and the likelihood ratio (sensitivity/1−specificity) was 2.03 for predicting postoperative asymptomatic VTE (Fig. 4). D-dimer levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour postoperatively (median: 1.9 μg/ml; interquartile range: 0.9 to 5.6; $P=0.39$; and median: 1.5 μg/ml; interquartile range: 0.9 to 3.4; $P=0.09$, respectively) (Fig. 3).

e-XDP

The e-XDP levels differed significantly at postoperative days 1 and 4 in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, significant increases in e-XDP levels were observed at postoperative day 1 (median: 12.2 μg/ml; interquartile range: 7.9 to 18.2) and day 4 (median: 6.7 μg/ml; interquartile range: 5.3 to 9.0) compared with the preoperative value (median: 3.8 μg/ml; interquartile range: 3.0 to 5.6; $P=0.01$ for both time points). In the no-thrombus group, significant increases in e-XDP levels were observed at postoperative day 1 (median: 7.0 μg/ml; interquartile range: 5.3 to 11.1) and day 4 (median: 7.0 μg/ml; interquartile range: 4.6 to 8.4) compared with the preoperative value (median: 3.1 μg/ml; interquartile range: 2.6 to 4.7; $P=0.01$ for both time points). With regard to the postoperative changes, the e-XDP level at postoperative day 1 was significantly higher in the thrombus group than that in the no-thrombus group ($P=0.01$; Fig. 3). With a cut-off e-XDP level of 8.2 U/ml, the sensitivity was 75%, the specificity was 59% and the likelihood ratio was 1.84 for predicting postoperative asymptomatic VTE (Fig. 4). The e-XDP levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour postoperatively (median: 3.3 U/ml; interquartile range: 2.0 to 5.1; $P=0.33$; and median: 2.7 U/ml; interquartile range: 2.0 to 4.4; $P=0.08$, respectively) (Fig. 3).

There was a significant difference in the minimum blood pressure, but no significant differences in age, sex, volume of intraoperative haemorrhage, operation time or other presurgical factors between the thrombus and no-thrombus groups (Table 1). Multiple logistic regression analyses revealed that the D-dimer level at postoperative day 4 differed significantly among the other factors and was an independent marker of postoperative new asymptomatic VTE by MDCT (odds ratio = 1.61, $P=0.01$; Table 2). Likewise, the e-XDP level at postoperative day 1 differed significantly among the other factors and was an independent marker of postoperative new asymptomatic VTE (odds ratio = 1.19, $P=0.04$; Table 2).

Discussion

Recently, MDCT has been used as a technique to identify VTE. In addition, MDCT is able to diagnose PE to the level of the subsegmental pulmonary arteries and can provide rapid and objective detection and

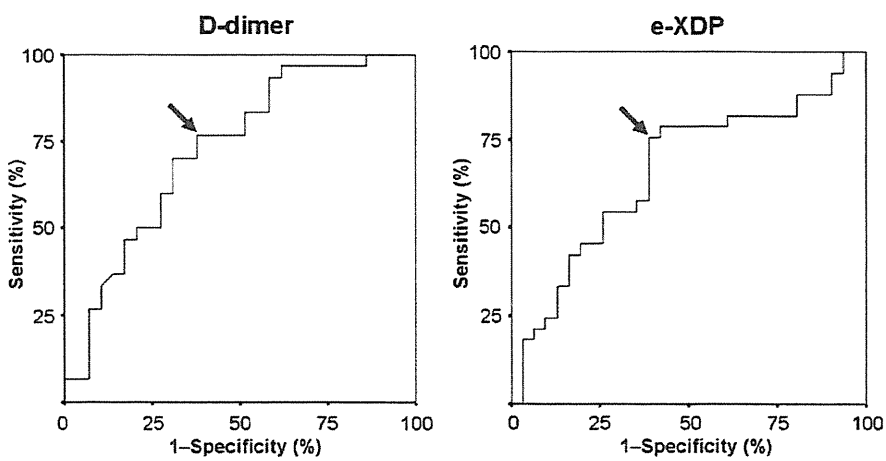

Fig. 4. Receiver-operator characteristic curves. Left: At a cut-off point of 7.5 μg/ml for the D-dimer level at postoperative day 4, the sensitivity is 75% (95% confidence interval: 73 to 77), the specificity is 63% (95% confidence interval: 60 to 66) and the likelihood ratio is 2.03. Right: At a cut-off point of 8.2 U/ml for the e-XDP level at postoperative day 1, the sensitivity is 75% (95% confidence interval: 73 to 77), the specificity is 59% (95% confidence interval, 56 to 62) and the likelihood ratio is 1.84.

Table 1
Patient characteristics.

		Thrombus group (n=36)	No-thrombus group (n=32)	P		95% Confidence interval
					(Odds ratio)	
Sex, male:female		1:35	5:27	0.09*	0.15§	0.02 to 1.40
Hypertension:normotension		18:18	14:18	0.61†	1.29¶	0.50 to 3.35
Diabetes mellitus:normoglycaemia		2:34	4:28	0.41*	0.41#	0.07 to 2.42
					*(Difference**)*	
Age	years	71 (68 to 73)††	72 (68 to 75)††	0.82‡	-0.5	-5 to 4
Intraoperative haemorrhage	ml	167 (0 to 344)††	175 (4 to 346)††	0.95‡	-7	-252 to 236
Operation time	min	161 (147 to 174)††	150 (140 to 160)††	0.23‡	10	-7 to 27
Before surgery						
Height	cm	148 (146 to 151)††	150 (148 to 153)††	0.25‡	-2	-6 to 1
Weight	kg	57 (54 to 60)††	57 (52 to 61)††	0.91‡	0	-5 to 5
Body mass index	kg/m^2	26 (25 to 27)††	25 (23 to 27)††	0.51‡	1	-2 to 3
Blood pressure (maximum)	mmHg	128 (124 to 133)††	127 (121 to 132)††	0.61‡	2	-5 to 9
Blood pressure (minimum)	mmHg	74 (71 to 77)††	67 (63 to 70)††	0.01‡	8	4 to 12
Pulse rate	/min	73 (70 to 76)††	71 (68 to 73)††	0.18‡	3	-1 to 7
Temperature	°C	36.5 (36.4 to 36.6)††	36.5 (36.4 to 36.7)††	0.58‡	-0.1	-0.2 to 0.1

*Fisher's exact test; †chi-square test; ‡unpaired t-test.
§Male/female; ¶hypertension/normotension; #diabetes mellitus/normoglycaemia; **thrombus-no thrombus; ††mean (95% confidence interval).

measurement of thrombi in both PE and DVT [20–24]. In patients in whom symptomatic PE is suspected, the usefulness of this modality is supported by its high sensitivity and specificity, which have been reported to be 100% and 89% for PE using pulmonary angiography as the reference standard [20], 100% and 96.6% for proximal DVT using doppler sonography as the reference standard [21] and 93% and 97% for distal and proximal DVT using doppler sonography and venography as the reference standard [22], respectively. Additionally, sensitivity and specificity have been reported to be 100% and 97% for distal DVT, 100% and 97% for proximal DVT, respectively, using conventional venography as the reference standard in patients in whom symptomatic DVT was suspected [23]. However, it is unknown how the sensitivity and specificity for only distal DVT of MDCT can be estimated, because there are few studies that have estimated sensitivity and specificity; overall, the sensitivity (ranges from 71—100%) and specificity (ranges from 93—100%) of MDCT are high [24]. A highly sensitive and specific imaging examination is necessary for early detection of VTE. However, there are problems with this approach that limit its practicality, such as exposure to radiation [25,26], invasive administration of contrast medium, potential for drug allergy, cost of equipment and the frequent imaging required to detect VTE, the occurrence of which is unpredictable. Therefore, initial evaluation of the presence of VTE using blood markers is preferable, with only high-risk patients examined by imaging techniques, to reduce the potential risks associated with radiation or contrast exposure and to improve the cost-effectiveness.

There were no blood coagulation markers for predicting early postoperative asymptomatic DVT after TKA until 1997 [27,28]. In 2000, Rever at al. [29] performed venography after TKA and reported that the SFMC level was significantly elevated in patients with asymptomatic DVT at postoperative days 3 and 6. However, they concluded that there was no clinically significant cut-off point. Similarly, the present study could not establish that the SFMC level was associated with asymptomatic VTE after TKA or an independent marker for predicting the postoperative occurrence of new asymptomatic VTE.

In 1998, Bounameaux et al. [30] performed venography and D-dimer measurements at day 3 after TKA. They found that the D-dimer level was significantly elevated in patients with asymptomatic DVT and that the sensitivity and specificity were 58.8% and 73.5%, respectively, at a cut-off level of 3000 μg/ml. In the present study, the D-dimer level at postoperative day 4 was significantly higher in the thrombus group than that in the no-thrombus group. The sensitivity, specificity and likelihood ratio of the D-dimer level at postoperative day 4 using a cut-off point of 7.5 μg/ml were 75%, 63% and 2.03, respectively, for predicting postoperative asymptomatic VTE. Furthermore, by multiple logistic regression analysis, the D-dimer level at postoperative day 4 was an independent marker for predicting postoperative asymptomatic VTE, whereas, except for the minimal blood pressure, there were no significant differences in age, sex, volume of intraoperative haemorrhage, operation time and presurgical factors or blood markers between the thrombus and no-thrombus groups.

Table 2
Multiple logistic regression analyses of blood coagulation markers and other factors.

		Postoperative day 1			Postoperative day 4		
		Odds ratio	95% Confidence interval	P	Odds ratio	95% Confidence interval	P
SMFC	μg/ml	1.00	0.98 to 1.02	0.84	1.00	0.97 to 1.04	0.88
D-dimer	μg/ml	0.97	0.93 to 1.02	0.29	1.61	1.12 to 2.20	0.01
e-XDP	U/ml	1.19	1.01 to 1.40	0.04	0.75	0.54 to 1.05	0.10
WBC	/μl	1.00	1.00 to 1.00	0.67	1.00	1.00 to 1.00	0.93
RBC	$\times 10^4/\mu l$	1.00	0.97 to 1.03	0.96	1.03	0.98 to 1.08	0.26
Hct	%	0.37	0.09 to 1.55	0.18	0.47	0.12 to 1.84	0.28
Hb	g/dl	13.49	0.30 to 604.51	0.18	3.34	0.06 to 239.21	0.52
Plt	$\times 10^4/\mu l$	1.05	0.94 to 1.17	0.40	1.16	0.97 to 1.38	0.10
Blood pressure (maximum)	mmHg	0.55	0.30 to 1.00	0.51	0.55	0.25 to 1.22	0.14
Blood pressure (minimum)	mmHg	2.31	0.84 to 6.40	0.11	2.73	0.93 to 8.00	0.07
Pulse rate	/min	0.91	0.42 to 2.00	0.82	0.75	0.29 to 1.94	0.55
Temperature	°C	1.2	0.46 to 3.08	0.72	12.47	0.88 to 177.10	0.06

WBC=white blood cell count; RBC=red blood count; Hct=haematocrit; Hb=haemoglobin; Plt, platelet count.