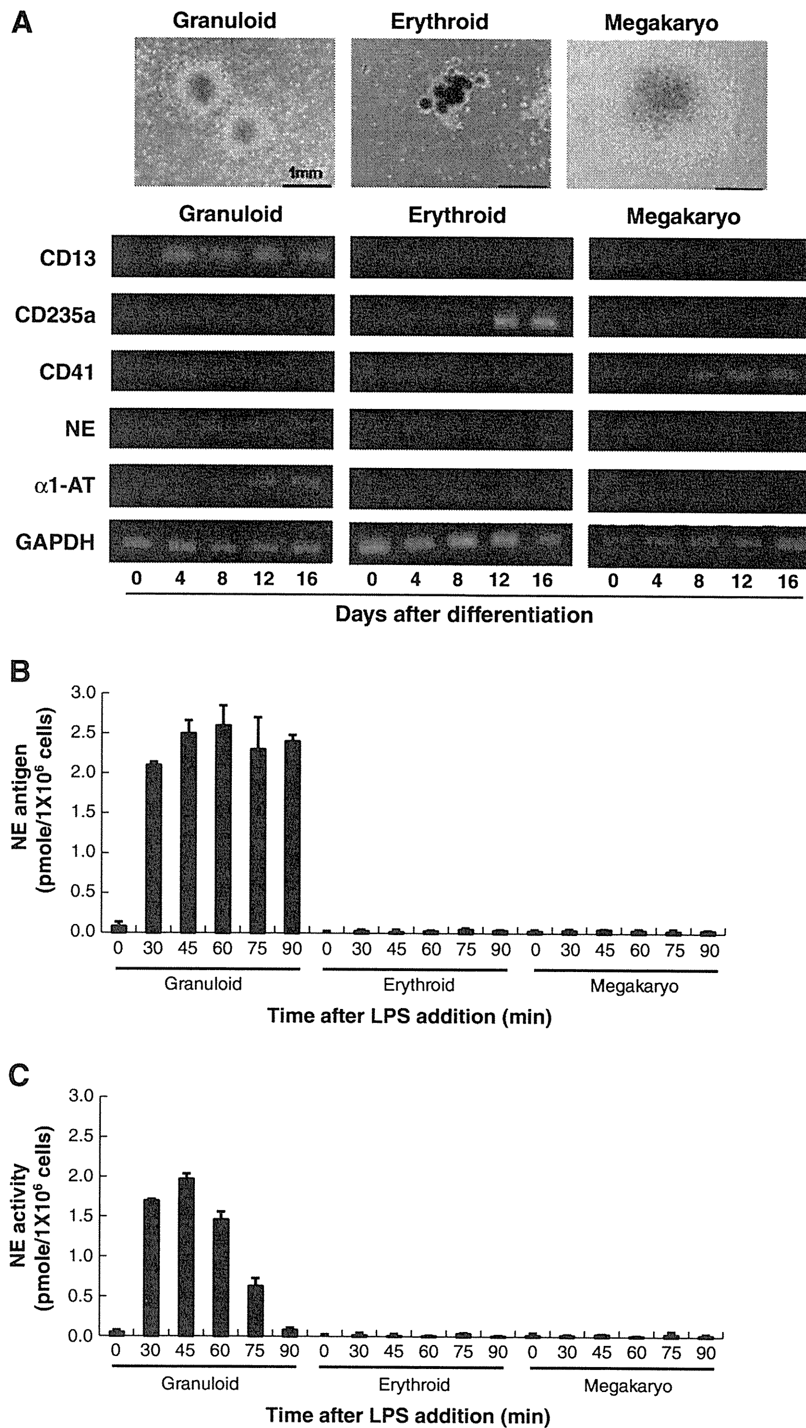

Fig. 6. *In vitro* differentiation of cord blood-derived CD34$^+$ progenitor cells towards three lineages of hematological cells and their responses against LPS or neutrophil elastase. A. Human cord blood-derived CD34$^+$ progenitor cells were differentiated *in vitro* into granulocytic cells (left panels), erythrocytic cells (middle panels) and megakaryocytic cells (right panels) as described in the Materials and Methods. Each cell type was harvested at the indicated time points, and subjected to RT-PCR analysis for CD13, CD235a, CD41, neutrophil elastase (NE), α1-antitrypsin (α1-AT) and GAPDH mRNA expression. Representative data from three independent experiments are shown. B - C. 1×10^5 differentiated granulocytic, erythrocytic and megakaryocytic cells were stimulated with 10 ng/mL LPS for 0–90 minutes. Neutrophil elatastase antigen levels (B) and activity (C) were measured as described in Materials and methods. D. Differentiated granulocytic (circles), erythrocytic (filled circles) and megakaryocytic cells (triangles) were cultured in the presence of 1 nM neutrophil elastase for 72 hours. Cell numbers were determined every 24 hours. E - F. 1×10^5 differentiated cells were cultured with 0, 0.3 and 1 nM neutrophil elastase for 24 hours and neutrophil elastase acitvity (E) and α1-antitrypsin antigen levels (F) were measured. 1×10^5 differentiated granulocytic cells were also cultured for 24 hours in the presence of 1 nM neutrophil elastase that had been treated with 100 nM sivelestat sodium, and neutrophil elastase activity was measured (E). G. Differentiated granulocytic (circles), erythrocytic (filled circles) and megakaryocytic cells (triangles) were cultured for 72 hours in the presence of granulocytic cell-derived culture medium that had been stimulated with 10 ng/mL LPS for 45 minutes. Viable cell numbers were determined every 24 hours. The values given are the mean ± SD; $n = 4$; *$p < 0.03$ and **$p < 0.01$ compared with controls.

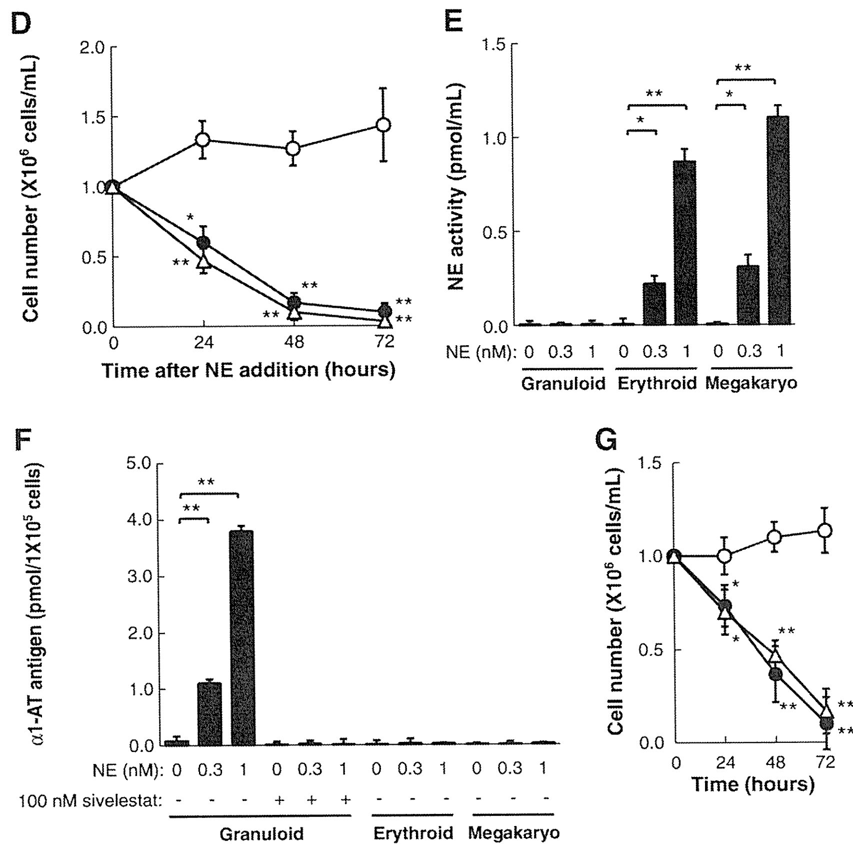

Fig. 6 (*continued*).

Leukocyte are known to release neutrophil elastase as well as α1-antitrypisn in response to LPS [28,36]. In our study, silencing of α1-antitrypsin expression with a short hairpin RNA lentiviral vector inhibited growth of HL-60 cells in the presence of neutrophil elastase (Fig. 4). In addition, HL-60 cells transduced with LentiLox-scrambled and -short hairpin α1-antitrypsin sequences secreted significant amounts of neutrophil elastase proteins shortly after stimulation with LPS (Fig. 5). The enzymatic activity of neutrophil elastase was only found in HL-60 cultures that had been transduced with LentiLox-short hairpin α1-antitrypsin which resulted in a significant reduction in cell growth. Moreover, K562 and MEG-01 cells did not express neutrophil elastase after LPS stimulation, and their proliferation was not significantly suppressed by LPS (Fig. 1). Taken together, our data suggests that LPS might stimulate secretion of neutrophil elastase, resulting in induction of neutrophil elastase-mediated apoptosis if its enzymatic activity is not efficiently neutralized by released α1-antitrypsin.

Although the cell surface receptor involved in neutrophil elastase has not been clearly identified, several candidates are suggested, such as protease-activated receptor (PAR)-2 and Toll-like receptor (TLR)-4 [8,37,38]. Additionally, the apoptosis induced by neutrophil elastase might be mediated through PAR-1 [39]. TLR-4 appears to be the principle receptor for LPS and mediates the activation of nuclear factor κB as well as the synthesis of proinflammatory cytokines such as interleukin-8 [8,40]. Tsujimoto *et al* showed that neutrophil elastase might be associated with expression of TLR-4 on monocytes and macrophages in the septic state [41]. In our study, TLR-4 was expressed in K562, MEG-01 and HL-60 cells [42], however, none of these hematological cells expressed PAR-1 or PAR-2 (data not shown), suggesting that the neutrophil elastase-induced apoptosis may be mediated through a signaling pathway similar to that for TLR-4.

Our *in vitro* differentiation models of hematopoiesis using $CD34^+$ progenitor cells showed that only granulocytic lineage cells expressed neutrophil elastase (Fig. 6), which is consistent with previous studies where levels of neutrophil elastase expression reach a maximum in the promyelocyte and are maintained until differentiation into a neutrophil [43,44]. Importantly, α1-antitrypsin was concomitantly expressed with neutrophil elastase during differentiation into a granulocytic lineage [34,45]. Additionally, α1-antitrypsin was secreted after stimulation by neutrophil elastase, and neutralized it to prevent growth inhibition. When we added inactive neutrophil elastase pretreated with sivelestat sodium to granulocytic lineage cells, we could not detect any α1-antitrypsin antigen (Fig. 6), suggesting that the enzymatic activity of neutrophil elastase might be crucial in secreting α1-antitrypsin. El Ouriaghli and coworkers reported that neutrophil elastase inhibits proliferation and induces apoptosis in $CD34^+$ cells along with degradation of G-CSF [17]. Although our *in vitro* hematopoietic differentiation system was free from G-CSF, concurrently secreted α1-antitrypsin may regulate neutrophil elastase activity. Granulocytic lineage cells released neutrophil elastase following stimulation with LPS, and active neutrophil elastase was neutralized for 90 minutes (Fig. 6). LPS stimulation did not affect erythrocytic or megakaryocytic lineage cells, however, the supernatant derived from granulocytic lineage cells 45 minutes after the addition of LPS significantly inhibited their proliferation as they could not inactivate neutrophil elastase. Erythropoiesis and megakaryopoiesis may be inhibited by neutrophil elastase due to the absence of α1-antitrypsin in these lineages in the inflammatory state. Thus, α1-antitrypsin released from granulocytes might play an important role for the maintenance of hematopoiesis in microenvironments such as the bone marrow niche where α1-antitrypsin might not be sufficiently supplied from plasma [36,46].

In conclusion, we demonstrated that hematological cells might be affected by neutrophil elastase which is regulated by endogenous α1-antitrypsin under the stimulation of lipopolysaccharide. We suggest that granulocytes could protect themselves from neutrophil elastase-induced cellular damage by efficiently neutralizing its activity with concomitant secretion of endogenous α1-antitrypsin. Extensive clinical studies would be required for understanding the precise mechanism of controlling neutrophil elastase activity by endogenous α1-antitrypsin in septic patients.

Authorship contribution

M. Dokai and S. Madoiwa designed and performed the research, analyzed the data, and wrote the paper; A. Yasumoto, Y. Kashiwakura, A. Ishiwata, A. Sakata and N. Makino performed experiments; S. Madoiwa, T. Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

Disclosure of conflicts of interest

The authors state that they have no conflict of interest to declare.

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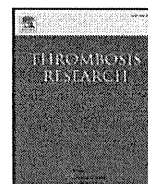

Regular Article

Degradation of cross-linked fibrin by leukocyte elastase as alternative pathway for plasmin-mediated fibrinolysis in sepsis-induced disseminated intravascular coagulation

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ABSTRACT

An alternative pathway for fibrinolysis that comprises leukocyte elastase and its interaction with the plasminogen activator-plasmin system has been suggested. Plasma levels of cross-linked fibrin degradation product by leukocyte elastase (e-XDP) were significantly increased in patients with sepsis induced disseminated intravascular coagulation (DIC) compared with healthy subjects (18.6 ± 19.9 vs 0.58 ± 0.47 U/mL, $p < 0.001$). Twenty seven unique spots were identified from e-XDP dominant patients by immune-purification and two-dimensional difference gel electrophoresis, and they contained fibrinogen Bβ-chain derived fragments Bβ Asp-164, Ser-200, Gln-301, Ala-354, Ile-484 and γ-chain derivatives γ Val-274 at their amino-termini by acquired and processed tandem mass spectrometer. The Sequential Organ Failure Assessment Scores in patients with e-XDPs levels 3-10 U/mL were significantly lower than those with e-XDPs levels -3 U/mL, 10-30 U/mL, and 30- U/mL. The adjusted odds for 28-day mortality rate in patients with e-XDP levels less than 3 U/mL (hazard ratio, 4.432; 95% CI, 1.557-12.615 [$p = 0.005$]) were significantly higher than those in patients with e-XDP levels of 3–10 U/mL. These data suggest that leukocyte elastase might contribute to the degradation of cross-linked fibrin in sepsis-induced DIC.

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Introduction

DIC is an acquired syndrome characterized by persistent activation of blood coagulation in the microvasculature, and is currently accepted to be a pathologic state that occurs over the course of a severe underlying disease [1–3]. Sepsis is a distressing disorder with systemic activation of the inflammatory and coagulation cascades in response to microbial infection, and may be the most common pathogenic state that leads to the development of DIC [4]. In spite of the apparent association between sepsis-induced DIC and the risk of death [5], it remains uncertain to what extent intravascular fibrin or coagulation proteases are critical factors in determining the clinical course.

The degradation of fibrin is usually performed by the serine protease plasmin, which is generated from plasminogen by plasminogen activators. However, suppression of the plasminogen activator-plasmin system is mediated by increased plasma plasminogen activator inhibitor-1 (PAI-1) levels in patients exhibiting systemic inflammatory response syndrome as well as sepsis-induced DIC [6,7]. Alternative systems for fibrinolysis that comprise proteases other than plasmin and their interactions with the plasminogen activator-plasmin system have been thought to play important roles in the digestion of fibrin [8,9]. Leukocytes are known to release intrinsic proteolytic enzymes, including leukocyte elastase as well as cathepsin G, in a variety of clinical conditions [10,11]. Although exposure to inflammatory mediators and interaction with leukocytes cause endothelial activation and damage, leukocyte elastase has been found to digest factor XIIIa mediated cross-linked fibrin and to yield different molecular species of cross-liked fibrin digests from those generated by plasmin [12,13].

Here, we demonstrate that the alternative pathway for fibrinolysis by leukocyte elastase is activated, and contribute to the degradation of cross-linked fibrin in sepsis-induced DIC.

Abbreviations: DIC, disseminated intravascular coagulation; FDP, fibrinogen and fibrin degradation product; XDP, cross-linked fibrin degradation product; e-XDP, cross-linked fibrin degradation product by leukocyte elastase; p-XDP, cross-linked fibrin degradation product by plasmin; TAT, thrombin-antithrombin complex; PIC, plasmin-α_2-plasmin inhibitor complex; SFMC, soluble fibrin monomer complex; PAI-1, plasminogen activator inhibitor-1; SOFA score, Sequential Organ Failure Assessment score.

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Materials and methods

Study population

All samples were obtained with informed consent from patients or their family members according to the Declaration of Helsinki. Blood was drawn from 117 patients with sepsis-induced DIC (Table 1). Samples from aged and sex matched healthy volunteers with consent (23 males and 23 females, 44.6 ± 10.5 years old) were also analyzed as normal controls. Sepsis was defined as infection plus systemic manifestations of infection based on the diagnostic criteria by 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference [14]. DIC was diagnosed according to the diagnostic criteria established by the Japanese Ministry of Health and Welfare (JMHW DIC criteria [15,16]). In brief, the presence of basic diseases, the clinical conditions (namely, bleeding symptoms and organ dysfunction), and the results of the examination (platelet counts, prothrombin time, fibrinogen, and fibrinogen and fibrin degradation products [FDP]) were quantified on a score basis (maximum = 13; minimum = 0). If the number was ≥ 7, DIC was established. In individuals with a DIC score of six points, two or more positive findings on supplementary tests (namely, soluble fibrin monomer complex [SFMC], D-Dimer, thrombin-antithrombin complex [TAT], and plasmin- α_2-plasmin inhibitor complex [PIC]) were needed to make a diagnosis of DIC. 75 patients (64.1%) diagnosed by JMHW DIC criteria were also diagnosed with overt DIC by the ISTH criteria [2], which coincided with the previous study by Wada H, et al [17].

Quantification of molecular markers of coagulation and fibrinolysis

The prothrombin time and the activated partial thromboplastin time were measured with coagulation-based activity assays. Plasma fibrinogen levels were measured by clotting methods using Fibrinogen Test Sankyo (Sankyo, Tokyo, Japan). FDP levels in sera were determined by the latex agglutination assay using LPIA-FDP (Mitsubishi Chemical Medience, Tokyo, Japan). Plasma PIC levels were measured by enzyme-linked immunosorbent assay (ELISA) (Kokusai-Shiyaku, Kobe, Japan). Plasma levels of TAT were also quantified by ELISA (Sysmex, Kobe, Japan). SFMC levels were determined by the latex agglutination assay and using monoclonal antibody IF-43 [18]. For the PAI-1 assay, we used a latex photometric immunoassay (LPIA-200; Mitsubishi Chemical Medience), as previously described [19]. The levels of XDPs by plasmin (p-XDPs) were measured by latex-agglutination assays utilizing JIF-23 [20]. Plasma levels of e-XDPs were also measured by the automated latex photometric immunoassay using IF-123 monoclonal antibody, which is specific for the fibrin fragment D species generated by granulocyte-elastase digestion as previously described [13,19]. The variance (CV) of e-XDP assay was 0.00 - 3.78% within-run, 0.00 - 3.35% between runs.

Table 1
Baseline demographics and disease characteristics.

	Septic DIC, n=117
Age (years)	61.7±15.4
Male / Female	72 / 45
Basic disease	
Respiratory infection	70 (59.8)
GI tract or biliary tract infection	18 (15.4)
Urinary tract infection	10 (8.6)
Other infection	19 (16.2)
Positive blood culture	44 (37.8)
Results of Gram's staining of bacterial pathogen	
Purely Gram-positive	43 (36.5)
Purely Gram-negative	33 (28.5)
Mixed	11 (9.5)
Culture negative or not obtained	30 (25.3)
Type of organism	
Gram-positive	
Staphylococcus aureus	39 (44.6)
Other *staphylococcus* species	3 (3.5)
Streptococcus pneumonia	5 (5.4)
Other Gram-positive	2 (1.7)
Gram-negative	
Escherichia coli	6 (7.1)
Klebsiella species	6 (7.1)
Pseudomonas species	14 (16.1)
Other Gram-negative	9 (10.5)
Fungus	3 (3.5)

The values are given in (%).

Two-dimensional difference gel electrophoresis (2D-DIGE) and image analysis

The fibrinogen and fibrin degradation products were isolated from septic DIC patients' samples using anti-fibrinogen polyclonal antibody (Dako, Carpinteria, California, USA) coupled Sepharose. 2D-DIGE was performed as described elsewhere [21,22]. Briefly, each sample was loaded onto a pH 3-10NL IPG Strip (BIO-RAD Laboratories, Hercules, California, USA) for 16 h at 25 °C. Isoelectric focusing was performed using the PROTEAN IEF cell (BIO-RAD). The second dimension of electrophoresis was then performed in 10% SDS-PAGE slab gels. After electrophoresis, the gels were stained with SYPRO Ruby Protein Stain, and the resulting protein spot patterns were analyzed using Molecular Imager FX Pro and PD Quest software (BIO-RAD).

In-gel digestion and nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Each protein spot was excised from 2D-DIGE gels with a spot cutter (ProteomeWorks, BIO-RAD). Each gel piece was washed with 50% v/v acetonitrile, 0.1% formic acid to extract the resulting peptides. Nano LC-MS/MS analysis was performed in positive ion mode on a Micromass Q-Tof Ultima Global mass spectrometer controlled by the software Mass LynxTM 4.0 (Waters-Micromass, Manchester, UK) coupled with an UltiMate Nano LC system (Dionex-LC Packings; GenTech, New York, NY, USA) [23,24]. Spectra were recorded under the condition of a source temperature 80 °C and cone voltage of 80 V. Acquired and processed MS/MS data were searched against the NCBInr database using the Mascot search program (MS/MS Ion Search; Matrix Science, Boston, MA, USA).

Statistical Analysis

The SPSS statistical software package (SPSS, Chicago, IL, USA) was used for all statistical analyses of data. Normally distributed variables are presented as the means ± SEM and were compared by means of the Student's t test. Variables not normally distributed were analyzed with the two-sided Mann-Whitney U test. A difference with $p<0.05$ was considered statistically significant. Multivariate logistic regression analyses were used to identify independent predictors of mortality in septic DIC cases. Kaplan-Meier product limits were computed for the freedom from endpoint, and the log-rank test was used to screen univariate group results regarding the outcomes. Multivariate Cox regression models were used to investigate the association of plasma e-XDPs levels with the 28-day mortality rate after DIC diagnosis.

Results

XDPs levels and their correlation with molecular markers of coagulation and fibrinolysis in sepsis-induced DIC patients

Baseline characteristics of sepsis-induced DIC patients are summarized in Table 1. Plasma concentrations of e-XDPs of patients with septic DIC were significantly higher than those of healthy subjects

(18.6 ± 19.9 vs 0.58 ± 0.47 U/mL, p<0.001, Fig. 1). We could not find any correlation between e-XDP levels and peripheral white blood cell counts (Fig. 2, A). However, there was a negative correlation between e-XDP levels and percentage of immature neutrophil ($rs = 0.046$, $p = 0.033$, Fig. 2, B). The e-XDPs levels showed a mild correlation with FDPs levels ($rs = 0.528$, $p<0.001$) and with p-XDPs levels ($rs = 0.547$, $p<0.001$) (Table 2). By contrast, there was not any correlation between e-XDPs levels and platelet counts, prothrombin time, levels of fibrinogen, TAT, SFMC, PAI-1, or PIC.

2D-DIGE and mass spectrometry analysis of XDPs isolated from patients with sepsis-induced DIC

We recovered XDPs from patients who showed marked elevation of e-XDPs levels with low levels of p-XDPs (patients e1 to e5, Fig. 3 A) and those who exhibited low levels of e-XDPs with remarkably increased levels of p-XDPs (patients p1 to p5, Fig. 3 A). Each of the isolated XDPs was analyzed by 2-D DIGE with fluorescent SYPRO Ruby staining. As shown in Fig. 3 B, twenty-seven spots were unique to the e-XDPs dominant group (red squares) and 19 spots were unique to the p-XDPs high group (green squares). Although the limited material available precludes identification of proteins corresponding to less intense spots given the detection limits, we could analyze only three spots (#5901, #5902 and #7601 in Fig. 3 C) unique to the e-XDP dominant group. Comparing acquired and processed MS/MS data against the NCBInr database using the Mascot search program showed that spot #5901 contained Bβ-chain-derived fragments possessing fibrinogen Bβ Asp-164, Ser-200, Gln-301, Ala-354 and Ile-484, and that spot #5902 had Bβ Asp-164 and Ile-484 at their amino-termini, respectively (Fig. 3 D). In addition, the spot #7601 was found to contain a fibrinogen γ-chain fragment corresponding to γ Val-274 at its amino-terminal.

Relationship between plasma e-XDPs levels, multiple organ failure and prognosis in sepsis-induced DIC

We found that Sequential Organ Failure Assessment (SOFA) scores were significantly higher in the patients with p-XDP levels of 3-10 μg/mL,10–30 μg/mL or greater than 30 μg/mL compared with those in the group with p-XDP levels less than 3 μg/mL (Fig. 4). By contrast, the SOFA scores in patients with e-XDPs levels 3–10 U/mL were significantly lower than those with e-XDPs levels -3 U/mL, 10-30 U/mL, and 30- U/mL. In addition, the survival rate to 28 days after DIC diagnosis in the group with e-XDP levels less than 3 U/mL was significantly lower than those in the groups with e-XDP levels of 3–10 U/mL, 10–30 U/mL and 30- U/mL (Fig. 5). Cox regression analyses of time-to-event data among patients according to e-XDPs levels at the time of DIC diagnosis revealed that the adjusted odds for the 28-day mortality rate in patients with e-XDPs levels less than 3 U/mL (hazard ratio, 4.432; 95% confidence interval, 1.557-12.615 [$p = 0.005$]; Table 3) were significantly higher than those in patients with e-XDPs levels 3–10 U/mL. Interestingly, plasma e-XDPs levels might be an independent factor predicting survival as revealed

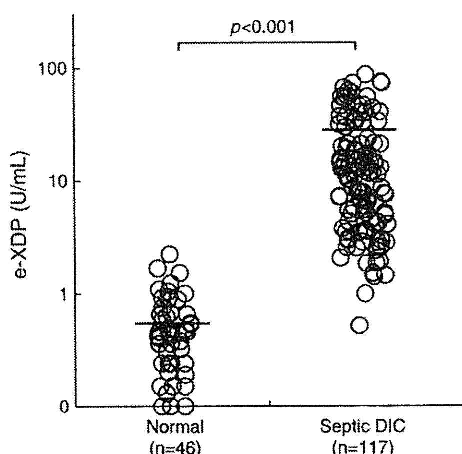


Fig. 1. Plasma e-XDPs levels in sepsis-induced DIC and healthy subjects. Plasma levels of e-XDPs of sepsis-induced DIC patients (n = 117) and normal healthy subjects (n = 46) are shown.

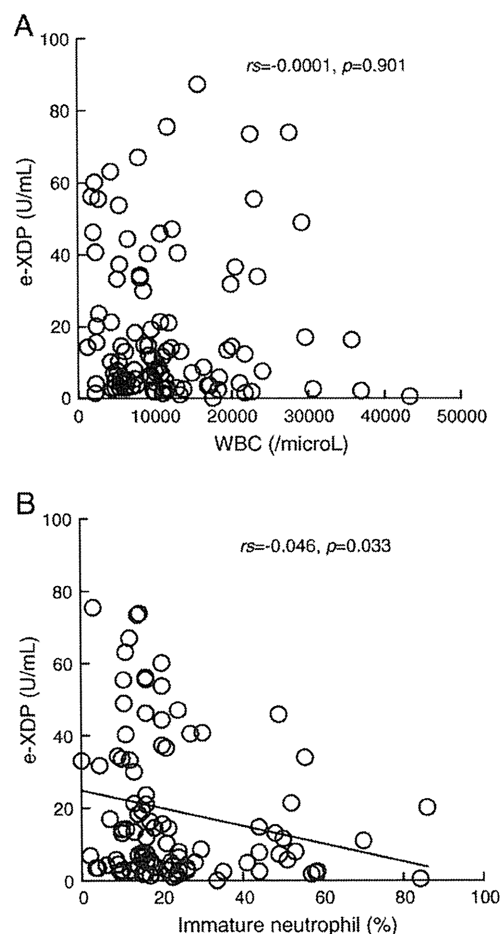
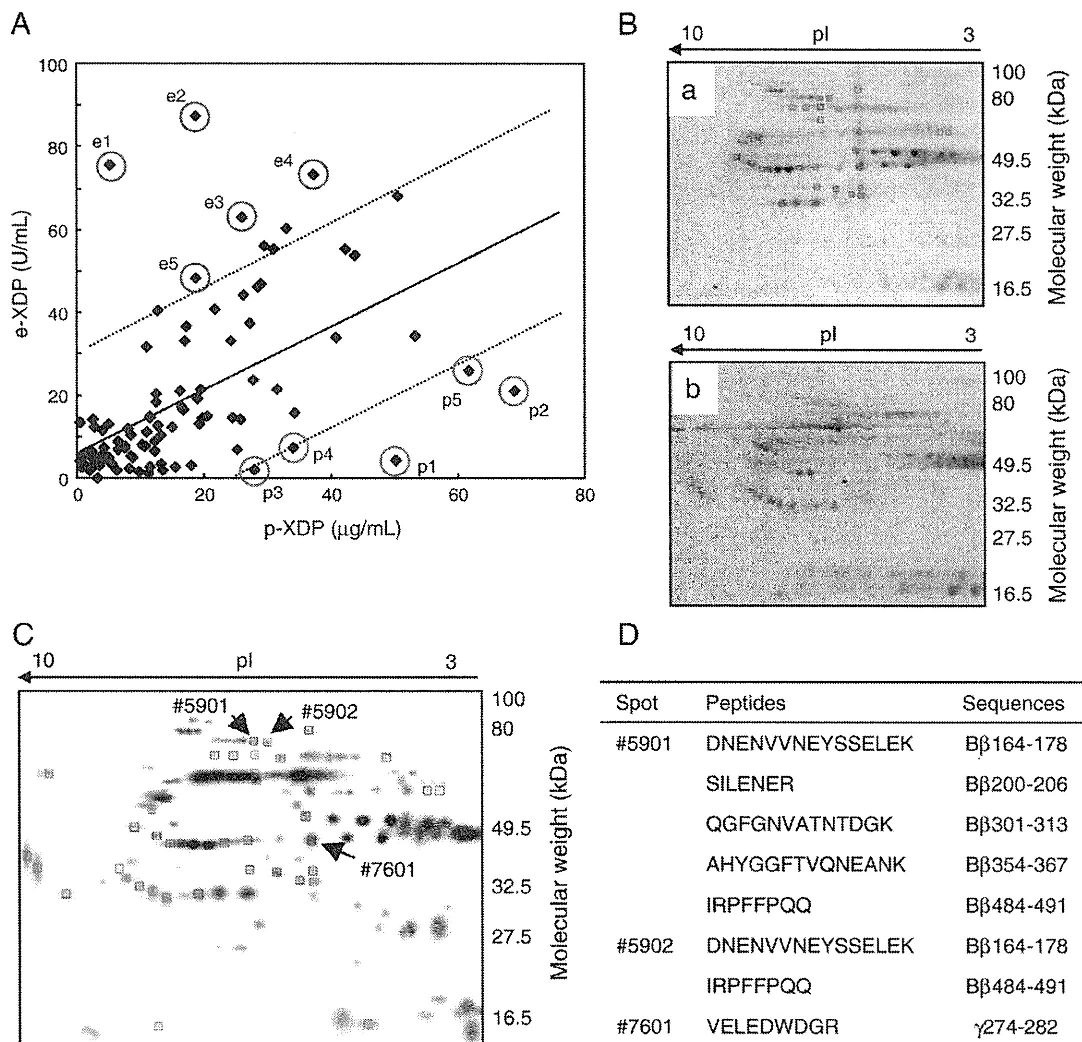


Fig. 2. Correlation between leukocyte and e-XDPs in patient with sepsis-induced DIC Correlations between peripheral white blood cell counts and plasma e-XDPs levels (A) and between immature neutrophil percentages and plasma e-XDPs levels (B) in patient with sepsis-induced DIC are shown. Values of *rs* are determined by Spearman rank correlation test.

Table 2
Correlation between XDP levels and molecular makers of DIC in patients with sepsis-induced DIC (n = 117).

Molecular markers	p-XDP		e-XDP	
	rs	*p*	*rs*	*p*
Platelet	-0.215	0.031	-0.056	0.575
PT	-0.133	0.183	-0.126	0.205
Fibrinogen	-0.222	0.025	-0.003	0.974
TAT	0.020	0.839	0.023	0.818
SFMC	0.119	0.233	0.147	0.140
PAI-1	0.045	0.654	0.043	0.666
PIC	0.348	<0.001	0.185	0.063
FDP	0.812	<0.001	0.528	<0.001
p-XDP	-	-	0.547	<0.001
e-XDP	0.547	<0.001	-	-

*Values of *rs* are determined by Spearman rank correlation test.



Spot	Peptides	Sequences
#5901	DNENVVNEYSSELEK	Bβ164-178
	SILENER	Bβ200-206
	QGFGNVATNTDGK	Bβ301-313
	AHYGGFTVQNEANK	Bβ354-367
	IRPFFPQQ	Bβ484-491
#5902	DNENVVNEYSSELEK	Bβ164-178
	IRPFFPQQ	Bβ484-491
#7601	VELEDWDGR	γ274-282

Fig. 3. 2D-DIGE analysis of fibrinogen and fibrin degradation products (FDPs) in sepsis-induced DIC patients. A. XDPs were recovered using anti-fibrinogen polyclonal antibody-coupled Sepharose from e-XDPs dominant (patients e1-e5) and p-XDPs dominant (patients p1-p5) patients with sepsis-induced DIC. B. Representative difference map comparison of five sets of e-XDPs dominant (a, red squqares) and p-XDPs dominant (b, green squares) subjects. Each sample was separated by isoelectric focusing (nonlinear gradient of pI 3 to 10) and SDS-PAGE (nominal range 16.5 kDa to 100 kDa) followed by Sypro Ruby-staining. C. Three spots (#5901, #5902 and #7601) were identified with MALDI-TOF, comparing acquired and processed MS/MS data by searching against NCBRInr databease using the Mascot search program. D. Sequences recovered from three spots.

by multivariate logistic regression analyses of molecular markers in sepsis-induced DIC patients (hazard ratio, 0.957; 95% confidence interval, 0.917-0.999 [$p = 0.045$]; Table 4), although platelet, PAI-1, PIC and FDP levels could also affect mortality.

Discussion

Leukocyte elastase may degrade major tissue constituent proteins, such as elastin and a variety of proteoglycans, as well as plasma proteins including fibrinogen and fibrin [8,25]. Gando and co-workers have demonstrated that leukocyte elastase mediated-fibrinolysis is activated to varying degrees depending on the amount of systemic inflammation such as major surgical procedure and sepsis [26–29]. The plasma levels of e-XDPs of sepsis-induced DIC patients were significantly higher than those of healthy controls (Fig. 1), and they showed no correlation with those of TAT or SFMC (Table 2). However, we did not measure anti-thrombin, protein C, or inflammatory cytokines levels in this study, we could not define any relationship between the fibrin formation and the leukocyte elastase-mediated fibrinolysis. We found that there was a negative correlation between e-XDP levels and percentage of immature neutrophil (Fig. 2). Previous reports showed that immature neutrophils are not fully developed as they have deficient phagocytic capacity, impaired bacterial killing, decreased chemotaxis and release of leukocyte elastase [30,31]. Thus, immature neutrophil could not effectively release leukocyte elastase to degrade cross-linked fibrin that might result in generation of e-XDP in sepsis-induced DIC patients.

The subsequent activity of leukocyte elastase is balanced by endogenous inhibitors, the predominant one being α_1-protease inhibitor [32]. In the clinical situation, leukocyte elastase level has been measured on the basis of the level of a leukocyte elastase and α_1-protease inhibitor complex [33]. Although the increase in the level of this complex in the plasma may be a marker of leukocyte elastase secretion from activated neutrophils, it may not be representative of actual leukocyte elastase-mediated proteolytic activity [34,35]. We could not find any correlation between e-XDP levels and the levels of elastase- α_1-protease inhibitor complexes ($rs = 0.095$, $p = 0.615$), even though they showed mild correlations with FDPs and p-XDPs levels (Table 2). Thus, monitoring the levels of degradation products

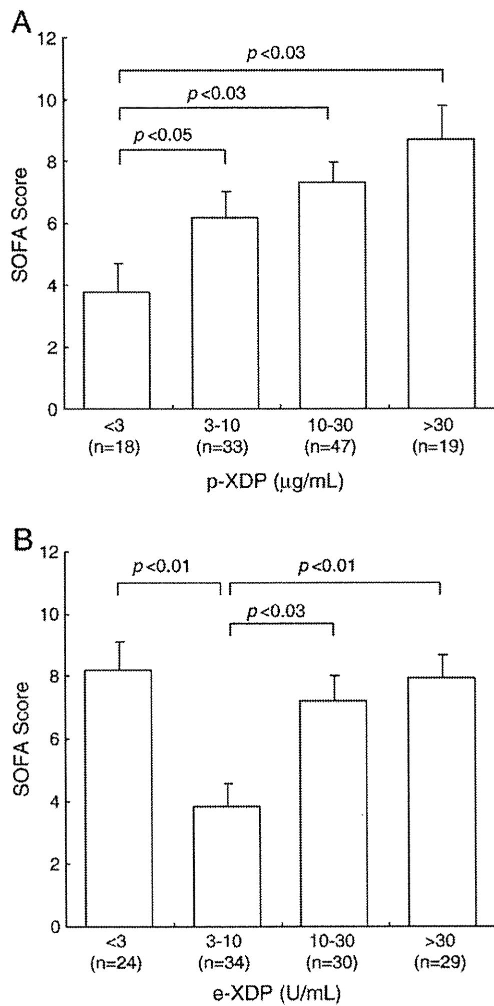


Fig. 4. Relationships between the SOFA score and XDPs levels in sepsis-induced DIC patients. A. Patients with sepsis-induced DIC were classified into four groups with p-XDPs levels (<3 μg/mL, 3-10 μg/mL, 10-30 μg/mL, >30 μg/mL) at the time of DIC diagnosed, and the groups were compared with respect to SOFA scores. B. Sepsis-induced DIC patients were classified into four groups according to e-XDPs levels (<3 U/mL, 3–10 U/mL, 10–30 U/mL, >30 U/mL) at the time of DIC diagnosis, and the groups were compared with respect to SOFA scores. Data are presented as means ± SEM.

of cross-liked fibrin produced by leukocyte elastase might be important to evaluate the status of local fibrinolysis by leukocyte elastase as well as by plasmin.

The discrimination of e-XDPs from p-XDPs is necessary for a better understanding of sepsis-induced DIC, because a variety of molecular species collectively termed fibrin degradation products are released into the circulation [36,37]. We demonstrated that cross-linked fibrin degradation products subjected to 2-D DIGE analysis showed major differences between e-XDPs dominant and p-XDPs dominant patients (Fig. 3). Three spots unique to the e-XDPs dominant patients with mass spectrometry were fibrinogen Bβ-chain-derived fragments and fibrinogen γ-chain derivatives, which might possess plasmin-cleavage P1' sites (Bβ Asp-164, Ser-200, and Ala-354) and leukocyte elastase-cutting ones (Bβ Gln-301, Ile-484, and γ Val-274) [38]. In addition, the epitope for IF-123 is located in the carboxyl-terminal region (residues 196–204) of the α-chain remnant of fragment D residing at both ends of e-XDPs [13]. Thus, the combination of elastase and plasmin could digest cross-linked fibrin molecules in patients manifesting extensively increased fibrinolysis and generate the XDPs species seen in sepsis-induced DIC patients.

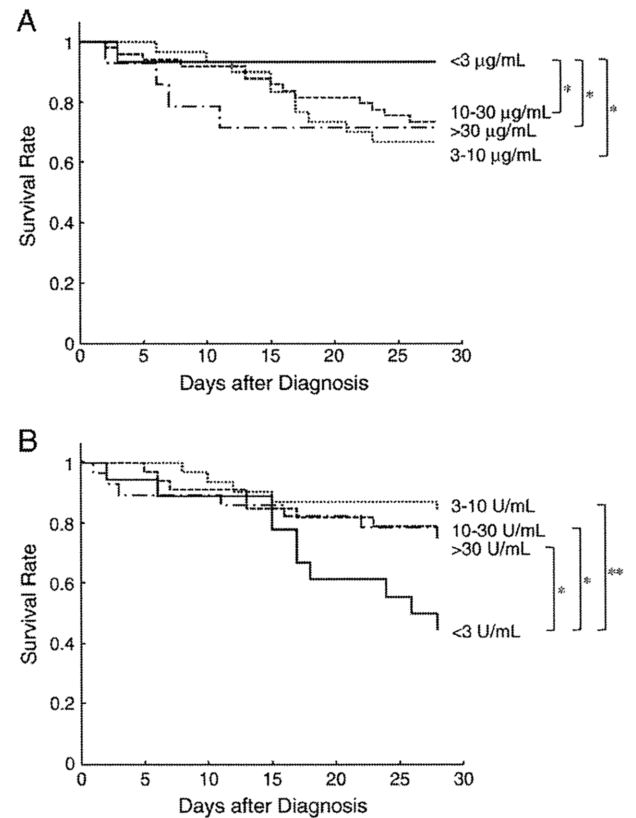


Fig. 5. The XDPs levels correlate with the mortality rate by 28 days after diagnosis in sepsis-induced DIC patients. A. Kaplan-Meier plots with respect to p-XDPs levels (<3 μg/mL [n = 18], 3-10 μg/mL [n = 33], 10-30 μg/mL [n = 47], >30 μg/mL [n = 19]) at the time of DIC diagnosis for sepsis-induced DIC patients. B. Kaplan-Meier plots with respect to e-XDPs levels (<3 U/mL [n = 24], 3–10 U/mL [n = 34], 10–30 U/mL [n = 30], >30 U/mL [n = 29]) at the time of DIC diagnosed for sepsis-induced DIC cases. *$p<0.05$; **$p<0.03$.

The SOFA scores were shown to be useful for evaluating organ failure in multicenter studies, and to be a valuable scoring system for predicting the outcome of DIC [39,40]. Several researchers have shown that plasma levels of e-XDPs are correlated with the level of sepsis-related organ dysfunction [28]. Interestingly, our study revealed that the SOFA scores in the group with e-XDPs levels of 3–10 U/mL were significantly lower than those with e-XDPs levels -3 U/mL, 10-30 U/mL, and 30- U/mL (Fig. 4). Gando et al. have demonstrated that leukocyte elastase-mediated fibrinolysis is activated in varying degrees depending on systemic inflammation [26]. Leukocyte elastase shows profibrinolyic effects, degrading fibrinogen and fibrin and inactivating PAI-1 [41]. In contrast, leukocyte elastase has antifibrinolytic potential to cleave fibrinolytic enzymes [42,43]. Recent study showed that neutrophil elastase can degrade tissue factor pathway inhibitor, which results in increasing blood clot formation [44]. Thus, the balance of all these interactions among the pro- and anti-fibrinolytic effects as well as

Table 3
Cox's regression analyses of time-to-event among patients according to e-XDP levels at the time of diagnosis of sepsis-induced DIC (n=117).

e-XDP (U/mL)	28-day mortality after DIC diagnosis	
	HR (95% CI)	p
<3	4.432 (1.557-12.615)	0.005
3-10	1.000	-
10-30	1.560 (0.510-4.770)	0.435
30<	1.450 (0.539-1.450)	0.539

HR: hazard ratio, CI: confidence interval.

Table 4
Logistic regression analyses of molecular markers for 28-day mortality after diagnosis of sepsis-induced DIC (n = 117).

Molecular markers	28-Day Mortality after DIC Diagnosis		
	Hazard Ratio	95% CI	p
Platelet, X10^3/μL	0.872	0.778-0.978	0.019
Prothrombin time, s	1.136	0.974-1.325	0.105
Fibrinogen, mg/dL	0.999	0.995-1.003	0.648
TAT, ng/mL	0.985	0.960-1.010	0.229
SFMC, μg/mL	1.032	1.004-1.060	0.023
PAI-1, ng/mL	1.012	1.003-1.021	0.011
PIC, μg/mL	0.737	0.547-0.993	0.045
FDP, μg/mL	1.046	1.003-1.091	0.035
p-XDP, μg/mL	1.013	0.942-1.088	0.731
e-XDP, U/mL	0.957	0.917-0.999	0.045

procoagulant action of neutrophil elastase may allow lysis to progress or not progress, or even enhancement of coagulation in clinical situation [26]. However, coagulation-related and inflammation-associated data are required to elucidate the clear mechanisms which cause the insufficient or sufficient activation of leukocyte elastase-mediated fibrinolysis in sepsis-induced DIC patients.

The group with e-XDP levels less than 3 U/mL showed significantly lower survival rates to 28 days after DIC diagnosis than patients with e-XDPs levels of 3–10 U/mL or 10–30 U/mL by Kaplan-Meier analyses (Fig. 5), and the adjusted odds for the mortality rate of this group were 4.432 (95% CI, 1.557-12.615, $p = 0.005$, Table 3). Multivariate logistic regression analyses showed that plasma e-XDP levels at DIC diagnosis might be an independent factor for 28-day mortality in sepsis-induced DIC patients (Table 4). However, it might not biologically be very relevant, as the hazard ratio was modest (0.957, 95% CI, 0.917-0.999). Collectively, the degree of the local activation of leukocyte elastase might contribute to organ damage as well as the actual prognosis in sepsis-induced DIC patients. Poor activation of leukocyte elastase (e-XDPs levels, <3 U/mL) might result in massive fibrin deposition when the plasminogen activator-plasmin system is suppressed, balanced activation (e-XDPs levels, 3–10 U/mL) could effectively degrade fibrin thrombi to protect against ischemic organ damage, and excessive activation (e-XDPs levels, >10 U/mL) evading local inhibitors might result in organ injury with proteolytic cleavage of tissue and plasma components [10,19,45].

In conclusion, we have demonstrated that leukocyte elastase could contribute to the degradation of cross-linked fibrin, and that e-XDPs levels at the time of diagnosis for DIC might predict the prognosis of patients with sepsis-induced DIC. The evaluation of leukocyte elastase-mediated fibrinolysis and control of its activity by specific inhibitors such as sivelestat could improve the poor outcome of septic DIC [46,47].

Authorship contribution

S. Madoiwa designed and performed the research, analyzed data, and wrote the paper. H. Tanaka, Y. Nagahama, M. Dokai, Y Kashiwakura, A. Ishiwata, A. Sakata, and A. Yasumoto performed experiments; S. Madoiwa, T. Ohmori, J. Mimuro, and Y. Sakata analyzed data and revised the paper.

Conflict of interest statement

The authors state that they have no conflict of interest.

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Original Article

Association of Platelet Aggregation with Lipid Levels in the Japanese Population: the Suita Study

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Aim: Platelets play a pivotal role in atherothrombotic diseases. Platelet aggregability induced by agonists has great interindividual variability; however, the factors influencing platelet aggregability variation have not been characterized in Asia.

Methods: To examine the confounding factors influencing platelet counts and responsiveness to agonists, we measured the platelet counts and platelet aggregability induced by 1.7 μM adenosine diphosphate (ADP) or 1.7 μg/mL collagen using a light transmittance aggregometer in the Japanese general population without medication or cardiovascular disease (387 men and 550 women) in the Suita Study.

Results: Platelet counts were negatively correlated with age in both men and women (Spearman's rank correlation coefficient: $r_s = -0.230$ and -0.227; $p < 0.01$, respectively). In women, platelet counts were correlated negatively with the high-density lipoprotein (HDL) cholesterol level and positively with the low-density lipoprotein (LDL) cholesterol/HDL cholesterol (L/H) ratio ($r_s = -0.135$ and 0.119; $p < 0.01$, respectively). In women, platelet aggregabilities by ADP and collagen were correlated with age ($r_s = 0.118$ and 0.143; $p < 0.01$, respectively), and collagen-induced platelet aggregability was correlated with the LDL cholesterol level, the L/H ratio, and the non-HDL cholesterol level ($r_s = 0.167$, 0.172, and 0.185; $p < 0.01$, respectively). Even after adjustment for age, systolic blood pressure, body mass index, and current smoking and drinking, the association of platelet counts with the L/H ratio in women and associations of collagen-induced platelet aggregability with the L/H ratio and the non-HDL cholesterol level remained.

Conclusion: Examination of platelet counts and platelet aggregability induced by ADP and collagen revealed gender, age and lipid levels as factors influencing inter-individual variability.

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Key words; LDL cholesterol, Lipid, Platelet aggregation, Platelet count

Introduction

Platelet thrombi form at the site of vascular injury or the site of a ruptured atherosclerotic plaque. Platelets contribute pivotally to atherothrombotic disease such as myocardial infarction and stroke; therefore, the suppression of platelet aggregability using anti-platelet drugs is widely recognized as a therapeutic means to prevent cardiovascular events, and these drugs show evidence of event prevention[1].

It is generally accepted that the response of platelets to agonists has large inter-individual variability within the population[2-7]. This interindividual responsiveness has a high degree of heritability[2, 4-6, 8-13]. In addition, increased platelet aggregability has been shown in women[14, 15]. Specifically, women showed higher platelet aggregability in response to collagen, adenosine diphosphate (ADP), arachidonic acid, and

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epinephrine in whole blood and platelet-rich plasma than in men[16]. Smoking is a common environmental factor that increases platelet function[17, 18]. In the context of this research, population-based research is helpful in providing information on the confounding factors for platelet responsiveness to agonists; however, these studies are very limited due to the difficulty of platelet aggregability measurement in a community setting. In the large population-based sample of the Framingham Heart Study, platelet responsiveness to agonists was associated with age, body mass index, triglyceride level, high-density lipoprotein (HDL) cholesterol, and diabetes[2]. In this study, higher fibrinogen levels were associated with increased epinephrine-induced aggregation and a tendency to word ADP-induced aggregation[8]. Evidence suggests that increased platelet reactivity could identify individuals at risk for atherothrombotic diseases; however, large cohort studies, including the Northwick Park Heart Study and the Caerphilly Prospective Study, did not show an association of platelet aggregability with cardiovascular events[3, 19].

Studies on the variability of platelet responsiveness to agonists have been mainly performed in the Caucasian population; studies in the Asian population are very limited. Since 1989, we have conducted the Suita Study, an epidemiological study of cerebrovascular and cardiovascular diseases, in a general urban population cohort in Japan[20-22]. The present study was undertaken to clarify the factors influencing the inter-individual variability of platelet responsiveness to agonists in a Japanese urban general population. This is a first step in unraveling systematically the complex interindividual variability of platelet responsiveness in our population.

Methods

Study Population

The study population of the Suita Study was based on samples randomly selected from 12,200 Japanese residents of Suita[20-22]. The participants had been visiting the National Cerebral and Cardiovascular Center every 2 years since 1989 for regular health checkups. Participants attended the National Cerebral and Cardiovascular Center from November, 2005 to December, 2007. A physician or nurse administered questionnaires covering medications, personal habits, and the personal history of cardiovascular diseases. Some cohort members of the study population were excluded from the study because they met one or more of the following criteria: past or present history of cardiovascular disease, failure to fast for at least 10 hours before venipuncture or missing data, age less than 39 years or more than 70 years, or use of any medications. After these exclusions, 937 individuals (men: 387, women: 550) remained in the study. Informed consent was obtained from all subjects. This study was approved by the Institutional Review Board of the National Cerebral and Cardiovascular Center.

Laboratory Measurements

Fasting (≥ 10 hours) blood samples for the platelet aggregation test were collected between 9 and 10 am from an antecubital vein through a needle into disposable, siliconized, evacuated plastic tubes containing 0.1 vol of 3.13% trisodium citrate, and blood collected in a second tube was used. The samples were centrifuged at 1,100 rpm for 10 minutes at room temperature within 1 hour of collection to obtain platelet-rich plasma. Platelet aggregation was measured using native platelet-rich plasma[23] by a single operator on a PA-200 platelet aggregometer (Kowa Company, Japan) using techniques based on the method of Born[24]. Incubation time was 5 minutes at 37℃, the stir bar speed was 1200 rpm, and sample run time was 7 minutes after addition of agonists. The agonists used were 1.7 μM ADP (Arkray Factory Inc., Japan) or 1.7 μg/mL equine-tendon-derived collagen (Arkray Factory)[25]. Percent platelet aggregation was expressed as the maximal percentage change in light transmission relative to that of platelet-poor plasma.

Glucose, total cholesterol, HDL cholesterol, and triglycerides were measured by enzymatic methods. Low-density lipoprotein (LDL) cholesterol was estimated using the Friedewald formula[26]. The LDL cholesterol /HDL cholesterol (L/H) ratio was obtained by dividing LDL cholesterol by HDL cholesterol. Non-HDL cholesterol was obtained by subtracting HDL cholesterol from total cholesterol. The subjects were classified as current smokers if they smoked at least one cigarette per day, and as non-smokers if they had never smoked or had stopped smoking. Similarly, subjects were classified as alcohol non-drinkers if they had never drunk or had drunk only in the past. Blood pressure (BP) was measured three times with subjects in a sitting position after 5 minutes of rest. Systolic BP (SBP) and diastolic BP (DBP) were taken to be the average of the second and third measurements recorded at least 1 minute apart by well-trained doctors. We measured height and weight in a fasting state. Body mass index was calculated as weight (kg) divided by the square of the height (m^2).

Statistical Analysis

For a comparison between gender groups, the

Table 1. Characteristics of study population by sex

	Men $n=387$	Women $n=550$	p value
Age, years	58.3 (7.1)	57.1 (7.1)	0.006
Systolic BP, mmHg	123.1 (17.3)	115.6 (17.0)	<0.001
Diastolic BP, mmHg	79.8 (11.2)	71.8 (10.6)	<0.001
Body mass index, kg/m^2	23.3 (2.6)	21.8 (2.9)	<0.001
Total cholesterol, mg/dL	200.6 (30.4)	218.8 (33.9)	<0.001
HDL cholesterol, mg/dL	57.1 (14.6)	67.1 (15.1)	<0.001
LDL cholesterol, mg/dL	120.4 (29.2)	134.4 (31.4)	<0.001
L/H ratio	2.23 (0.79)	2.11 (0.74)	0.015
non-HDL cholesterol, mg/dL	143.5 (31.4)	151.8 (34.9)	0.003
Platelet count, $\times 10^3/\mu L$	247 (62)	256 (60)	0.011
ADP-induced platelet aggregation, %	68.2 (12.0)	72.2 (10.6)	<0.001
Collagen-induced platelet aggregation, %	77.4 (9.1)	79.5 (8.4)	<0.001
Current smoking, %	33.7	20.7	<0.001
Current drinking, %	66.1	39.7	<0.001

Values are the means (standard deviation) or percent. BP, blood pressure; ADP, adenosine diphosphate; L/H ratio, LDL cholesterol/HDL cholesterol ratio.

Mann-Whitney U test was used. The association between the platelet count or level of platelet aggregations and the analyzed parameters was assessed by Spearman correlation analysis. We used ANCOVA to investigate whether plasma levels of total cholesterol, LDL cholesterol and HDL cholesterol were positively and independently associated with the platelet count or level of platelet aggregation. We performed adjustments for age, body mass index, SBP, and lifestyle factors (current smoking and drinking) for each gender. Differences of $p<0.05$ were considered to be significant. All analyses were performed with SAS statistical software (release 8.2; SAS Institute Inc.).

Results

Characteristics of Populations

After exclusion of individuals with cardiovascular disease and medications, 937 individuals (men: 387, women: 550), aged from 40 to 69 years, were eligible (**Table 1**). Mean ages (standard deviations, SD) of men and women were 58.3 (7.1) and 57.1 (7.1), respectively. SBP, DBP, body mass index, and habits of smoking and drinking were higher in men than in women (**Table 1**). Total cholesterol, HDL cholesterol, LDL cholesterol, and non-HDL cholesterol were higher in women than in men. We calculated the L/H ratio as a new parameter of the lipid profile. This ratio was higher in men than in women. Platelet counts were higher in women than in men. Platelet aggregabilities induced by ADP and collagen were both higher in women than in men (**Table 1**).

Correlates of Platelet Counts and Platelet Aggregation in Response to ADP and Collagen with Age and Other Covariates

In men, the correlations between platelet count and ADP- and collagen-induced aggregations were $r_s=0.050$ and $r_s=0.022$, respectively. The correlation between ADP- and collagen-induced aggregations was $r_s=0.559$. In women, these correlations were $r_s=0.086$, $r_s=0.051$, and $r_s=0.590$, respectively.

Spearman's rank correlation coefficients of the platelet count, ADP- or collagen-induced aggregation with age and other factors are listed in **Table 2**. Platelet counts were negatively correlated with age in both sexes. Platelet counts were negatively correlated with HDL cholesterol and positively correlated with the L/H ratio in women. ADP- and collagen-induced aggregation was correlated with age in women. Collagen-induced aggregability was correlated with LDL cholesterol, the L/H ratio, and non-HDL cholesterol in women. Smoking was correlated with platelet counts and ADP- and collagen-induced aggregation in both sexes. Drinking was correlated with platelet counts in both sexes.

Age-Related Changes of Platelet Counts and Platelet Aggregation in Response to ADP and Collagen

Platelet counts and platelet aggregabilities induced by ADP and collagen are shown according to the decade of life and sex in **Table 3**. Platelet counts

Table 2. Correlation of platelet count and platelet aggregation with age and other covariates

	Men (n=387)			Women (n=550)		
	Platelet count	ADP-induced platelet aggregation	Collagen-induced platelet aggregation	Platelet count	ADP-induced platelet aggregation	Collagen-induced platelet aggregation
Age, years	−0.230**	−0.059	0.029	−0.227**	0.118**	0.143**
Systolic BP, mm Hg	−0.070	0.005	0.066	0.064	0.027	0.027
Diastolic BP, mm Hg	−0.045	−0.004	0.046	0.062	0.002	0.017
Body mass index, kg/m^2	−0.050	−0.062	−0.019	0.106*	0.058	0.034
Total cholesterol, mg/dL	0.092	0.044	0.033	0.013	0.050	0.137*
HDL cholesterol, mg/dL	−0.004	−0.057	−0.127*	−0.135**	−0.091*	−0.101*
LDL cholesterol, mg/dL	0.072	0.044	0.054	0.026	0.070	0.167**
L/H ratio	0.051	0.068	0.092	0.119**	0.101*	0.172**
non-HDL cholesterol, mg/dL	0.104*	0.047	0.076	0.054	0.087*	0.185**
Glucose, mg/dL	−0.038	−0.030	0.055	0.002	0.061	0.094*
Hemoglobin A1c,%	0.102*	−0.002	0.077	0.105*	0.055	0.047
Current smoking	0.145**	0.109*	0.146**	0.212**	0.237**	0.220**
Current drinking	0.191**	0.098	0.072	0.131**	0.105*	0.080

Data indicate Spearman's rank correlation coefficient. *$p<0.05$, **$p<0.01$. BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ADP, adenosine diphosphate; L/H ratio, LDL cholesterol/HDL cholesterol ratio.

Table 3. Age-related changes of platelet counts and ADP- and collagen-induced platelet aggregation by sex

	Age group, years	40-49	50-59	60-69
Men	Numbers of individuals	62	138	187
	Platelet count, × 10^3/μL	261.0 (8.1)	256.0 (5.3)	235.9 (4.6)**
	ADP-induced platelet aggregation, %	68.0 (1.6)	69.2 (1.0)	67.6 (0.9)
	Collagen-induced platelet aggregation, %	76.3 (1.2)	77.1 (0.8)	78.0 (0.7)
Women	Numbers of individuals	99	234	217
	Platelet count, × 10^3/μL	287.1 (5.9)	257.4 (3.8)**	241.4 (4.0)**
	ADP-induced platelet aggregation, %	71.9 (1.1)	71.8 (0.7)	73.0 (0.7)
	Collagen-induced platelet aggregation, %	77.7 (0.9)	79.5 (0.5)*	80.5 (0.6)**

*$p<0.05$, **$p<0.01$ compared with 40-49 age-group in the same sex. Values are the means (standard errors).

decreased in individuals aged 60-69 compared to aged 40-49 in both sexes. ADP-induced aggregability in individuals aged 60-69 was not different from aged 40-49 in both sexes; however, collagen-induced aggregability in individuals aged 50-59 and 60-69 was higher than aged 40-49 in women, but not men.

Multivariate Analysis of Lipid Levels According to the Quartile Rank of Platelet Counts or Platelet Aggregation

We divided platelet counts and platelet aggregability induced by ADP or collagen into quadripartite rank by sex and compared lipid levels among the quartiles after adjustment for age, SBP, body mass index, and lifestyle (current smoking and drinking).

In men, increased total cholesterol and an increased ratio of LDL cholesterol to HDL cholesterol were observed in the highest (Q4) platelet-count quartile (**Table 4**). Non-HDL cholesterol was associated with the platelet count (p for trend, 0.042). In women, the L/H ratio was associated with the platelet count (p for trend, 0.037).

In analysis of the quadripartite rank of ADP-induced platelet aggregability, a weak increment of HDL cholesterol in the Q2 rank was observed in women, but no other parameters showed significant differences among quartiles (**Table 5**).

In contrast to ADP-induced platelet aggregability, LDL cholesterol and non-HDL cholesterol and the L/H ratio in women were increased in the highest (Q4) quartile of collagen-induced platelet aggregability (**Table 6**). The L/H ratio and non-HDL choles-

Table 4. Lipid levels according to quadripartite rank of platelet counts by sex

	Rank	Q1	Q2	Q3	Q4	p for trend
Men	Platelet count, $\times 10^3/\mu L$	39-210	211-244	245-283	284-824	
	Total cholesterol, mg/dL	194.3 (3.2)	202.5 (3.1)	200.2 (3.1)	205.5 (3.1)**	0.085
	HDL cholesterol, mg/dL	57.3 (1.4)	56.6 (1.4)	58.4 (1.4)	56.3 (1.4)	0.679
	LDL cholesterol, mg/dL	115.0 (3.0)	122.7 (2.9)	120.0 (2.9)	124.0 (2.9)	0.155
	L/H ratio	2.13 (0.08)	2.30 (0.07)	2.16 (0.07)	2.35 (0.07)*	0.118
	non-HDL cholesterol, mg/dL	137.0 (3.2)	145.9 (3.1)	141.8 (3.1)	149.2 (3.1)	0.042
Women	Platelet count, $\times 10^3/\mu L$	75-210	211-244	245-283	284-569	
	Total cholesterol, mg/dL	218.0 (3.0)	217.1 (2.9)	217.4 (2.8)	222.5 (2.9)	0.514
	HDL cholesterol, mg/dL	69.3 (1.2)	67.6 (1.2)	65.4 (1.2)*	66.1 (1.2)	0.114
	LDL cholesterol, mg/dL	132.7 (2.7)	133.3 (2.6)	133.4 (2.6)	138.0 (2.6)	0.487
	L/H ratio	2.00 (0.06)	2.08 (0.06)	2.16 (0.06)	2.23 (0.06)*	0.037
	non-HDL cholesterol, mg/dL	148.6 (2.9)	149.5 (2.8)	152.1 (2.8)	156.4 (2.9)	0.231

Values are the means (standard errors) adjusted for age, systolic blood pressure, body mass index, and lifestyle factors (current smoking and drinking). * $p<0.05$, ** $p<0.01$, compared with Q1. HDL, high-density lipoprotein; LDL, low-density lipoprotein; L/H ratio, LDL cholesterol/HDL cholesterol ratio.

Table 5. Lipid levels according to quadripartite rank of ADP-induced platelet aggregation by sex

	Rank	Q1	Q2	Q3	Q4	p for trend
Men	ADP-induced platelet aggregation, %	23-62	63-71	72-77	78-93	
	Total cholesterol, mg/dL	200.6 (3.2)	198.3 (3.4)	202.3 (2.9)	200.8 (3.0)	0.859
	HDL cholesterol, mg/dL	57.3 (1.4)	57.6 (1.5)	57.8 (1.3)	55.9 (1.3)	0.758
	LDL cholesterol, mg/dL	119.1 (3.0)	117.8 (3.2)	123.4 (2.7)	120.4 (2.9)	0.577
	L/H ratio	2.18 (0.08)	2.19 (0.08)	2.25 (0.07)	2.29 (0.07)	0.669
	non-HDL cholesterol, mg/dL	143.3 (3.2)	140.7 (3.5)	144.5 (2.9)	144.8 (3.1)	0.818
Women	ADP-induced platelet aggregation, %	29-62	63-71	72-77	78-96	
	Total cholesterol, mg/dL	217.5 (3.1)	219.8 (2.9)	218.7 (2.7)	219.1 (2.9)	0.957
	HDL cholesterol, mg/dL	66.1 (1.3)	69.7 (1.2)*	67.1 (1.1)	65.4 (1.2)	0.072
	LDL cholesterol, mg/dL	134.0 (2.8)	133.0 (2.6)	135.3 (2.4)	134.9 (2.6)	0.927
	L/H ratio	2.12 (0.06)	2.04 (0.06)	2.13 (0.05)	2.18 (0.06)	0.377
	non-HDL cholesterol, mg/dL	151.3 (3.0)	150.1 (2.9)	151.5 (2.7)	153.7 (2.8)	0.841

Values are the means (standard errors) adjusted for age, systolic blood pressure, body mass index, and lifestyle factors (current smoking and drinking). * $p<0.05$, compared with Q1. HDL, high-density lipoprotein; LDL, low-density lipoprotein; L/H ratio, LDL cholesterol/HDL cholesterol ratio.

terol in women were associated with collagen-induced platelet aggregability (p for trend; 0.005 and 0.036, respectively).

Discussion

In the present study, we found gender differences in the platelet count and platelet aggregability and revealed the correlation of these parameters with some lipid levels. The interindividual variability of the platelet count and platelet responsiveness to ADP and collagen appeared to be partly explained by gender, age and lipid levels.

We found in the present study that women had higher platelet counts and platelet aggregability in response to ADP and collagen than men. These results were consistent with previous findings that women show higher platelet responsiveness to agonists in whole blood and platelet-rich plasma than men[16]. This gender difference in the platelet aggregability may be related to marked changes of the lipid profile in postmenopausal women. Both total cholesterol and

Table 6. Lipid levels according to quadripartite rank of collagen-induced platelet aggregation by sex

	Rank	Q1	Q2	Q3	Q4	p for trend
Men	Collagen-induced platelet aggregation, %	8-73	74-78	79-82	83-95	
	Total cholesterol, mg/dL	196.9 (3.4)	202.6 (3.2)	202.1 (3.0)	200.5 (2.9)	0.619
	HDL cholesterol, mg/dL	58.8 (1.5)	58.9 (1.4)	57.1 (1.3)	54.7 (1.2)*	0.088
	LDL cholesterol, mg/dL	115.6 (3.2)	122.6 (3.0)	121.2 (2.9)	121.4 (2.7)	0.400
	L/H ratio	2.11 (0.08)	2.22 (0.08)	2.25 (0.07)	2.32 (0.07)	0.296
	non-HDL cholesterol, mg/dL	138.1 (3.4)	143.7 (3.2)	145.0 (3.1)	145.8 (2.9)	0.342
Women	Collagen-induced platelet aggregation, %	7-73	74-78	79-82	83-97	
	Total cholesterol, mg/dL	212.7 (3.1)	218.9 (2.9)	221.2 (2.7)*	220.7 (2.8)	0.179
	HDL cholesterol, mg/dL	68.3 (1.3)	68.7 (1.2)	65.8 (1.1)	66.0 (1.2)	0.223
	LDL cholesterol, mg/dL	127.9 (2.9)	133.3 (2.6)	137.8 (2.4)*	136.5 (2.5)*	0.050
	L/H ratio	1.97 (0.06)	2.03 (0.06)	2.23 (0.05)*	2.20 (0.06)*	0.005
	non-HDL cholesterol, mg/dL	144.5 (3.1)	150.2 (2.9)	155.4 (2.6)**	154.7 (2.8)*	0.036

Values are the means (standard errors) adjusted for age, systolic blood pressure, body mass index, and lifestyle factors (current smoking and drinking). $^{*}p<0.05$, $^{**}p<0.01$, compared with Q1. HDL, high-density lipoprotein; LDL, low-density lipoprotein; L/H ratio, LDL cholesterol/HDL cholesterol ratio.

LDL cholesterol are markedly increased in postmenopausal women[20], and hypercholesterolemia is associated with hyperaggregability. In the present study, the mean age of women was 57.1 year old and thus most were postmenopausal. Furthermore, we found that platelet counts were negatively correlated with age and positively correlated with smoking in both men and women. We also found that smoking correlated with platelet aggregability which was not in agreement with the Framingham Heart Study[2]. The discrepancy in terms of smoking between the two studies is not clear; however, it might have been caused by the difference in the frequency of smokers.

Beside cellular interactions of platelets with other blood cells and vascular cells, interactions of platelets with lipoproteins seem to be quite important, and circulating lipoproteins in blood directly or indirectly influence platelet properties[27]. LDL is an atherogenic lipoprotein and increases platelet activation. Platelets are directly associated with LDL in blood[28]. LDL is modified to oxidative LDL. Oxidative LDL induces platelet activation followed by quick changes in shape and aggregation contributing to thrombus formation after plaque rupture. In contrast with LDL, HDL particles have several antiatherogenic activities, including anti-inflammatory, antithrombotic, antioxidative, and vasodilatory properties[29]. Lowering LDL cholesterol or raising HDL cholesterol therapy has well-established benefits in the primary and secondary prevention of atherothrombotic diseases[30-33]. Actually, platelet aggregation evaluated with a thrombus area on the aorta in an *ex vivo* superfusion chamber under 1,000 s^{-1} has been inversely correlated with HDL cholesterol levels[34]. Infusion of reconstituted HDL to humans showed a transient inhibition of platelet aggregation induced by arachidonic acid and collagen[35]. These findings also suggested that HDL has antiplatelet actions. In our study, HDL cholesterol was negatively associated with collagen-induced platelet aggregation. This negative association was also observed in the Framingham Heart Study[2].

In the present study, collagen-induced platelet aggregability was associated with the L/H ratio in women, even after adjustment for age, systolic blood pressure, body mass index, and current smoking and drinking. Recently, the L/H ratio has been considered to be a clinically useful marker, because it is more closely associated with the occurrence of cardiovascular events than the levels of LDL cholesterol or HDL cholesterol[36]. Therefore, our findings suggest that increased collagen-induced platelet aggregation in women is potentially associated with early atherosclerotic conditions.

There have been few studies on the prediction of cardiovascular events by platelet tests. Two small studies have suggested that platelet aggregability assessed by a light transmittance aggregometer could be predictive of cardiovascular events[37, 38]; however, the Northwick Park Heart Study, a large cohort study consisting of 740 men followed up for 10.1 years, found no association of ADP-induced aggregation with ischemic heart disease events[19]. In the Caerphilly Prospective Study, consisting of 2000 elderly men followed up for 10 years, the aggregative response to ADP in platelet-

rich plasma, that to ADP in whole blood measured using an impedance method, and platelet aggregation induced in whole blood by high-shear flow did not show an association with myocardial infarction[3].

In this study, we found that the platelet count and platelet aggregation are affected by factors such as gender, age, and lipid levels in the Japanese population. Furthermore, increased platelet aggregation by collagen in women is closely associated with the LDL-C/HDL-C ratio and LDL-C as risk factors for atherosclerotic disease. Therefore, this study offers modest support for the hypothesis that increased platelet aggregation by collagen even within the normal range might be associated with atherosclerosis in middle-aged women. However, future studies are necessary to establish whether platelet aggregation by collagen is a useful marker to predict coronary events and mortality. We are now following the occurrence of cardiovascular disease events in the Suita Study. Moreover, the response of platelets to agonist may have inter-individual variability within the population that is partly due to genetics. We are now genotyping the DNA polymorphisms of the study participants using a candidate gene approach.

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ORIGINAL ARTICLE

Genetic analysis of patients with deep vein thrombosis during pregnancy and postpartum

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Abstract Deep vein thrombosis (DVT) is a serious pregnancy-related complication. Recent studies indicate that the genetic background for DVT differs with ethnicity. In our study, we enrolled 18 consecutive Japanese patients who had developed DVT during pregnancy and postpartum. We performed a genetic analysis of three candidate genes for DVT, protein S, protein C and antithrombin, in these patients. We found that four patients had missense mutations in the protein S gene, including the K196E mutation in two patients, the L446P mutation in one patient, and the D79Y and T630I mutations in one patient, as well as one patient with the C147Y mutation in the protein C gene. All five patients with genetic mutations had DVT in their first two trimesters. Nine of the patients without genetic mutations developed DVT in the first two trimesters, and four in the postpartum period. Thus, genetic mutations in the protein S gene were predominant in pregnant Japanese DVT women, and DVT in pregnant women with genetic mutations occurred more frequently at the early stage of pregnancy than postpartum. Considering the rapid decrease in protein S activity during pregnancy, we may need to assess thrombophilia in women before pregnancy.

Keywords Deep vein thrombosis · Protein S · Thrombophilia · Pregnancy

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1 Introduction

Venous thromboembolism is the leading cause of maternal deaths in Western countries [1]. The incidence of pregnancy-related venous thromboembolism was 13 per 10,000 deliveries [2]. A 30-year population-based study reported that the unadjusted incidence of deep vein thrombosis (DVT) was 151.8 per 100,000 woman-years [3]. Most studies have found that the risk for thrombosis were 3–12 times higher in postpartum than during pregnancy [3, 4]. One study, however, reported twice as many events antenatally as postpartum [5]. Most of these studies involved patients in Western countries. A study in Japan showed that pulmonary thromboembolism occurred in 0.02% of total births, and the mortality rate was 2.5 per 100,000 deliveries [6]. Women with pregnancy-related thrombosis tend to have inherited thrombophilia, thus the prevention of DVT during pregnancy and postpartum is important for pregnant women. Therefore, the identification of inherited or acquired thrombophilia in pregnant women is urgently needed for the prevention of pregnancy-related thrombosis.

In Caucasian populations, two thrombotic mutations, the factor V Leiden mutation and the prothrombin G20210A mutation, for venous thromboembolism are widely distributed, with 30–60% of women with pregnancy-related thrombosis having these mutations [7, 8]. Both mutations are well-established risk factors for venous thromboembolism during pregnancy and postpartum in Caucasian populations. Several prophylactic therapies for pregnant women, such as heparin administration in the perinatal period, are recommended based on the type of thrombophilia and history of thrombosis [9]. However, these two genetic mutations are not found in the Japanese population [10, 11]. Thus, Caucasians and Japanese have clear genetic differences for thrombosis [12].

Deficiencies in protein S, protein C, and antithrombin are well-known risk factors for DVT [13]. The frequency of protein C deficiency and antithrombin deficiency in the general Japanese population was estimated to be 0.13 and 0.15%, respectively, and was comparable to the Caucasian population [12, 14–16]. The frequency of protein S deficiency in Japanese, however, seemed to be higher than that in Caucasians [17, 18], although the assays for plasma protein S levels differed among the studies. Actually, the frequency of protein S deficiency in 2,690 individuals randomly selected from the general Japanese population was estimated to be 1.12%, higher than reported in Caucasian populations (0.03–0.13%) [17, 18]. In a study of Japanese patients with venous thromboembolism, the frequency of inherited protein S deficiency was higher than that in Caucasian patients [19, 20]. It was recently reported that 17% of Japanese patients with venous thromboembolism had genetic mutations in the protein S gene [20]; this was much higher than in selected Caucasian patients with thromboembolism (1.4–8.6%) [13]. Furthermore, we and others reported the significant association with a missense mutation, K196E, in the protein S gene and venous thromboembolism in Japanese populations [19, 21, 22]. The carriers of this mutation showed low protein S activity [19, 23]. The prevalence of this mutant allele in the general Japanese population was about 0.009, suggesting that a substantial proportion of the Japanese population carried the protein S E-allele and was at risk of developing DVT [12, 19, 21, 22, 24]. This mutation seems to be ethnically specific, because it has not so far been identified in Caucasians.

It is well recognized that plasma levels of protein S activity and antigen are significantly reduced during pregnancy [25] and in oral contraceptive users [26]. The activities of protein S, protein C, and antithrombin can be affected at the acute stage of thrombotic events or after antithrombotic therapies. Therefore, the plasma assay may have an intrinsic limitation for the diagnosis of thrombophilia, and alternative ways to diagnose thrombophilia are expected. Genetic analysis might fulfill this requirement if it is applicable.

In this study, we performed DNA analysis for the genes of protein S, protein C, and antithrombin in patients with DVT during pregnancy and postpartum. We measured their plasma activities of protein S, protein C, and antithrombin. Based on these analyses, we described the clinical characteristics of the DVT events in patients with genetic mutation.

2 Materials and methods

2.1 Study patients

In this study, 18 consecutive patients with DVT during pregnancy and postpartum were enrolled from two tertiary perinatal centers: the National Cerebral and Cardiovascular Center and the Osaka Medical Center and Research Institute for Maternal and Child Health. Both centers are located in the Osaka Prefecture, which has the third-largest population in Japan. Postpartum was defined as the first 3 months after delivery. DVT was diagnosed by ultrasonography, venography, or magnetic resonance imaging angiography. We enrolled only patients with symptomatic DVT. Each patient's age, body mass index, gestational weeks of DVT onset, complications of pregnancy, delivery mode, and other information were reviewed.

The protocol of this study was approved by the Ethics Review Committee of the National Cerebral and Cardiovascular Center and by that of the Osaka Medical Center and Research Institute for Maternal and Child Health. Only those who had given written informed consent for genetic analyses were included.

2.2 Activity measurements of protein S, protein C, antithrombin, and antiphospholipid syndrome screening

The plasma samples were obtained after at least 3 months' postpartum and at least 3 months without the use of warfarin. Samples were subjected to a thrombophilia screening, including prothrombin time, activated partial prothrombin time, and activities of protein S, protein C, and antithrombin. Protein S activity was measured as cofactor activity for activated protein C on the basis of the activated partial thromboplastin time assay using Staclot protein S (Diagnostica Stago, Asnieres, France) [18]. Protein C amidolytic activity was measured using S-2366 as a chromogenic substrate and Protac derived from *Agkistrodon contortrix* venom as the activator [16]. Antithrombin activity was measured as a heparin cofactor activity using chromogenic substrate S-2238 (Chromogenix AB, Stockholm, Sweden) [16, 27]. Samples were also subjected to an antiphospholipid syndrome screening of