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Regular Article

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

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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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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.

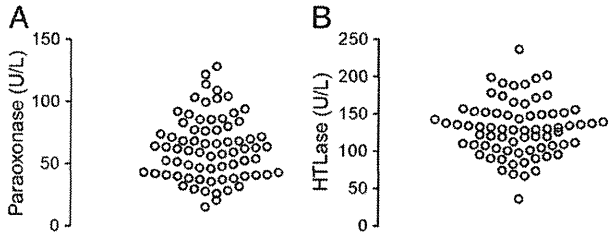

Fig. 1. Serum paraoxonase and HTLase activities in the study population.

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*

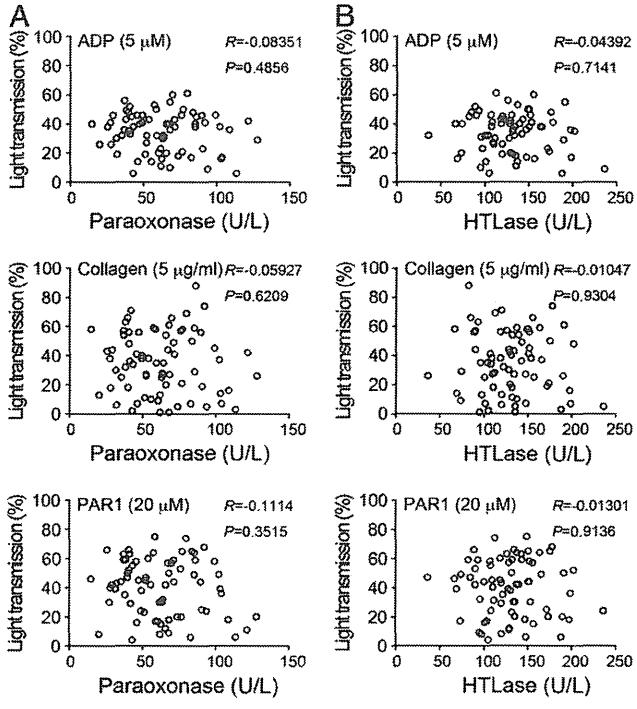

Fig. 2. Association between PON1 activities and platelet aggregation. Platelet aggregation induced by 5 μM ADP, or 5 μg / ml of collagen, or 20 μM TRAP was assessed by aggregometry, and was expressed as light transmission (%). Serum paraoxonase activities (U / L) (A) and HTLase activities (U / L) (B) 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. *$P<0.05$.

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.

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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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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.

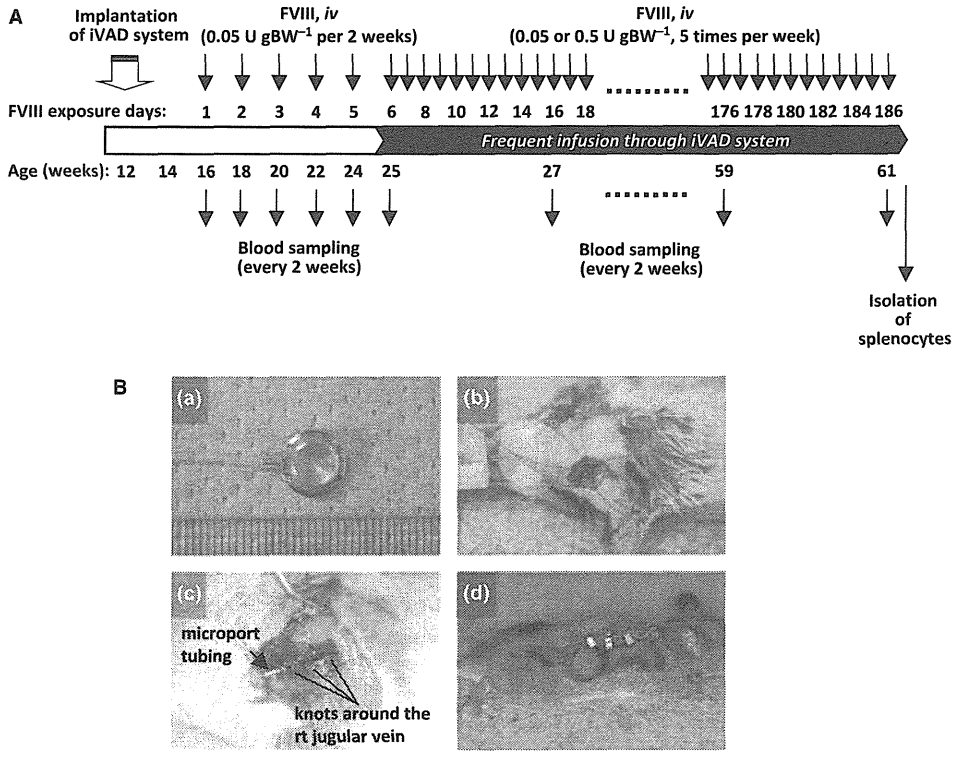

Fig. 1. Protocol for serial intravenous administration of FVIII through an implantable venous access device (iVAD) system in haemophilia A mice. A. Haemophilia A mice were implanted an iVAD system at age 12 weeks, then they were preimmunized with intravenous injection of 0.05 U g^{-1} BW recombinant 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

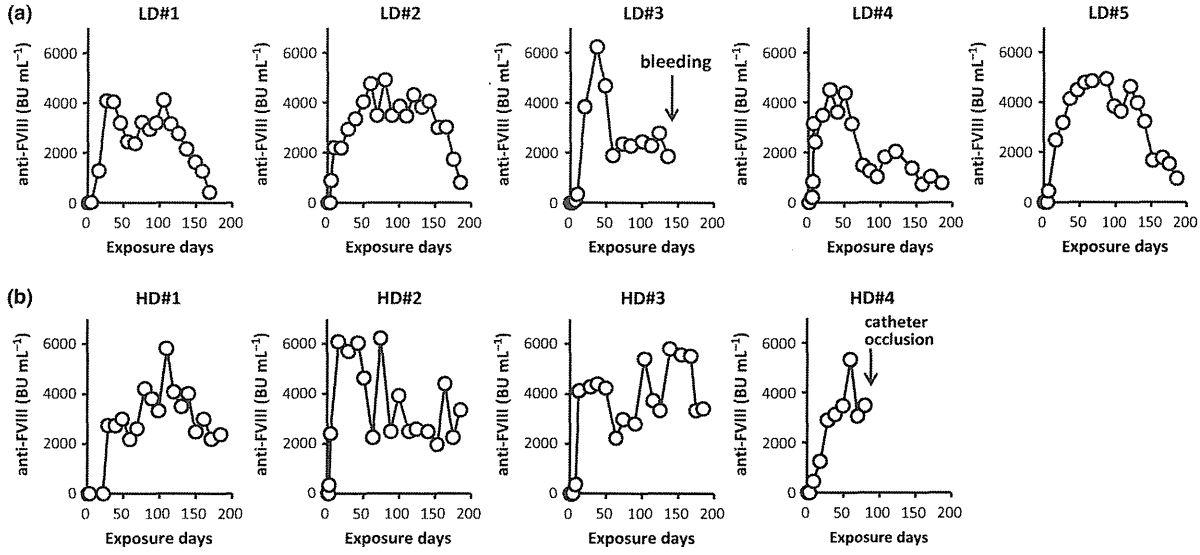

Fig. 2. Effect of serial intravenous administration of FVIII on anti-FVIII inhibitory antibody formation in preimmunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies 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.

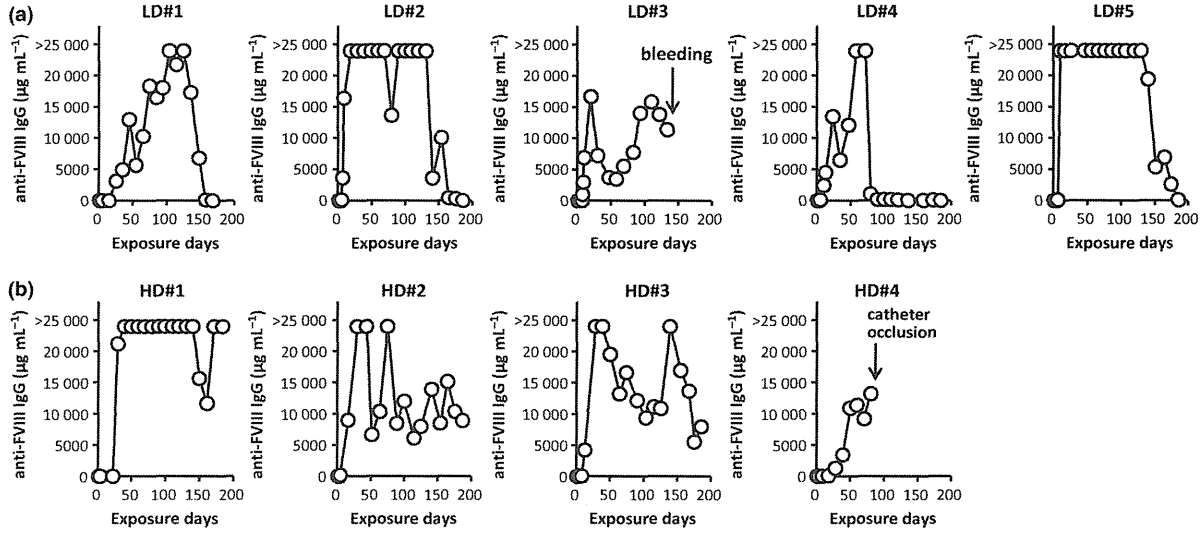

Fig. 3. Effect of repeated intravenous infusion of FVIII on FVIII-specific IgG formation in haemophilia A mice with inhibitors. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized 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

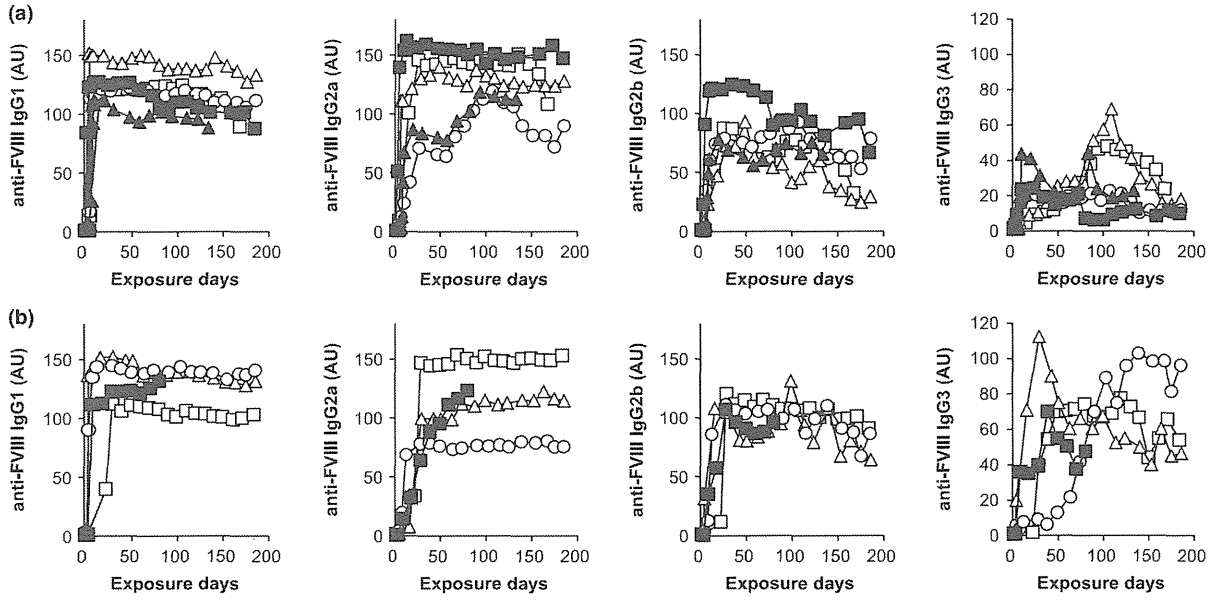

Fig. 4. Effect of serial intravenous injection on anti-FVIII IgG subclasses formation in preimmunized haemophilia A mice. (a) Haemophilia A mice (LD1, open squares; LD#2, open triangles; LD#3, closed triangles; LD#4, closed squares; LD#5, open circles) were intravenously immunized with 0.05 U g^{-1} 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

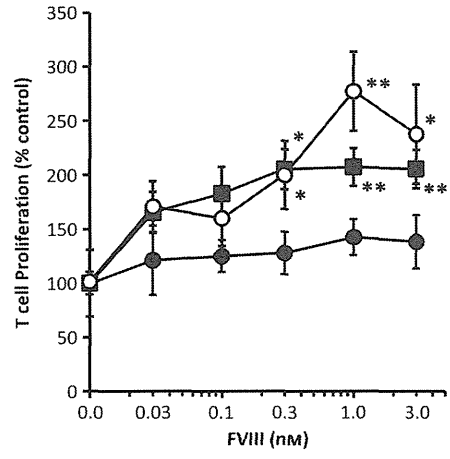

Fig. 5. Effect of repeated administration of FVIII on anti-factor VIII T-cell proliferation of haemophilia A mice. Haemophilia A mice were given intravenous injection of 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice 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.

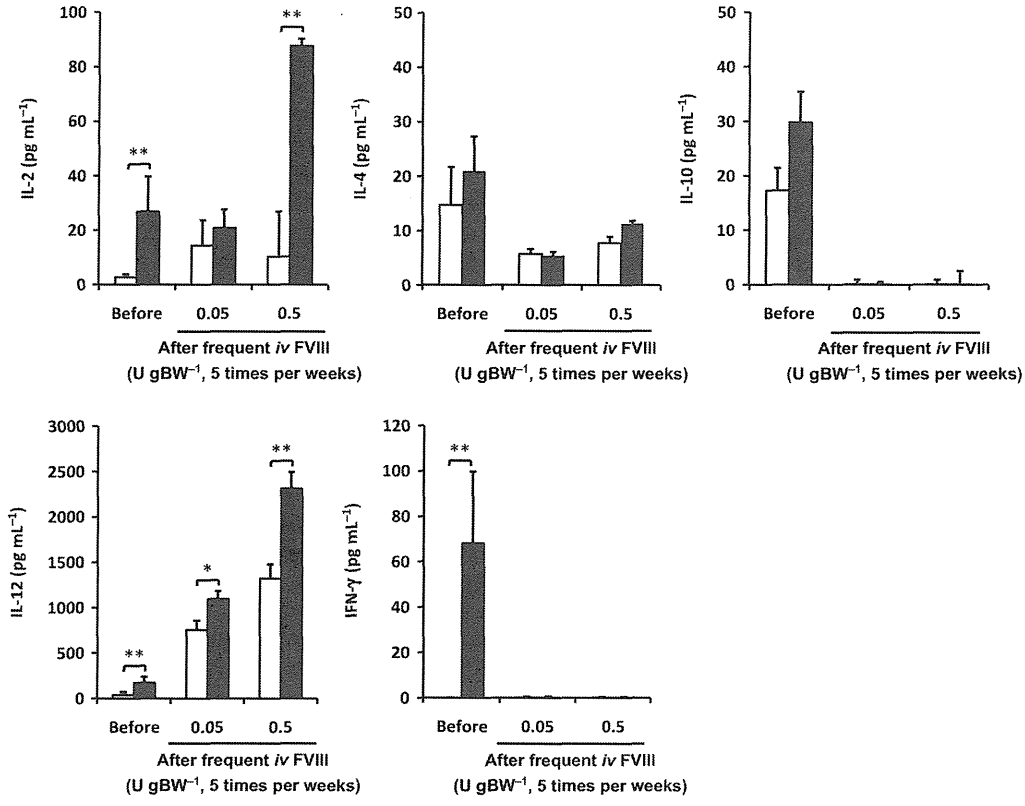

Fig. 6. Effect of serial infusion of FVIII on cytokine response of pre-immunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were frequently 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.

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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.

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Large infarct and high mortality by cerebral ischemia in mice carrying the factor V Leiden mutation

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Factor V Leiden (FVL) mutation (R506Q mutation) is an established risk factor for venous thromboembolism due to a hypercoagulable state through the resistance to activated protein C [1]. The FVL mutation also exerts a modest effect on arterial ischemic diseases. It is associated with ischemic stroke in children [2] and in young adults [3] and appears to be weakly associated with ischemic stroke in the general adult population [4]. A mouse model carrying a targeted homologous mutation at R504 to Q in FV has been developed [5] and studied under various stimulations or pathophysiologic conditions [6–8]. However, the role of the FVL mutation in ischemic stroke has not been confirmed using the mouse model. In this study, we applied brain ischemia-reperfusion injury in FVL mice using the three-vessel occlusion technique [9]. This technique produces a constant infarcted lesion limited to within the neocortex with small variances and does not require intraluminal thread insertion, which might activate the coagulation system during ischemia.

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Male young adult mice (7–18 weeks old) of wild-type $Fv^{+/+}$, heterozygous $Fv^{Q/+}$ and homozygous $Fv^{Q/Q}$ (Jackson Labs, Bar Harbor, ME, USA) were used for the experiments. All animal procedures were approved by the Animal Care and Use Committees of the National Cerebral and Cardiovascular Center. Temporary focal ischemia was induced using the three-vessel occlusion technique as described previously [9]. Briefly, we electrocauterized the distal M1 portion of the left middle cerebral artery, peripheral to the perforating arteries of the basal ganglia, and made temporal occlusions on the bilateral common carotid arteries for 15 min using vascular clips. After 24 h, neurological deficits were assessed using a scoring scale (from 0 to 4) as described [10,11], and the brains were excised and stained with 2, 3, 5-triphenyl tetrazolium chloride. The infarct and total hemispheric volumes were measured as described [10]. The infarct volume was adjusted for edema by dividing the volume by the edema index (left hemisphere volume/right hemisphere volume) [10,11]. The regional cerebral blood flow (rCBF) at the penumbra-like area of the left hemisphere was monitored using a laser-Doppler blood flowmeter TBF-LN1 (Unique Medical, Tokyo, Japan) [9,10]. In separate experimental groups the survival of the mice was monitored for 7 days after ischemia and graphed using a Kaplan–Meyer plot. Data were analyzed by the one-way ANOVA test followed by the post hoc Bonferroni's multiple comparison test. Survival rates were analyzed by the Mantel-Cox log-rank test. Data were expressed as the means ± standard deviation. P-values < 0.05 were considered significant.

Infarct volumes 24 h after ischemia in $Fv^{Q/+}$ and $Fv^{Q/Q}$ mice were significantly larger than those in $Fv^{+/+}$ mice (15.9 ± 5.3 mm^3 in $Fv^{+/+}$, n = 11; 26.7 ± 4.8 mm^3 in $Fv^{Q/+}$, n = 12; 29.9 ± 4.1 mm^3 in $Fv^{Q/Q}$ mice, n = 8; $Fv^{+/+}$ vs. $Fv^{Q/+}$, $P < 0.001$; $Fv^{+/+}$ vs. $Fv^{Q/Q}$, $P < 0.001$) (Fig. 1A,B). The edema index (1.06 ± 0.03 in $Fv^{+/+}$, 1.05 ± 0.02 in $Fv^{Q/+}$, 1.04 ± 0.04 in $Fv^{Q/Q}$) and the neurological deficit score (2.82 ± 0.40 in $Fv^{+/+}$, 2.58 ± 0.67 in $Fv^{Q/+}$, 2.75 ± 0.46 in $Fv^{Q/Q}$) were not different among the groups. The rCBF during ischemia in $Fv^{Q/Q}$ mice (9.9 ± 1.2%, n = 7) was more severely decreased than in either $Fv^{+/+}$ (21.2 ± 0.5%, n = 7) or $Fv^{Q/+}$ mice (19.5 ± 2.0%, n = 7) ($P < 0.001$) (Fig. 1C). In every group, the rCBF was recovered to the preischemic normal level immediately after removal of the vascular clips, ensuring sufficient reperfusion of the left middle cerebral artery territory. $Fv^{Q/Q}$ mice started to die from day 2, and only two out of seven mice survived for 7 days (Fig. 1D). $Fv^{Q/+}$ mice started to die from day 4, and only two mice survived. Two $Fv^{+/+}$ mice died on day 7, and the remaining five survived. The 7-day survival in $Fv^{Q/+}$ and $Fv^{Q/Q}$ mice was significantly lower than that in $Fv^{+/+}$ mice ($P < 0.05$).

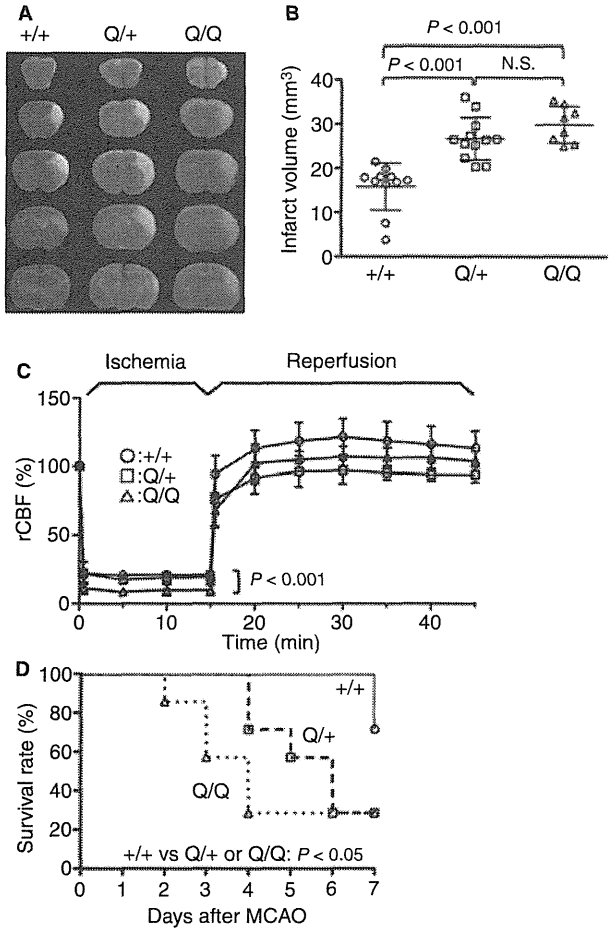

Fig. 1. Effects of factor V Leiden (FVL) on focal ischemia. (A) Representative images of coronal sections of $Fv^{+/+}$, $Fv^{Q/+}$ and $Fv^{Q/Q}$ mouse brains. *Red* areas represent vital brain tissue and *white* areas represent cerebral infarction. (B) Infarct volumes of $Fv^{+/+}$ (○), $Fv^{Q/+}$ (□) and $Fv^{Q/Q}$ (△) mice. *Bars* represent the means ± standard deviation. N.S., not significantly different ($P > 0.05$). (C) rCBF of $Fv^{+/+}$, $Fv^{Q/+}$ and $Fv^{Q/Q}$ mice during 15-min ischemia and 30-min reperfusion. The rCBFs were expressed as percentages of the baseline flow obtained before middle cerebral artery occlusion. Data are the means ± standard deviation of seven mice per group. (D) Seven-day survival of $Fv^{+/+}$, $Fv^{Q/+}$ and $Fv^{Q/Q}$ mice after temporary ischemia (n = 7/group).

We demonstrated that both $Fv^{Q/+}$ and $Fv^{Q/Q}$ mice showed increased infarct volumes and decreased long-term survival compared with $Fv^{+/+}$ mice after the temporary focal ischemia-reperfusion stress. Although the three-vessel occlusion technique does not activate the coagulation system during ischemia, the infarct lesion was larger in $Fv^{Q/+}$ or $Fv^{Q/Q}$ mice than in $Fv^{+/+}$ mice. The reperfusion process after transient cerebral ischemia is known to induce many cellular events, including the loss of normal permeability in the blood brain barrier, which deteriorates cerebral metabolism, increases cerebral damage [12], and induces ischemic cerebral damage-related secondary activation of the coagulation system with subsequent thrombus formation around the infarct lesion [13]. The hypercoagulable state of FVL, which can enhance reperfusion injury after ischemia, is considered to be the primary cause of both the enlargement of infarct lesions and the vulnerability of the brain after ischemic stroke. The other possible pathophysiology is that FVL may have affected some of the intrinsic vascular and/or neuronal protection systems.

The findings that both $Fv^{Q/+}$ and $Fv^{Q/Q}$ mice showed larger infarct volumes and lower survival rates than $Fv^{+/+}$ mice after temporary focal ischemia-reperfusion stress support a direct causal relationship between the FVL mutation and increased susceptibility to ischemic stroke in young adult individuals.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Binding of von Willebrand factor cleaving protease ADAMTS13 to Lys-plasmin(ogen)

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The metalloprotease ADAMTS13 affects platelet adhesion and aggregation through depolymerization of von Willebrand factor (VWF) multimers. Identification of ADAMTS13-binding proteins would reveal the hitherto unrecognized mechanisms underlying microvascular thrombus. To identify ADAMTS13-binding proteins, we performed a yeast two-hybrid screen using the Cys-rich and spacer domains of ADAMTS13, the critical regions for the binding and cleavage of VWF, as a bait region. We identified Lys-plasminogen, an amino-terminal truncated form of plasminogen, as the binding protein to ADAMTS13. Intact Glu-plasminogen did not bind to ADAMTS13. Active-site blocked Lys-plasmin bound to ADAMTS13. Domain truncation of ADAMTS13 and elastase digest of plasminogen indicated that the Cys-rich and spacer domains of ADAMTS13 and the kringle 5 and protease domains of plasminogen served as the main binding sites. Biacore measurements revealed that Lys-plasminogen bound to ADAMTS13 with a K_d of $1.9 \pm 0.1 \times 10^{-7}$ M and Glu-plasminogen exhibited a significantly lower affinity to ADAMTS13. Specific activity measurements revealed that ADAMTS13 and Lys-plasmin were still active even after the binary complex was formed. The binding of ADAMTS13 to Lys-plasminogen may play an important role to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

Keywords: ADAMTS13/fibrinolysis/plasminogen/thrombotic thrombocytopenic purpura/von Willebrand factor.

Abbreviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; APMSF, *p*-amidinophenyl methanesulfonyl fluoride; CUB, complement components C1r and C1s/urinary epidermal growth factor/bone morphogenic protein-1; Glu-Pg, Glu-plasminogen; HRP, horseradish peroxidase; Lys-Pg, Lys-plasminogen; mAb, monoclonal antibody; mini-Pg, mini-plasminogen; VWF, von Willebrand factor.

Platelet thrombus formation is dependent on the multimeric sizes of von Willebrand factor (VWF) under shear stress conditions. VWF multimers are depolymerized by plasma metalloprotease ADAMTS13. Thus, ADAMTS13 regulates the VWF-dependent platelet thrombus formation. Congenital or acquired deficiency of ADAMTS13 can cause thrombotic thrombocytopenic purpura that is characterized with thrombocytopoenia and microangiopathic haemolytic anaemia, sometimes accompanied with transient neurological dysfunction (*1–4*). ADAMTS13 has multiple discrete domains, comprising a metalloprotease domain (M), a disintegrin-like domain (D), a first thrombospondin type-1 repeat (T), a Cys-rich region (C), a spacer domain (S), seven consecutive T repeats and two CUB (Complement components C1r and C1s/urinary epidermal growth factor/bone morphogenic protein-1) domains (*5–7*).

ADAMTS13 cleaves a single specific peptide bond of Tyr^{1605}–Met^{1606} within the A2 domain of VWF under shear stress conditions *in vivo* or under denatured conditions *in vitro*. This restricted substrate specificity can be defined by several structural features in ADAMTS13. The C and S domains in ADAMTS13 play a critical role on the binding and cleavage of VWF, and the S domain seems to be highly important for the recognition of VWF (*8,9*). Studies using ADAMTS13 mutants and VWF peptides indicated cooperative and modular interaction of discrete segments of VWF with ADAMTS13 (*10–13*). The crystal structures of the DTCS domains showed three VWF-binding exosites on the linearly aligned discontinuous surfaces of the D, C and S domains (*14,15*). Two C-terminal CUB domains are also important for regulation of VWF cleavage *in vitro* as well as *in vivo* (*16–20*). Thus, the interaction between ADAMTS13 and VWF has been intensively investigated; however, the binding proteins for ADAMTS13 are not well known.

Fibrinolytic system in blood is involved in dissolution of blood clots and maintains a patent vascular system. The key component of the fibrinolytic system is plasmin that degrades fibrin clots. Plasmin is

generated from the inactive proenzyme, plasminogen, by cleavage of the Arg561–Val562 peptide bond. Two distinct physiological plasminogen activators, tissue type- or urokinase type-plasminogen activator, convert plasminogen to active plasmin on the fibrin or cell surface. Native plasminogen has N-terminal glutamic acid, designated Glu-plasminogen (Glu-Pg). Lys-plasminogen (Lys-Pg), an amino-terminal truncated form of plasminogen, is formed by the release of a 76-amino acid pre-activation peptide from intact Glu-Pg by the action of plasmin. Because Lys-Pg shows a more open conformation than Glu-Pg, plasminogen activators preferentially cleave Lys-Pg than Glu-Pg. To inhibit the fibrinolytic system, a plasminogen activator inhibitor-1 or α_2-plasmin inhibitor forms an inactive complex with plasminogen activator or plasmin, respectively (*21*).

In the present study, we performed a yeast two-hybrid screen using the critical regions, the C and S domains, for the VWF binding as a bait. The co-immunoprecipitation analysis, the far-western blotting and the Biacore measurement indicated that Lys-Pg is the binding protein to ADAMTS13. ADAMTS13 and Lys-plasmin were active even after the binary complex was formed. The binding of ADAMTS13 to Lys-Pg may play an important role to localize these two proteases at sites of thrombus formation or vascular injury where the fibrinolytic system is activated.

Materials and Methods

Yeast two-hybrid screen

The Matchmaker Two-hybrid System 3 (Clontech, Palo Alto, CA, USA) was used according to the manufacturer's instructions. A fragment encoding the C and S domains of human ADAMTS13 (amino acids 440–685) was used as the bait. cDNA libraries (Clontech) constructed from human liver and brain mRNA (1.3×10^8 and 1.4×10^7 clones, respectively) were screened. Insert DNA of positive clones was sequenced, and the sequence homologies were searched by basic local alignment search tool (BLAST).

Binding of ADAMTS13 to immobilized candidate proteins

The binding of ADAMTS13 (3 μg/ml) to immobilized proteins (9 μg/ml) was examined using microtiter plates. Bound ADAMTS13 to immobilized proteins was detected using anti-ADAMTS13 monoclonal antibody (mAb) WH2-22-1A, which recognizes the disintegrin-like domain (*22*), and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. Bound HRP activity was detected at 450 nm with a reference wavelength of 650 nm using 3,3′,5,5′-tetramethylbenzidine substrate (KPL, Gaithrburg, MD, USA) and a Multiskan Ascent microplate reader (Thermo, Waltham, MA, USA).

Co-immunoprecipitation analysis of ADAMTS13 with Glu-Pg or Lys-Pg

Human ADAMTS13 with a FLAG tag (ADAMTS13-FLAG) and two mutants, MD-FLAG constituting the M and D domains with the FLAG tag and MDTCS-FLAG constituting the M, D, T, C and S domains with the FLAG tag, were expressed in the culture medium using HeLa cells, as previously described (*8*). Culture medium containing each of those recombinant proteins was incubated with intact Glu-Pg (Calbiochem, Madison, WI, USA) and/or Lys-Pg (Calbiochem) in Tris-buffered saline (TBS: 50 mM Tris, 100 mM NaCl, pH 7.5) and immunoprecipitated with anti-FLAG M2 mAb-immobilized gel (Sigma-Aldrich, St. Louis, MO, USA). After washing with TBS containing 0.5% Tween-20 (TBS-T), proteins were eluted by the FLAG peptide, and subjected to SDS–PAGE for western blotting using anti-FLAG M2 mAb (Sigma) or anti-Pg mAb MAB2596 (R&D Systems, Minneapolis, MN, USA). Alternatively, we used the anti-Pg mAb and protein G-agarose (Sigma) for the co-immunoprecipitation analysis of purified ADAMTS13 (*22*) with Glu-Pg, Lys-Pg or *p*-amidinophenyl methanesulfonyl fluoride (APMSF)-treated Lys-plasmin (Calbiochem). Bound proteins were eluted with 100 mM glycine-HCl, pH 2.5, and then subjected to SDS–PAGE for western blotting using anti-ADAMTS13 mAb WH10, which recognizes the fourth thrombospondin type-1 repeat (*22*) or anti-Pg mAb MAB2596. Immunoblots were probed with HRP-conjugated anti-mouse IgG antibody. Protein bands were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer, Waltham, MA, USA) on an image analyser LAS3000 (Fujifilm, Tokyo, Japan).

Identification of ADAMTS13 binding region in Lys-Pg

Lys-Pg (0.1 mg) was digested with porcine pancreatic elastase (5 μg; Sigma). The resulting mini-plasminogen (mini-Pg), a functionally active zymogen containing the kringle 5 and protease domains, and fragments containing the kringle 1–4 domains were obtained in the unbound and bound fractions, respectively, using a lysine-Sepharose column (GE Healthcare, Little Chalfont, UK) (*23*). Proteins were subjected to SDS–PAGE for N-terminal sequence analysis and transferred onto polyvinylidene difluoride membranes for far-western blotting. Proteins on the membranes were incubated with ADAMTS13. Bound ADAMTS13 was detected with the HRP-conjugated anti-ADAMTS13 polyclonal antibody (*22*) prepared using Peroxidase Labeling Kit-NH_2 (Dojindo, Kumamoto, Japan) and visualized using Western Lightning Chemiluminescence Reagent Plus on the image analyzer LAS3000.

Lys-Pg binding to ADAMTS13 using Biacore

The binding of Glu-Pg or Lys-Pg to ADAMTS13 was examined using a Biacore 2000 (GE healthcare, Piscataway, NJ, USA). ADAMTS13 was immobilized on a CM5 sensor chip with an amino coupling kit (GE healthcare) according to manufacturer's instructions. Approximately 500–600 resonance units (RU) of ADAMTS13 were covalently attached onto the chip. Lys-Pg (0.05, 0.1, 0.2, 0.4 and 0.8 μM) or Glu-Pg (0.4, 0.8, 1.6 and 3.2 μM) in 50 mM Tris, 100 mM NaCl, pH 7.5, containing 0.005% Tween-20 and 5 mM $CaCl_2$ was injected over the ADAMTS13-immobilized sensor chip at a flow rate of 20 μl/min for 2 min.

The sensor chip was regenerated with 50 mM Tris, 1 M NaCl, pH 7.5, containing 0.005% Tween-20 and 5 mM $CaCl_2$ for 1 min. The dissociation constants (K_d) at the equilibrium were obtained using several ligand concentrations with the BIA evaluation software. Each K_d value was obtained from four or three independent experiments using Lys-Pg or Glu-Pg, respectively.

Activity measurements of ADAMTS13 and plasmin in the complex

ADAMTS13 activity was measured using VWF (*24*) and synthetic fluorogenic substrate FRETS-VWF73 (Peptide Institute, Osaka, Japan) (*25*). For VWF assay, ADAMTS13 (15 ng/ml) was mixed with Glu-Pg (0.1 mg/ml), Lys-Pg (0.1 mg/ml) or bovine serum albumin (0.1 mg/ml) and incubated with guanidine-pretreated VWF multimers (2 mg/ml) for 30, 60 or 120 min at 37°C (*24*). The cleaved fragment with a molecular weight of 200 kDa was assessed by western blotting using HRP-conjugated anti-human VWF polyclonal antibody (DAKO, Carpinteria, CA, USA). For FRETS-VWF73 assay, ADAMTS13 (6.6×10^{-1} nM) was mixed with Glu-Pg (11, 110, 1100 nM) or Lys-Pg (12, 120, 1200 nM). After addition of FRETS-VWF73 (2 μM) to the mixture, increase in fluorescence was measured using Mx3000P System (Stratagene, La Jolla, CA, USA) with 340-nm excitation and 450-nm emission (*25*). The reaction rate was calculated by linear regression analysis of fluorescence over time from 0 min to 10 min using the PRISM software (GraphPad Software, San Diego, CA, USA). The relative activities were estimated from the activity of ADAMTS13 without Glu-Pg or Lys-Pg. To assess the plasmin activity, plasmin (20 nM) was preincubated with ADAMTS13 (40, 80, 200 nM) for 30 min at room temperature followed by the addition of S-2251 (1 mM). Plasmin activity was recorded as a change in absorbance at 405 nm with a reference wavelength of 492 nm during 30 min using the Multiskan Ascent microplate reader.

Results

Yeast two-hybrid screen for ADAMTS13

A yeast two-hybrid screen enabled us to identify more than 500 positive clones. A BLAST search for the insert DNA sequences identified approximately 200 genes, and 36 genes were categorized as membrane or secretory proteins. For further analysis, among these candidate genes, we selected nine secretory proteins that were commercially available or generously donated: Glu-Pg, biglycan (bovine), collagen type I, collagen type III, decorin (bovine), fibrinogen, laminin, histidine-rich glycoprotein and zinc-α2-glycoprotein. We found that ADAMTS13 was bound to immobilized Glu-Pg but not to the others (Fig. 1). The positive clone of human Pg contained a 676-bp cDNA fragment encoding the C-terminal 150 amino acids (amino acid residues 661–810) of the protease domain. ADAMTS13 also bound to Lys-Pg, an amino-terminal truncated form of Glu-Pg (Fig. 1).

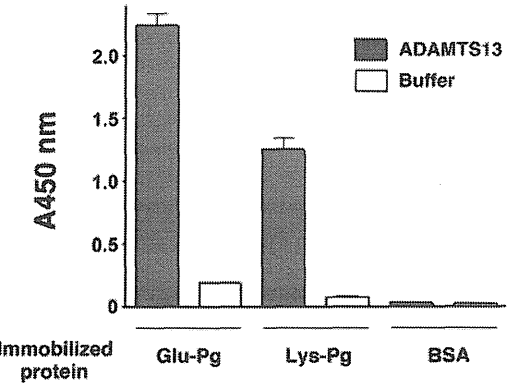

Fig. 1 Binding of ADAMTS13 to immobilized Glu-Pg and Lys-Pg. Microtitre wells were coated with Glu-Pg, Lys-Pg or BSA (each 9 μg/ml) and then incubated with or without ADAMTS13 (3 μg/ml). Bound ADAMTS13 was detected using anti-ADAMTS13 mAb WH2-22-1A (1 μg/ml) and HRP-conjugated anti-mouse IgG (0.25 μg/ml). After incubation with 3,3′,5,5′-tetramethylbenzidine substrate for 20 min, bound HRP activity was detected at 450 nm with a reference wavelength of 650 nm by a Multiskan Ascent microplate reader. The binding was expressed as the mean ± SD ($n = 3$). Grey bar, with ADAMTS13; white bar, without ADAMTS13.

ADAMTS13 binding to Pg

The binding of ADAMTS13 to Pg was examined by co-immunoprecipitation analysis. Anti-FLAG antibody immunoprecipitated ADAMTS13-FLAG with Lys-Pg but not with Glu-Pg (Fig. 2A). Next, anti-Pg antibody was used for the co-immunoprecipitation analysis. Again, ADAMTS13 was co-immunoprecipitated with only Lys-Pg but not with Glu-Pg (Fig. 2B). We found that APMSF-treated Lys-plasmin could be co-immunoprecipitated with ADAMTS13 (Fig. 2C). These results showed that Lys-Pg and Lys-plasmin but not Glu-Pg could bind to ADAMTS13. It is known that Glu-Pg and Lys-Pg have different conformational states in solution (*26*). We assumed that immobilized Glu-Pg had, in part, the conformational change on the plate surface. Microheterogeneity of Pg with or without carbohydrates attached to Asn289 is known (*27*). Doublets of Glu-Pg and Lys-Pg shown in Fig. 2 are likely explained by the carbohydrate difference.

Pg-binding domains in ADAMTS13

Because the C and S domains of ADAMTS13 were used as the bait, the Pg-binding regions would reside in the C and S domains. The co-immunoprecipitation analysis using MD-FLAG and MDTCS-FLAG of ADAMTS13 indicated that both could bind to Lys-Pg but not to Glu-Pg (Fig. 3A). The intensity of bound Lys-Pg was apparently lowest in MD-FLAG and highest in full-length ADAMTS13-FLAG, indicating the gradual loss of affinity in domain truncation. The dose-dependent binding experiments showed that Lys-Pg bound to MDTCS-FLAG at a lower concentration (4 nM) than MD-FLAG (Fig. 3B). Although the Lys-Pg binding to MDTCS-FLAG was saturated at 40 nM, the binding to MD-FLAG was not saturated at the same concentration. The results of the yeast two-hybrid screen and the co-immunoprecipitation