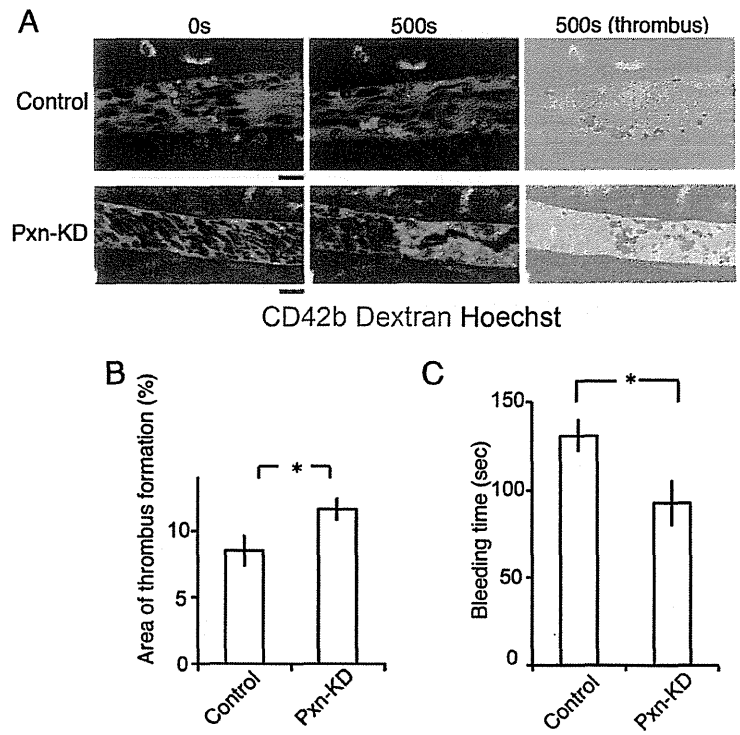

Figure 6 Pxn-KD in platelets expedites thrombus formation in vivo. (A) Intravital imaging of thrombus formation by laser irradiation of mesenteric arterioles in mice with control or Pxn-KD platelets. Thrombus formation was increased in mice with Pxn-KD platelets following laser irradiation. Bar, 10 μm. The right panel shows the results of quantification of the thrombus area. **(B)** Percentage areas of thrombus within blood vessels after laser irradiation. Columns and error bars represent the mean ± s.e.m. ($n = 40$ vessels in five mice/group). **(C)** Tail bleeding times were assessed as described in the Methods. Columns and error bars represent the mean ± s.e.m. (n = four mice/group). Statistical significance was determined using Student's *t*-test. $^*P < 0.05$ vs. control.

in signaling of integrin αIIbβ3 were increased in Pxn-KD platelets, even though paxillin failed to interact with platelet-specific integrin αIIb [19]. It is possible that other signaling pathways in platelets are modulated by paxillin, which is independent of direct interactions with integrins.

An issue that remains unresolved is the precise mechanism governing the negative regulatory function of paxillin in platelet activation. As described above, Rathore et al. previously reported that integrin αIIbβ3-dependent platelet aggregation induced tyrosine phosphorylation of paxillin and Hic-5 in platelets, leading to the binding of Csk, which controls activation of the Src family of tyrosine kinases [17]. Csk preferentially binds to paxillin in murine platelets that coexpress paxillin and Hic-5 [17]. Furthermore, the interaction abolishes the activity of Lyn, but not Fyn or Src. It is possible that paxillin acts as a negative feedback regulator of outside-in signaling by modulating Lyn activity after ligand binding to integrin αIIbβ3 [17]. However, this mechanism does not fully explain the functional roles of paxillin in platelets. Our data suggest that paxillin controls additional proximal signaling pathways for platelet activation. Pxn-KD did not directly augment the conformational changes of integrin αIIbβ3 expressed on Chinese hamster ovary cells (Additional file 9), tyrosine phosphorylation, or calcium mobilization induced by phosphoinositide turnover. These data suggest that paxillin negatively controls downstream signaling of calcium mobilization or a calcium-independent signaling pathway. In addition, calcium mobilization was rather reduced by Pxn-KD. It is therefore possible that negative feedback exists to prevent further activation of Pxn-KD platelets, or phosphoinositide turnover is directly modulated by Pxn-KD.

Our data suggest that several mechanisms may increase platelet activation by Pxn-KD. Notably, calcium-independent actions by Pxn-KD appear to exist, because P-selectin expression elicited by an agonist was still observed in Pxn-KD platelets even in the presence of BAPTA-AM. A previous report has suggested that coordinated signaling through both $G_{12/13}$ and G_i causes integrin αIIbβ3 activation, despite a small increase in intracellular calcium [33]. In addition, $G_{12/13}$ and G_i signaling activates integrin αIIbβ3 in Gq-deficient mice [34]. It is possible that paxillin modify the calcium-independent signaling

pathway leading to release reaction and integrinαIIbβ3 activation. Additional studies are needed to investigate how paxillin regulates platelet activation, and to assess whether these roles of paxillin in control of cellular signaling are common mechanisms in other cell types. We are now interested in further investigation of the precise mechanisms, and additional experiments are currently underway in our laboratory.

Another interesting finding of our study is that Pxn-KD resulted in an enlargement of platelet volume. CLP36, a member of the LIM domain family, was recently reported to play some roles in platelet activation [35]. Platelets from mice lacking the LIM domain of CLP36 show a slight increase in size and hyperactivation in response to a GPVI agonist [35]. The phenotypes of CLP36-deficient or mutant platelets are similar to those of Pxn-KD platelets in our study, although G protein-coupled receptor signaling is not affected in CLP36-deficient or mutant mice. Accordingly, the expression of LIM domain proteins may determine platelet size and reactivity.

To extrapolate the implications of our study to the biology and pathophysiology of humans, we must consider the differential expression pattern of paxillin-related proteins in platelets among species. Murine platelets express paxillin, Hic-5, and leupaxin, whereas human platelets only express Hic-5 [17]. Hagmann et al. reported that a switch from paxillin to Hic-5 expression should occur during the late phase of megakaryopoiesis in humans [15]. A recent report has described platelet functions in Hic-5-deficient mice [36]. Hic-5-deficient mice exhibit prolonged bleeding times, and the loss of Hic-5 in platelets slightly impairs integrin αIIbβ3 activation induced by thrombin, but not other agonists including convulxin, U46619, and ADP [36]. Although the hemostatic defect in Hic-5-deficient mice, as assessed by tail bleeding, is not fully explained by a mild defect in platelet function, it is possible that the structurally related proteins paxillin and Hic-5 play opposing roles in the regulation of platelet function in murine platelets. Leupaxin, another LIM protein that is predominantly expressed in leukocytes, has been reported to play an inhibitory role in B cell receptor signaling [37], which is similar to the role of paxillin reported in this study. In human platelets, which only express Hic-5, it will be necessary to elucidate whether Hic-5 acts as a positive regulator of integrin αIIbβ3 activation.

In summary, we have shown that paxillin is a negative regulator of platelet activation in mouse platelets. Modulation of platelet activation by Pxn-KD may originate in the augmentation of common signaling pathways, leading to integrin αIIbβ3 activation, release reactions, and Tx biosynthesis. Modulation of the LIM protein function might be an attractive candidate therapeutic target capable of strongly suppressing unexpected platelet activation in thrombotic disorders. The next challenge will be elucidating the precise mechanism by which paxillin regulates the signaling pathway in platelet activation.

Additional files

Additional file 1: Schematic diagrams of the lentiviral vector used in this study. (A) Schematic diagram of the lentiviral vector. (B) Locations of the oligonucleotides encoding the shRNAs in the mouse *paxillin* (*Pxn*) gene. (C) Mouse embryonic fibroblasts were transduced with a lentiviral vector containing the control, Pxn-1, Pxn-2, or Pxn-3 shRNA sequences at MOIs of 1, 3, or 10. Protein expression was determined by immunoblotting at 48 h after transduction. Data are representative of three independent experiments.

Additional file 2: Oligonucleotide sequences of siRNA cloned into LentiLox.

Additional file 3: Pxn-KD does not affect granule contents. Bone marrow cells transduced with LentiLox-sh-control-GPIbα (Control) or LentiLox-sh-paxillin-GPIbα (Pxn-KD) at an MOI of 5 were transplanted into lethally irradiated recipient mice. (A) The morphology of control and Pxn-KD platelets was examined by transmission electron microscopy, and the areas of granules and cytoplasm in each platelet were independently quantified using ImageJ software for Macintosh. Columns and error bars represent the mean ± s.d. ($n = 53–70$). (B–C) Washed platelets were lysed to measure the concentrations of platelet factor 4 (PF4) (B) and serotonin (C). Columns and error bars represent the mean ± s.d. ($n = 4$). Statistical significance was determined using Student's *t* test. ***$P < 0.001$ vs. control.

Additional file 4: Expression levels of platelet-specific glycoproteins. Description of data: (A) Expression levels of GPIIb/IIIa (integrin αIIbβ3) (left panel), GPIb (middle panel), and GPVI (right panel) in control (dark gray) and paxillin-knockdown platelets (light gray). (B) Columns and error bars represent the mean ± s.d. of the mean fluorescence intensity (MFI) of antibody binding ($n = 5$). Statistical significance was determined using Student's *t* test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control.

Additional file 5: Effects of apyrase and SQ29548 on agonist-induced integrin αIIbβ3 activation and P-selectin expression in control and Pxn-KD platelets. Platelets pretreated without or with 5 U/mL apyrase and 10 μmol/L SQ29548 were stimulated with the indicated agonists. JON/A binding (A) and P-selectin expression (B) on GFP-positive platelets were assessed by flow cytometry. Column and error bars represent the mean ± s.d. of the mean fluorescence intensity (MFI) ($n = 3–4$). Statistical significance was determined using Student's *t* test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control.

Additional file 6: Intravital imaging of thrombus formation by laser irradiation of mesenteric arterioles in mouse with control platelets.

Additional file 7: Intravital imaging of thrombus formation by laser irradiation of mesenteric arterioles in mice with Pxn-KD platelets.

Additional file 8: Thrombus formation in femoral arteries induced by FeCl3. (A) Intravital imaging of thrombus formation 5 mins after FeCl3 treatment in femoral arteries in mice with control or paxillin knock-down platelets (Pxn-KD). The black arrows indicate the direction of blood flow, and triangles show the developed thrombus. Bar, 100 μm. (B) Areas of thrombus within arteries 20 mins after laser irradiation. Columns and error bars represent the mean ± s.e.m. (n = 8 arteries in four mice/group).

Additional file 9: Knock-down of paxillin does not affect talin-dependent activation of integrin αIIbβ3 in CHO cells. (A) Schematic representation of the lentiviral vectors used in this experiment. (B–D) αIIbβ3-CHO cells were transduced with lentiviral vectors expressing a control shRNA sequence and GFP (Control), the paxillin shRNA sequence and GFP (Pxn-KD), a control shRNA sequence and the GFP-Talin FERM domain (Control-FERM), or the paxillin shRNA sequence and the GFP-Talin FERM domain (Pxn-KD-FERM). (B) Lysates obtained from the transduced cells were immunoblotted with anti-GFP polyclonal antibody, anti-paxillin monoclonal antibody, and anti-vinculin monoclonal antibody. (C) PAC-1 binding after transduction in the presence or absence of 1 mmol/L GRGDS was assessed by

flow cytometry. Data are representative of four independent experiments. (D) Columns and error bars represent the mean ± s.d. of PAC-1 binding (n = 4). *Statistical significance was determined using Student's t test.*

Abbreviations

Pxn-KD: Paxillin-knockdown; Tx: Thromboxane; shRNA: Short hairpin RNA; GRGDS: Gly-Arg-Gly-Asp-Ser; ROS: Reactive oxygen species; ITAM: Immunoreceptor tyrosine-based activation motif; ITIM: Immunotyrosine-based inhibitory motif.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Contribution: AS, TO, and SN designed the study, performed the experiments, analyzed the data, and wrote the manuscript; HS performed the experiments and wrote the manuscript; SM, JM, KK, and YS analyzed the data and revised the manuscript. All authors read and approved the final manuscript.

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CRITICAL CARE

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Combination of thrombin-antithrombin complex, plasminogen activator inhibitor-1, and protein C activity for early identification of severe coagulopathy in initial phase of sepsis: a prospective observational study

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Abstract

Introduction: Current criteria for early diagnosis of coagulopathy in sepsis are limited. We postulated that coagulopathy is already complicated with sepsis in the initial phase, and severe coagulopathy or disseminated intravascular coagulation (DIC) becomes overt after progressive consumption of platelet and coagulation factors. To determine early diagnostic markers for severe coagulopathy, we evaluated plasma biomarkers for association with subsequent development of overt DIC in patients with sepsis.

Methods: A single-center, prospective observational study was conducted in an adult ICU at a university hospital. Plasma samples were obtained from patients with sepsis at ICU admission. Fourteen biomarkers including global markers (platelet count, prothrombin time, activated partial thromboplastin time, fibrinogen and fibrin degradation product (FDP)); markers of thrombin generation (thrombin-antithrombin complex (TAT) and soluble fibrin); markers of anticoagulants (protein C (PC) and antithrombin); markers of fibrinolysis (plasminogen, α_2-plasmin inhibitor (PI), plasmin-α_2-PI complex, and plasminogen activator inhibitor (PAI)-1); and a marker of endothelial activation (soluble E-selectin) were assayed. Patients who had overt DIC at baseline were excluded, and the remaining patients were followed for development of overt DIC in 5 days, and for mortality in 28 days.

Results: A total of 77 patients were enrolled, and 37 developed overt DIC within the following 5 days. Most patients demonstrated hemostatic abnormalities at baseline with 98.7% TAT, 97.4% FDP and 88.3% PC. Most hemostatic biomarkers at baseline were significantly associated with subsequent development of overt DIC. Notably, TAT, PAI-1 and PC discriminated well between patients with and without developing overt DIC (area under the receiver operating characteristic curve (AUROC), 0.77 (95% confidence interval, 0.64 to 0.86); 0.87 (0.78 to 0.92); 0.85 (0.76 to 0.91), respectively), and using the three together, significantly improved the AUROC up to 0.95 (vs. TAT, PAI-1, and PC). Among the significant diagnostic markers for overt DIC, TAT and PAI-1 were also good predictors of 28-day mortality (AUROC, 0.77 and 0.81, respectively).

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Conclusions: Severe coagulation and fibrinolytic abnormalities on ICU admission were associated with subsequent development of overt DIC. A single measurement of TAT, PAI-1, and PC activity could identify patients with ongoing severe coagulopathy, early in the course of sepsis.

Introduction

Sepsis is frequently complicated with coagulopathy [1]. The severity of sepsis-associated coagulopathy is variable, ranging from subclinical abnormalities that are detectable by a mild decrease in platelet count and prolongation of global clotting times, to severe forms of coagulopathy or disseminated intravascular coagulation (DIC) [2]. The incidence of DIC is up to 25 to 50% in patients with sepsis [3].

Septic DIC is characterized by systemic intravascular activation of coagulation, and microvascular endothelial injury with impaired anticoagulation and insufficient fibrinolysis, which leads to widespread thrombosis in microvasculature. In sepsis, DIC has a feature of vascular endothelial dysfunction, as well as being an etiological factor in the failure of other organs: excessive thrombin generation and subsequent fibrin deposition exacerbate inflammation and ischemia, contributing to organ damage [4]. A number of studies have reported that DIC is an independent risk factor for organ dysfunction and mortality in patients with sepsis [2,3,5]. DIC might, therefore, be an important therapeutic target in the management of sepsis, and the development of reliable methods for early identification of DIC is a high priority.

However, the early diagnosis of sepsis-associated coagulopathy and evaluation of its severity is still challenging [6]. Currently, the overt DIC criteria of the International Society on Thrombosis and Haemostasis (ISTH) are the diagnostic standard for severe coagulopathy in sepsis [4]. Although the ISTH criteria for overt DIC are simple and widely used, and shown to be associated with organ failure and mortality, they have limited application in the early phase of sepsis to improve outcome [7,8]. The ISTH overt DIC criteria use global markers, such as prothrombin time (PT) and platelet count for scoring. The coagulation factors and platelets are consumed and decrease over time because of progressive thrombin generation and endothelial injury, thus it takes several days to reveal their abnormalities and fulfill the overt DIC criteria in the course of sepsis [9,10]. Furthermore, introduction of the concept of pre-DIC, which is considered as the stage prior to overt DIC, has failed to predict disease progression. An ISTH subcommittee defined non-overt DIC as compensated coagulopathy, or pre-stage DIC, for the early diagnosis of overt DIC [4]. However, previous studies have shown that only 10 to 30% of patients with non-overt DIC progressed to overt DIC, although the mortality rates were similar between patients with non-overt and overt DIC [6,11].

In the past decade, there has been increasing evidence that inflammation and coagulation play pivotal roles in the pathogenesis of sepsis [12,13]. Pro-inflammatory cytokines produced by the host response against infection stimulate tissue factor expression and lead to activation of coagulation. An activated coagulation system in turn modulates inflammatory activity through specific receptors, such as protease-activated receptors. Considering that excessive crosstalk between inflammation and coagulation is ongoing from the onset of sepsis, severe coagulopathy may have developed early in the course.

The objective of this study was to identify hemostatic biomarkers that can be used for early diagnosis of severe coagulopathy in patients with sepsis. We postulate that severe coagulopathy has already developed in the initial phase of sepsis, and is related to the subsequent fulfillment of the criteria for overt DIC [14]. We, therefore, evaluated the association between plasma biomarkers measured at the time of intensive care unit (ICU) admission and development of overt DIC in the following five days. We also investigated the hemostatic biomarkers as predictors for 28-day mortality.

Material and methods

Study design and setting

This was a single-center, prospective observational study, that was conducted in a 12-bed medicosurgical ICU at a university hospital from January 2012 to June 2013. The study was approved by the Institutional Research Ethics Committee of Jichi Medical University, and informed consent was obtained from the patients or their families.

The consecutive patients who were admitted to the ICU because of sepsis, and without overt DIC on ISTH criteria at the time of ICU admission, were enrolled. Sepsis was defined according to the 2001 International Sepsis Definitions Conference [15]. Exclusion criteria were: age <18 years, presence of decompensated cirrhosis (Child-Pugh class B or C), hematological disorders, chronic renal failure on hemodialysis, and history of therapeutic anticoagulation or blood transfusion during the preceding four weeks.

Clinical and demographic data, including age, sex, comorbidity and Acute Physiology and Chronic Health Evaluation (APACHE) II scores [16], were recorded on ICU admission. Sequential Organ Failure Assessment (SOFA) score [17] except for coagulation (platelet count),

and overt DIC score on ISTH criteria were determined daily. ISTH non-overt DIC score, and acute DIC score established by the Japanese Association for Acute Medicine (JAAM) [18] were also calculated daily as early diagnostic systems for DIC.

The primary endpoint was the development of overt DIC within the first five days of ICU stay. A score ≥5 on the ISTH criteria was defined as overt DIC. The secondary endpoint was 28-day all-cause mortality. Plasma samples were drawn from the eligible patients within 6 h of ICU admission, and the patients were followed for 5 days for overt DIC score and 28 days for mortality.

Biomarker measurements

Plasma biomarkers were measured at the time of ICU admission (Day 0) as baseline, and on days 1 to 3. We classified 14 biomarkers into five categories: global markers (platelet count, prothrombin time (PT) and PT-international normalized ratio (PT-INR), activated partial thromboplastin time, fibrinogen, fibrin degradation product (FDP)); markers of thrombin generation (thrombin–antithrombin complex (TAT), soluble fibrin (SF)); markers of anticoagulants activity (protein C (PC), antithrombin (AT)); markers of fibrinolytic activity (plasminogen, α_2-plasmin inhibitor (PI), plasminogen activator inhibitor (PAI)-1, plasmin–α_2-PI complex (PIC)); and a marker of endothelial activation (soluble E-selectin (sES)).

Blood samples were collected heparin-free and centrifuged at 2,500 rpm at 4°C in citrated tubes. Global markers, TAT, PC, AT, plasminogen, α_2-PI and PIC were assayed using the CS-2100i automatic coagulation analyzer (Sysmex, Hyogo, Japan) immediately after the samples were collected. Berichrom assays (Siemens Healthcare Diagnostics, Tokyo, Japan) were used for PC, AT, plasminogen and α2-PI activities, and TAT/PIC test F enzyme immunoassay (Sysmex) were used for measurements of TAT and PIC levels, respectively. SF, PAI-1 and E-selectin were measured with the stored samples, which were frozen at −80°C within 2 h of collection, using iatroSF, tPAI test and sES latex photometric immunoassay, respectively (Mitsubishi Chemical Medience, Tokyo, Japan).

Patient management

Our facility provides 24-h coverage by attending ICU physicians. Management of patients followed the Surviving Sepsis Campaign Guidelines (SSCG) with the goal of initial resuscitation and infection control [19]. Patients received mechanical prophylactic treatment without concomitant low-dose heparin, until no active bleeding or severe coagulopathy was confirmed. Antithrombin substitution therapy was at the discretion of the ICU physicians, limited for the patients with AT activity <50% after the plasma samples at baseline were collected. The patients with bleeding risk or complications were transfused with platelet concentrate or fresh frozen plasma as decided by the ICU physicians.

Data analysis

The study population was grouped according to the development of overt DIC. Statistical differences between the groups were analyzed using Wilcoxon rank-sum test for non-normally distributed variables, and the χ^2, or Fisher's exact test for categorical variables as appropriate. Biomarker abnormalities were defined as values higher than the upper limit of normal, or lower than the lower limit of normal, which were used in practice at our institution. Receiver operating characteristic (ROC) curve analysis was performed to calculate the area under the receiver operating characteristic curve (AUROC) of the 14 biomarkers at baseline for the development of overt DIC, and of those at baseline and at Day 2 for 28-day mortality. The AUROC for APACHE II score and pre-DIC scores (by ISTH non-overt DIC, and JAAM acute DIC criteria) at baseline were also calculated for comparison. The best cutoff values were calculated to maximize the sum of sensitivity and specificity. Positive predictive value (PPV) and negative predictive value (NPV) were also calculated. To assess the bivariable association among biomarkers, Spearman rank correlation coefficients (r value) along with the associated P-value were calculated, and r <0.5 was considered as no evidence of collinearity. A multivariate logistic regression model based on a forward stepwise method was used to identify the best combination to discriminate the development of overt DIC. To assess the impact of biomarkers on survival, Kaplan-Meier estimates were used to illustrate trends in 28-day mortality and the log-rank test was performed. All P-values were two-tailed, and P <0.05 was considered statistically significant. Data were analyzed using JMP version 10 (SAS Institute, Tokyo, Japan).

Results

Patient characteristics and outcomes

One hundred, eleven patients were admitted to the ICU because of sepsis during the study period. Thirty-four patients were excluded according to the study criteria, and the remaining 77 patients were enrolled. The baseline characteristics and prognosis of the study population are described in Table 1. Of 77 patients with sepsis, 37 (48.1%) developed overt DIC within five days of their ICU stay. Patients who newly developed overt DIC were more severely ill with a higher APACHE II score, maximum SOFA scores and 28-day mortality, compared with patients who did not develop overt DIC. No therapeutic heparin was administered during the study period. Prophylactic low-dose heparin was used more frequently in patients without DIC than in those who developed overt DIC (50.0 vs. 10.8%, $P = 0.0001$). Platelet concentrate, fresh frozen plasma and antithrombin were more frequently

Table 1 Baseline characteristics and outcomes of the 77 patients with sepsis

	All patients (n = 77)	Develop DIC (n = 37)	No DIC (n = 40)	*P*-value*
Demographics				
Age (years)	69.9 ± 12.9	70.7 ± 13.2	69.1 ± 12.7	0.58
Male	42 (54.5)	16 (43.2)	26 (65.0)	0.069
Source of sepsis				
Pulmonary infection	15 (19.5)	7 (18.9)	8 (20.0)	0.91
Abdominal infection	43 (55.8)	22 (59.5)	21 (52.5)	0.54
Urinary tract infection	5 (6.5)	3 (8.1)	2 (5.0)	0.58
Soft tissue infection	11 (14.3)	3 (8.1)	8 (20.0)	0.13
Blood stream infection	2 (2.6)	2 (5.4)	0 (0.0)	0.084
Comorbidities				
IHD	7 (9.1)	2 (5.4)	5 (12.5)	0.27
CHF	2 (2.6)	0 (0.0)	2 (5.0)	0.11
Arrhythmia	3 (3.9)	3 (8.1)	0 (0.0)	0.033
COPD	6 (7.8)	1 (2.7)	5 (12.5)	0.094
CKD	10 (13.0)	6 (16.2)	4 (10.0)	0.42
CVD	3 (3.9)	2 (5.4)	1 (2.5)	0.51
Severity of illness				
APACHE II score	25.4 ± 7.9	28.8 ± 8.2	22.2 ± 6.1	0.0002
Organ dysfunction (days 0 to 5)				
max SOFA score**	9 (7 to 11)	10 (9 to 14)	7 (4 to 9)	0.0001
Prognosis				
ICU-free days	18 (10 to 21)	16 (0 to 19)	21 (17 to 23)	0.0001
28-day mortality	15 (19.5)	13 (35.1)	2 (5.0)	0.0005

Data are expressed as mean ± SD, median (interquartile range), or No. (%).
APACHE, acute physiology and chronic health evaluation; CHF, chronic heart failure; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; IHD, ischemic heart disease.
*Comparison of groups with and without subsequent development of overt DIC.
**maximum SOFA scores except for score of coagulation (platelet count) during the first five days of ICU stay.

administered to patients who developed overt DIC than to those who did not (24.3 vs. 0%, $P = 0.0001$; 29.7 vs. 7.5%, $P = 0.012$; 56.8 vs. 5.0%, $P < 0.0001$, respectively).

Evidence of biomarker abnormalities at baseline and subsequent changes over time

The majority of the 77 patients with sepsis presented with plasma biomarker abnormalities at the time of admission (Day 0), as indicated by elevated TAT (98.7% of patients) and FDP (97.4% of patients), and decreased activity of PC (88.3% of patients, Table 2). In contrast, decreased platelet count, prolonged PT-INR or decreased level of fibrinogen was not frequently observed on Day 0 in patients with sepsis.

Plasma biomarkers of platelet, PT-INR, FDP, TAT, PAI-1 and PC over time (days 0 to 3) in patients with and without subsequent development of overt DIC are shown in Figure 1 (other studied biomarkers are shown in Additional file 1). On Day 0, there were marked increases in TAT and PAI-1, and decreases in PC, plasminogen and α_2-PI activities, which were particularly marked in patients with subsequent development of overt DIC. Notably, TAT and PAI-1 were the highest on Day 0 and gradually returned to normal in patients who developed overt DIC (TAT on Day 0 vs. Day 2, $P = 0.013$; PAI-1, $P = 0.0035$), whereas platelet count and PT-INR were around the normal range on Day 0 and exacerbated until days 2 to 3 (platelet on Day 0 vs. Day 2, $P < 0.0001$; PT-INR, $P = 0.0043$).

Discrimination capacity of plasma biomarkers at baseline for subsequent development of overt DIC

We conducted ROC curve analysis to evaluate the ability of biomarkers to discriminate among patients who subsequently developed overt DIC and those who did not. The AUROCs and best calculated cutoff values, PPV and NPV, are shown in Table 3. The AUROCs and PPVs for the development of overt DIC were high for TAT, PC, AT, plasminogen, α2-PI and PAI-1. For the comparison between discrimination abilities of plasma biomarkers and those of severity of illness, and pre-DIC scores at

Table 2 Plasma biomarkers at baseline (Day 0) in patients with sepsis

	Normal range	All patients		Develop DIC	No DIC	
		Median level	Abnormal patients (%)	Median level	Median level	*P*-value*
Global markers						
Platelet (× 10^3/μL)	130 to 369	163 (118 to 205)	33.8[a]	140 (108 to 184)	176 (136 to 228)	0.036
PT-INR	0.9 to 1.2	1.25 (1.15 to 1.37)	55.8[b]	1.29 (1.17 to 1.38)	1.21 (1.13 to 1.31)	0.091
APTT (sec)	23.1 to 36.3	39.5 (32.2 to 48.9)	62.3[b]	42.7 (35.4 to 49.5)	37.7 (31.3 to 42.7)	0.096
Fibrinogen (mg/dL)	129 to 371	395 (249 to 544)	25.9[a]	299 (225 to 481)	419 (319 to 565)	0.041
FDP (μg/mL)	0 to 5.0	16.7 (10.4 to 28.9)	97.4[b]	20.5 (11.7 to 44.1)	15.6 (8.4 to 22.1)	0.011
Thrombin generation						
TAT (ng/mL)	<2.4	12.5 (7.2 to 20.1)	98.7[b]	19.5 (10.5 to 25.8)	8.4 (5.7 to 12.9)	<0.0001
SF (μg/mL)	<7.0	10.5 (5.3 to 24.2)	66.2[b]	13.9 (7.9 to 29.3)	7.4 (3.2 to 17.3)	0.013
Anticoagulant activity						
PC (%)	67 to 129	46.2 (34.1 to 59.5)	88.3[a]	36.6 (28.1 to 44.9)	59.1 (46.7 to 65.6)	<0.0001
AT (%)	75 to 125	51.8 (38.4 to 63.2)	88.3[a]	42.8 (31.3 to 54.8)	58.2 (48.1 to 72.5)	0.0001
Fibrinolytic activity						
Plasminogen (%)	85 to 120	60.2 (43.6 to 73.7)	85.7[a]	48.1 (30.3 to 66.1)	67.0 (57.1 to 84.6)	0.0001
α2-PI (%)	83 to 115	67.3 (52.1 to 82.8)	74.1[a]	54.1 (40.5 to 67.6)	78.6 (67.1 to 88.7)	<0.0001
PAI-1 (ng/mL)	<50.0	154.7 (60.7 to 533.1)	81.8[b]	531.6 (191.1 to 992.6)	77.6 (40.8 to 154.7)	<0.0001
PIC (μg/mL)	<0.9	1.0 (0.7 to 1.8)	54.5[b]	1.0 (0.6 to 2.7)	1.1 (0.8 to 1.5)	0.99
Endothelial activation						
sES (ng/mL)	<29.7	55.2 (35.9 to 101.1)	83.1[b]	65.3 (34.8 to 144.8)	49.5 (36.9 to 72.9)	0.17

α2-PI, α2-plasmin inhibitor activity; APTT, activated partial thromboplastin time; AT, antithrombin activity; DIC, disseminated intravascular coagulation; FDP, fibrin degradation products; PAI-1, plasminogen activator inhibitor-1; PC, protein C activity; PIC, plasmin-α2-plasmin inhibitor complex; PT-INR, prothrombin time-international normalized ratio; sES, soluble E-selectin; SF, soluble fibrin; TAT, thrombin-antithrombin complex.
*Comparison of groups with and without subsequent development of overt DIC. [a]Percentage of patients with values lower than the lower limit of normal. [b]Percentage of patients with values higher than the upper limit of normal.

baseline, the AUROCs and PPVs were also calculated for APACHE II scores (AUROC, 0.72, (95% confidence interval, 0.61 to 0.82); PPV, 0.62), ISTH non-overt DIC scores (AUROC, 0.71 (0.59 to 0.80); PPV, 0.58), and JAAM DIC scores (AUROC, 0.68 (0.55 to 0.78); PPV, 0.62) with relatively low PPV values.

Correlation and multivariate analysis to identify significant diagnostic biomarkers for subsequent development of overt DIC

To identify efficient diagnostic markers for the development of overt DIC, we undertook further analysis of significant biomarkers with AUROC >0.7 and PPV >0.7, which were superior to the results of APACHE II scores or pre-DIC scores. First, we calculated Spearman rank correlation coefficients for TAT, PC, AT, plasminogen, α_2-PI and PAI-1 to rule out collinearity among the significant biomarkers. We found a strong and significant correlation with r >0.5 between each pair of PC, AT, plasminogen and α_2-PI values (Additional file 2). However, TAT and PAI-1 were not so highly correlated with PC.

Next, we conducted a multivariate stepwise logistic regression analysis, and found that TAT, PAI-1 and PC were the best combination to discriminate between patients with and without development of overt DIC. These three biomarkers remained significantly associated with overt DIC, even after adjustment for APACHE II score in separate models (TAT, $P = 0.0002$; PAI-1, $P = 0.0001$; PC, P <0.0001, respectively). Furthermore, the combination of TAT, PAI-1 and PC substantially improved discrimination of the development of overt DIC, compared with each marker alone (AUROC 0.95 (vs. TAT, $P = 0.0004$; vs. PAI-1, $P = 0.033$; vs. PC, $P = 0.025$), Figure 2).

Plasma biomarkers on days 0 and 2 as predictors of 28-day mortality

Univariate analysis revealed that only TAT and PAI-1 at baseline were significant predictors of 28-day mortality among the biomarkers that had good discriminative power for the development of overt DIC (Table 4 and Additional file 3). Based on the best calculated cutoff values, cutoff points at baseline were set at 18 ng/mL for TAT and 270 ng/mL for PAI-1. The Kaplan-Meier survival curve for patients with sepsis demonstrated that TAT >18 ng/mL and/or PAI-1 >270 ng/mL on admission were significantly correlated with higher mortality ($P = 0.0024$, Figure 3).

Most of the studied Day 2 markers had higher AUROCs for prediction of 28-day mortality compared with

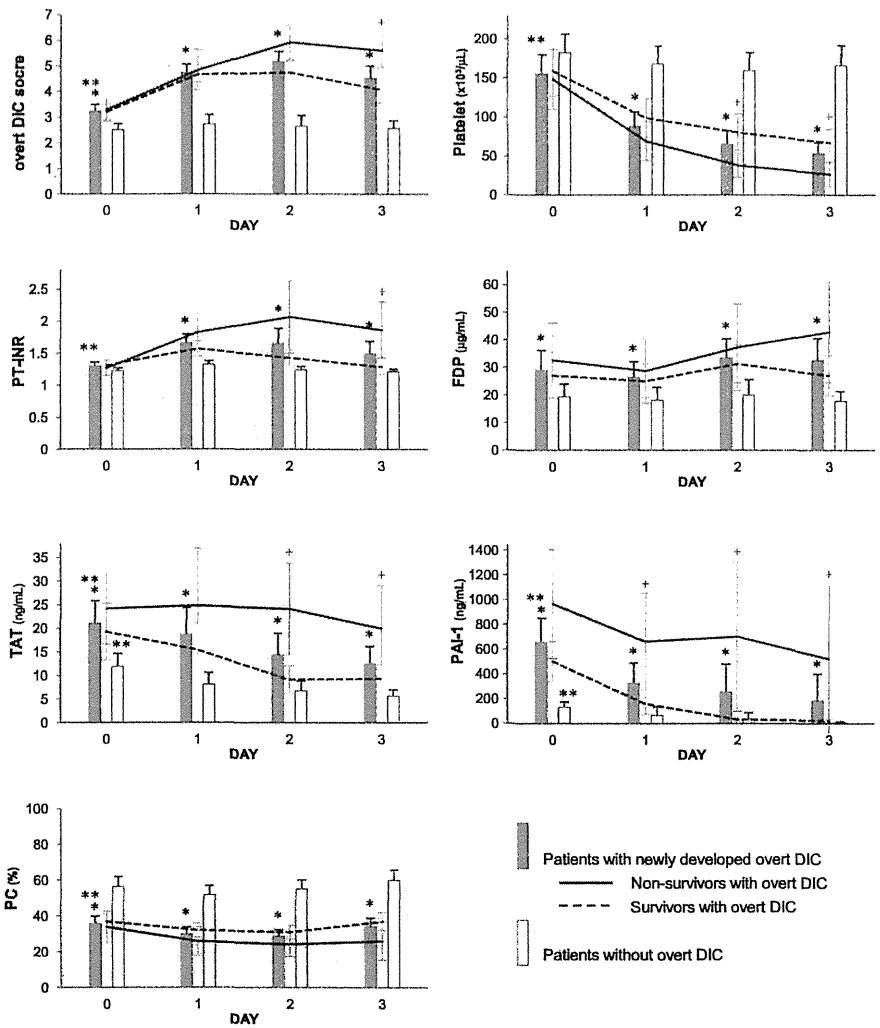

Figure 1 Time course of overt DIC scores and hemostatic biomarkers from baseline to Day 3. Overt disseminated intravascular coagulation (DIC) scores, platelet count, prothrombin time-international normalized ratio (PT-INR), fibrin degradation product (FDP), thrombin-antithrombin complex (TAT), plasminogen activator inhibitor-1 (PAI-1) and protein C (PC) for patients with and without subsequent development of overt DIC (gray vs. white bars), and for survivors (dotted line) and non-survivors (solid line) among patients with overt DIC. Data are expressed as mean and 95% CI. **P* <0.05 between patients with and without overt DIC on the same day. ***P* <0.05 between patients on Day 0 versus Day 2. ^{+}P <0.05 between survivors and non-survivors with overt DIC on the same day.

Day 0 markers (Table 4 and Additional file 3). Among the Day 2 biomarkers, TAT, SF and PAI-1 remained statistically significant for prediction of 28-day mortality after adjustment for APACHE II score ($P = 0.0016$, P <0.0001, P <0.0001, respectively).

Discussion

The main findings of our study were as follows. 1) Coagulopathy developed in the initial phase of sepsis, and the severity of hemostatic biomarker abnormalities on the day of admission was associated with the subsequent development of overt DIC. 2) Among all the studied biomarkers, TAT, PAI-1 and PC had the best discriminative power for the patients who newly developed overt DIC. 3) However, only TAT and PAI-1 on Day 0 were significant predictors of 28-day mortality among the diagnostic biomarkers for the development of overt DIC. In contrast, Day 2 markers had higher predictive power for 28-day mortality compared with Day 0 markers, suggesting that persistence of severe coagulopathy was correlated with mortality.

Inflammation and coagulation constitute two host defense systems with complementary roles against infection [13], which means that an overwhelming systemic inflammatory reaction in sepsis is accompanied by severe coagulopathy, and both may contribute to tissue damage in the early phase of sepsis. In our study, most patients with

Table 3 Area under the ROC curves of biomarkers at baseline for prediction of overt DIC

Biomarkers (Day 0)	AUC (95% CI)	Cutoff values*	Sensitivity	Specificity	PPV	NPV
Global markers						
Platelet	0.65 (0.51 to 0.76)	158 (× 10^3/μL)	0.62	0.65	0.62	0.65
PT-INR	0.61 (0.48 to 0.73)	1.3	0.62	0.63	0.61	0.64
APTT	0.61 (0.48 to 0.73)	42 (sec)	0.54	0.75	0.67	0.64
Fibrinogen	0.64 (0.51 to 0.76)	310 (mg/dL)	0.54	0.78	0.69	0.65
FDP	0.67 (0.54 to 0.78)	28 (μg/mL)	0.43	0.88	0.76	0.63
Thrombin generation						
TAT	0.77 (0.64 to 0.86)	15 (ng/mL)	0.67	0.85	0.81	0.72
SF	0.67 (0.54 to 0.78)	7.9 (μg/mL)	0.77	0.54	0.61	0.72
Anticoagulant activity						
PC	0.85 (0.76 to 0.91)	46 (%)	0.81	0.79	0.79	0.82
AT	0.76 (0.63 to 0.85)	46 (%)	0.60	0.85	0.78	0.69
Fibrinolytic activity						
Plasminogen	0.76 (0.63 to 0.85)	52 (%)	0.60	0.79	0.73	0.67
α2-PI	0.79 (0.67 to 0.88)	70 (%)	0.81	0.67	0.70	0.79
PAI-1	0.87 (0.78 to 0.92)	269 (ng/mL)	0.72	0.92	0.89	0.78
PIC	0.49 (0.36 to 0.63)	1.9 (μg/mL)	0.35	0.89	0.76	0.59
Endothelial activation						
sES	0.59 (0.45 to 0.72)	67 (ng/mL)	0.51	0.72	0.62	0.61

α2-PI, α2-plasmin inhibitor activity; APTT, activated partial thromboplastin time; AT, antithrombin activity; AUC, area under the curve; CI, confidence interval; FDP, fibrin degradation products; NPV, negative predictive value; PAI-1, plasminogen activator inhibitor-1; PC, protein C activity; PIC, plasmin-α2-plasmin inhibitor complex; PPV, positive predictive value; PT-INR, prothrombin time-international normalized ratio; ROC, receiver operating characteristic; sES, soluble E selectin; SF, soluble fibrin; TAT, thrombin- antithrombin complex. *Cutoff values were calculated to maximize the sum of sensitivity and specificity.

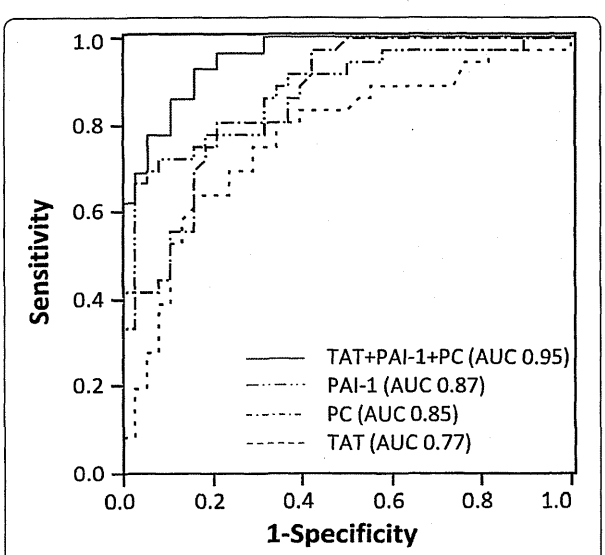

Figure 2 ROC curves of TAT, PAI-1 and PC activity for prediction of overt DIC. Area under the receiver operating characteristic curve (AUROC) for thrombin-antithrombin complex (TAT), 0.77 (95% CI, 0.64 to 0.86), plasminogen activator inhibitor-1 (PAI-1), 0.87 (0.78 to 0.92), protein C (PC), 0.85 (0.76 to 0.91), and combination of these biomarkers are described. Combination of TAT, PAI-1 and PC was superior to each marker alone (AUROC, 0.95 (vs. TAT, $P = 0.0004$; vs. PAI-1, $P = 0.033$; vs. PC, $P = 0.025$)).

sepsis exhibited coagulation and fibrinolytic abnormalities at the time of ICU admission, which is consistent with the data from the PROWESS trial [1]. In addition, most hemostatic biomarkers measured on ICU admission were associated with subsequent fulfillment of overt DIC criteria. These results support the hypothesis that coagulopathy is present in the initial phase of sepsis, and the strategy to identify markers of acute ongoing coagulopathy, rather than to detect pre-DIC state, may be necessary for the early diagnosis of septic DIC.

The pathogenesis of DIC is primarily due to excess production of thrombin [20]. In sepsis, anticoagulation impairment and insufficient fibrinolysis also contribute to thrombin generation and fibrin deposition. Anticoagulation pathways such as the antithrombin and protein C systems are impaired because of increased consumption, decreased protein synthesis, extravasation and degradation by several proteolytic enzymes such as neutrophil elastase [21,22]. The fibrinolytic system is largely suppressed by increased production of PAI-1, which is a principal inhibitor of this system [23,24]. In our study, increased levels of TAT and PAI-1, and decreased PC activity, were observed at the time of ICU admission and each independently discriminated the patients who developed overt DIC from those who did not. Our findings indicate that activation of coagulation, anticoagulation impairment and insufficient

Table 4 Area under ROC curves of Day 0 and Day 2 biomarkers for prediction of mortality

Biomarkers	ICU day	AUC (95% CI)	Cutoff values*	Sensitivity	Specificity	PPV	NPV
Global markers							
Platelet	Day 0	0.58 (0.41 to 0.74)	117 ($\times 10^3$/μL)	0.41	0.79	0.32	0.84
	2	0.81 (0.64 to 0.91)	66 ($\times 10^3$/μL)	0.81	0.79	0.48	0.94
PT-INR	Day 0	0.53 (0.34 to 0.72)	1.2	0.53	0.71	0.31	0.86
	2	0.68 (0.47 to 0.84)	1.5	0.61	0.81	0.43	0.89
FDP	Day 0	0.61 (0.42 to 0.76)	21 (μg/mL)	0.61	0.65	0.29	0.87
	2	0.61 (0.41 to 0.77)	22 (μg/mL)	0.67	0.65	0.31	0.89
Thrombin generation							
TAT	Day 0	0.77 (0.62 to 0.87)	18 (ng/mL)	0.81	0.77	0.46	0.94
	2	0.83 (0.65 to 0.93)	16 (ng/mL)	0.67	0.92	0.67	0.92
Anticoagulant activity							
PC	Day 0	0.64 (0.45 to 0.79)	37 (%)	0.53	0.75	0.35	0.87
	2	0.76 (0.53 to 0.89)	22 (%)	0.61	0.97	0.82	0.91
Fibrinolytic activity							
Plasminogen	Day 0	0.64 (0.45 to 0.79)	61 (%)	0.81	0.52	0.29	0.91
	2	0.75 (0.57 to 0.87)	50 (%)	0.81	0.67	0.38	0.93
PAI-1	Day 0	0.81 (0.64 to 0.91)	269 (ng/mL)	0.85	0.71	0.38	0.96
	2	0.91 (0.79 to 0.96)	81.4 (ng/mL)	0.69	0.97	0.82	0.94

AUC, area under the curve; CI, confidence interval; FDP, fibrin degradation products; NPV, negative predictive value; PAI-1, plasminogen activator inhibitor-1; PC, protein C activity; PPV, positive predictive value; PT-INR, prothrombin time-international normalized ratio; ROC, receiver operating characteristic; TAT, thrombin-antithrombin complex.
*Cutoff values were calculated to maximize the sum of sensitivity and specificity.

fibrinolysis develop early in the course of sepsis, and these three mechanisms should be evaluated individually for the diagnosis of DIC in patients with sepsis.

In this study, we found that TAT, a marker of thrombin generation, and PAI-1, which is induced by pro-inflammatory cytokines, were highest at baseline and improved when diagnosis of DIC was made in patients who developed overt DIC. These significant trends were obvious in survivors with overt DIC. In non-survivors with overt DIC, elevated levels of TAT and PAI-1 persisted during the study period. Similar trends in those biomarkers were observed in an experimental model of sepsis and in clinical studies [25,26]. TAT and PAI-1 have short half-lives and they are produced early in the course of septic coagulopathy, while other biomarkers, such as platelets, PT-INR or PC, are the markers of consumption. The differences in those biomarkers over time between survivors and non-survivors indicate that TAT and PAI-1 may well reflect disease progress in septic coagulopathy.

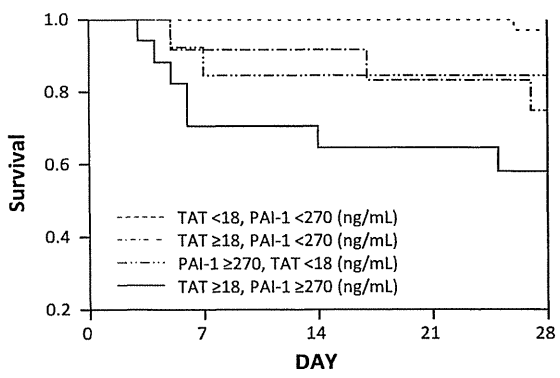

Figure 3 Kaplan-Meier survival curves for patients grouped by cutoff points of TAT and PAI-1 at baseline. The cutoff points were set at 18 ng/mL for thrombin-antithrombin complex (TAT) and 270 ng/mL for plasminogen activator inhibitor-1 (PAI-1), based on the best calculated cutoff values that maximize the sum of sensitivity and specificity for 28-day mortality.

Current criteria for early diagnosis of DIC have some potential limitations. Considering easy implementation, most criteria, including ISTH non-overt DIC and JAAM acute DIC criteria, use readily available coagulation tests for scoring. However, it is clear that global coagulation tests, such as PT and platelet count, primarily reflect the result of consumption and impaired synthesis rather than direct ongoing coagulopathy. Kinasewitz *et al.* [27] and Dhainaut *et al.* [28] established a simple diagnostic scoring system for the acute phase of septic coagulopathy, but these systems depend partly on worsening trends of global markers, which take at least two days to identify.

Several hemostatic molecular biomarkers, including AT, PC, TAT, PIC and PAI-1, have also been evaluated in patients with sepsis, but the reported results were inconsistent [1,24,25,28-30]. Several possible explanations could account for these conflicting results. First, we demonstrated dynamic changes in the biomarkers within a few days in the initial phase of sepsis, which is consistent with previous studies [1,25]; therefore, the timing of biomarker measurement is important for interpretation of the results. Second, the cutoff value is another factor that influences the diagnostic ability of biomarkers. Oh *et al.* [6] and Egi *et al.* [31] evaluated the cutoff value of the lower limit of normal (70%) in AT levels for ISTH non-overt DIC criteria, and showed that the diagnostic ability for overt DIC did not improve by adding AT to non-overt DIC criteria. In our study, AT activity, as well as PC, was decreased below the lower limit of normal, even in most of the patients without overt DIC, and the cutoff value of AT level to discriminate patients with and without overt DIC was much lower (46.1%). Last, most of the previous studies evaluated the impact of hemostatic biomarkers on prognosis in patients with sepsis. We found that some plasma biomarkers at baseline were good predictors for the development of overt DIC, but were less predictive for 28-day mortality compared with Day 2 markers, which indicates that persistence of coagulopathy, rather than just the development of it, influences the prognosis in patients with sepsis. In addition, multiple interactive systemic factors other than coagulopathy would be involved in the pathogenesis of organ failure and the risk of mortality. We, therefore, evaluated diagnostic and prognostic values of biomarkers individually.

There were some potential limitations to our study. First, this was a prospective observational study conducted in a single center with a relatively small population size. Although the overall rate of DIC matched that in previous studies [3,32], our cohort included fewer pneumonia patients, who often die from respiratory failure rather than multiple organ failure, including DIC. A large validation study is needed to confirm our results. Second, there is no gold standard for diagnosis of or the criteria for intervention in sepsis-associated coagulopathy. We used the ISTH overt DIC criteria as the diagnostic standard, considering coagulopathy that fulfilled these criteria would be severe enough to be eligible for intervention. Third, although our management of sepsis followed the SSCG guidelines, and did not deviate from standard care, prophylactic anticoagulation and interventions, such as blood transfusion as well as AT substitution, may have influenced the levels of hemostatic biomarkers except for baseline profile, and their relationship with the scores of overt DIC. Last, our study lacked explanations about why TAT, PAI-1 and PC were the best diagnostic markers for overt DIC. We found a strong correlation among AT, PC, plasminogen and α_2-PI at baseline. Considering that the same mechanism of consumption might be the main reason for decreased activity of those biomarkers [33], it is unclear why PC had superior diagnostic ability. Of particular interest is the contrast between the diagnostic value of TAT and another thrombin generation marker, SF. One possible explanation is the differences in half-life or mechanisms of clearance, where TAT has a shorter half-life (10 to 15 minutes), compared with SF (several hours). Further study is needed to better understand the processes of these biomarkers, and for the development of new therapeutic strategies in septic DIC.

Conclusions

The results of our study provide evidence that almost half of the patients developed severe coagulopathy in the initial phase of sepsis, which was demonstrated by baseline abnormalities in hemostatic biomarkers and their strong association with subsequent fulfillment of overt DIC criteria. In particular, a single determination of TAT, PAI-1 and PC activity at ICU admission allowed early identification of severe coagulopathy, or DIC, leading to early intervention for patients with sepsis.

Key messages

- The present study showed that coagulopathy was frequently observed in the initial phase of sepsis, and severe coagulation and fibrinolytic abnormalities were strongly associated with subsequent development of overt DIC.
- Among the 14 plasma biomarkers evaluated, TAT, PAI-1 and PC activity on ICU admission were the best combination to discriminate between patients with and without overt DIC.
- In terms of predicting mortality, only TAT and PAI-1 were significant predictors of 28-day mortality at the time of ICU admission.

Additional files

Additional file 1: Figure S1. Time course of biomarkers from baseline to Day 3. Fibrinogen, soluble fibrin (SF), plasminogen, α_2-plasmin inhibitor (α2-PI), plasmin-α_2-plasmin inhibitor complex (PIC) and soluble E-selectin (sES) for patients with and without subsequent development of overt disseminated intravascular coagulation (DIC) (gray vs. white bars), and for survivors (dotted line) and non-survivors (solid line) among patients with overt DIC. Data are expressed as mean and 95% CI. **P* <0.05 between patients with and without overt DIC on the same day. ***P* <0.05 between patients on Day 0 versus Day 2. ^{+}P <0.05 between survivors and non-survivors with overt DIC on the same day.

Additional file 2: Figure S2. Correlation of plasma biomarkers at baseline with each other. The correlation graphs and Spearman rank correlation coefficients (*r* value) are shown here.

Additional file 3: Table S1. Area under ROC curves of Day 0 and Day 2 biomarkers for prediction of mortality.

Abbreviations

α_2-PI: α_2-plasmin inhibitor; APACHE: Acute Physiology and Chronic Health Evaluation; AT: Antithrombin; AUROC: Area under the receiver operating curve; DIC: Disseminated intravascular coagulation; FDP: Fibrin degradation product; ISTH: International Society on Thrombosis and Haemostasis; JAAM: Japanese Association for Acute Medicine; NPV: Negative predictive value; PAI-1: Plasminogen activator inhibitor-1; PC: Protein C; PIC: Plasmin-α_2-plasmin inhibitor complex; PPV: Positive predictive value; PT-INR: Prothrombin time-international normalized ratio; ROC: Receiver operating characteristic; sES: soluble E-selectin; SF: Soluble fibrin; SOFA: Sequential Organ Failure Assessment; TAT: Thrombin-antithrombin complex.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KK conceived and designed the study. KK and SN prepared the data for analysis. KK conducted the data analysis. SM assisted with interpretation of the results. YS, JM and SN supervised the study. KK and SM drafted the article. All authors read and approved the manuscript. KK and SM take responsibility for the paper as a whole.

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Letter to the Editors-in-Chief

Addition of recommendations for the use of recombinant human thrombomodulin to the "Expert consensus for the treatment of disseminated intravascular coagulation in Japan"

Dear Editors,

When we published the "Expert consensus for the treatment of disseminated intravascular coagulation in Japan" [1], recombinant human thrombomodulin (rhTM) had not been marketed; therefore, recommendations regarding the use of rhTM were not stated at that time. A phase III trial of rhTM [2] subsequently showed the usefulness of this agent in treating disseminated intravascular coagulation (DIC), and the results of the postmarketing surveillance of rhTM guided by the Japanese Society on Thrombosis and Hemostasis (JSTH) confirmed the results of that trial [3]. In addition, the results of a phase II international trial of rhTM in patients with sepsis were also recently published [4]. Therefore, the JSTH would like to add a recommendation for the use of rhTM in the "Expert consensus for the treatment of disseminated intravascular coagulation in Japan."

Recommendation for the Use of rhTM

General: B1, asymptomatic type: B2, bleeding type (mild: B1, severe: C), organ failure type: B1, complication type: B2

B1: Treatment has moderately high quality of evidence, or it has high quality of evidence but the clinical usefulness is not significant.

B2: Treatment does not have a high quality of evidence, but it has few deleterious effects and it is carried out clinically.

C: Treatment does not have a high quality of evidence or the clinical usefulness is not clear.

Mechanisms of rhTM

Thrombomodulin (TM) exists on vascular endothelial cells and combines with thrombin. The thrombin-TM complex does not cleave fibrinogen, although it changes protein C into activated protein C (APC) [5]. APC inhibits the activity of coagulation factor VIII (FVIII) and FV by cleaving activated FVIII and FV using the protein S as a coenzyme. In addition, the thrombin-TM complex activates thrombin-activatable fibrinolysis inhibitor (TAFI) to inhibit fibrinolysis [6]. Since TM inhibits the complement system, it is considered to have an anti-inflammatory effect. In patients with sepsis, the expression of TM on vascular endothelial cells is down-regulated to a markedly low level by LPS and inflammatory cytokines. rhTM is a medication developed as a soluble protein containing an extracellular domain required to perform the activity of TM [7,8] and is considered to have an anticoagulant effect via APC production as well as antifibrinolytic and anti- inflammatory effects.

Evidence

In a Japanese phase III double-blind randomized control trial (RCT) of rhTM vs unfractionated heparin (UFH) in subjects with DIC [2], including 227 DIC patients with 125 hematological malignancies and 102 infections, the rate of resolution of DIC was 66.1% vs 49.9%, for absolute risk reduction of 16.2% (95%CI: 3.3% to 29.1%). The rate of disappearance of bleeding conditions was 35.2% in the rhTM group and 20.9% in the UFH group, for a difference of 14.3% (1.2% to 27.4%). In addition, the 28-day mortality among the patients with infection was 28.0% in the rhTM group and 34.6% in the UFH group, for a difference of 6.6% (-24.6% to 11.3%). The frequency of adverse events of bleeding was up to 7 days after the start of infusion significantly higher in the UFH group (56.5%) than in the rhTM group (43.1%), with no significant differences in other adverse events between the groups. In the present retrospective subanalysis of 80 patients with DIC secondary to infection [9] among the full analysis sample [2], the rate of resolution of DIC was 63.2% in the UFH group and 73.2% in the rhTM group, for a difference of 10.0% (95%CI:-10.5% to 30.5%). Furthermore, the 28-day mortality was 21.4% in the rhTM group and 31.6% in the UFH group, for a difference of 10.2% (-9.1% to 29.4%).

In an international double-blind placebo-controlled RCT [4] of 750 septic patients with suspected DIC, the 28- day mortality was 17.8% in the rhTM group and 21.6% in the placebo group, thus indicating a trend toward a low value, although the difference was not significant ($p = 0.273$), in the rhTM group. Furthermore, the values of hemostatic markers, such as D-dimer prothrombin fragment F1+2 and thrombin antithrombin complex, were lower in the rhTM group than in the placebo group, while there were no significant differences in the levels of inflammatory marker or rates of organ failure, bleeding, thrombosis or new infection. In the post hoc analysis, the greatest benefit from rhTM was seen in the patients with at least one site of organ system dysfunction and an international normalized ratio greater than 1.4 at baseline. This trial subsequently shifted to a phase III trial.

In a domestic postmarketing surveillance of rhTM among 3,548 patients with DIC (2,516 cases of infection and 1,032 cases of hematological malignancy)[3], the DIC scores were significantly decreased after treatment with rhTM in both groups ($p < 0.001$). The frequency of adverse drug reactions of critical bleeding was 2.6% in the infection group and 2.4% in the hematological malignancy group, with survival rates at 28 days after the last rhTM administration of 64.1% in the infection group and 70.7% in the hematological malignancy group.

Disclosure of Conflicts of Interest

The authors declare no potential conflicts of interest.

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Living donor liver transplantation from an asymptomatic donor with mild coagulation factor IX deficiency: Report of a case

Sanada Y, Sasanuma H, Sakuma Y, Morishima K, Kasahara N, Kaneda Y, Miki A, Fujiwara T, Shimizu A, Hyodo M, Hirata Y, Yamada N, Okada N, Ihara Y, Urahashi T, Madoiwa S, Mimuro J, Mizuta K, Yasuda Y. (2014) Living donor liver transplantation from an asymptomatic donor with mild coagulation factor IX deficiency: Report of a case. *Pediatr Transplant*, 18: E270–E273. DOI: 10.1111/petr.12358.

Abstract: The use of donors with coagulation FIX deficiency is controversial, and there are no current protocols for peri-transplant management. We herein describe the first reported case of a pediatric LDLT from an asymptomatic donor with mild coagulation FIX deficiency. A 32-yr-old female was evaluated as a donor for her 12-month-old daughter with biliary atresia. The donor's pretransplant coagulation tests revealed asymptomatic mild coagulation FIX deficiency (FIX activity 60.8%). Freeze-dried human blood coagulation FIX concentrate was administered before the dissection of the liver and 12 h afterwards by bolus infusion (40 U/kg) and was continued on POD 1. The bleeding volume at LDLT was 590 mL. On POD 1, 3, 5, and 13, the coagulation FIX activity of the donor was 121.3%, 130.6%, 114.6%, and 50.2%, respectively. The donor's post-transplant course was uneventful, and the recipient is currently doing well at 18 months after LDLT. The FIX activity of the donor and recipient at nine months after LDLT was 39.2% and 58.0%, respectively. LDLT from donors with mild coagulation FIX deficiency could be performed effectively and safely using peri-transplant short-term coagulation FIX replacement and long-term monitoring of the plasma FIX level in the donor.

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Key words: coagulation factor IX deficiency – coagulation factor IX activity – freeze-dried human blood coagulation factor IX concentrate – living donor – living donor liver transplantation

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The use and safety of expanded-criteria donors have become accepted in clinical practice because of the scarcity of organs for transplantation. However, the use of grafts from donors with coagulation FIX deficiency has not been reported. There is a safety concern related to such donors because coagulation FIX deficiency exposes patients to greater risks of bleeding complications during the peri-transplant period; there is no consensus as to whether grafts from donors with coagulation FIX deficiency should be used.

Although the efficacy of FIX administration to patients with hemophilia undergoing surgical interventions has been shown (1) and some experiences of liver transplantation for hemophiliac patients with end-stage liver disease have been reported (2–13), there are no current protocols for the peri-transplant management of donors with coagulation FIX deficiency.

We describe the first reported case of pediatric LDLT from an asymptomatic donor with mild coagulation FIX deficiency.

Abbreviations: Alb, albumin; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; FVIII, factor VIII; FIX, factor IX; FFP, fresh frozen plasma; Hb, hemoglobin; Hct, hematocrit; LDLT, living donor liver transplantation; Plt, platelet; POD, postoperative day; POM, postoperative month; PT-INR, prothrombin time-international normalized ratio; TB, total bilirubin.

Case report

Donor

A 32-yr-old woman was evaluated as a donor for her 12-month-old daughter with biliary atresia.

Asymptomatic mild coagulation FIX deficiency was diagnosed during the pretransplant examination for LDLT based on the prolonged APTT. The blood test results were as follows: Hb 13.1 g/dL; Hct 39.2%; Plt 254 000/μL; Alb 4.2 g/dL; TB 0.89 mg/dL; AST 16 mU/mL; ALT 11 mU/mL; PT-INR 1.02; APTT 37.0 s; FVIII activity 91.4%; FIX activity 60.8%; von Willbrand factor >134.0%.

Pretransplant liver volumetry, as measured using Synapse Vincent (FUJIFILM Medical Co., Ltd., Tokyo, Japan), showed that the donor's whole liver volume was 1001 mL and that the left lateral segment volume was 180 mL. Therefore, the post-transplant predictive FIX activity of the donor was calculated as 49.9% (60.8% × 821/1001), assuming that the remnant liver would elaborate FIX.

The donor underwent left lateral segmentectomy for ABO-identical LDLT. The length of the operation was four h 47 min, and the volume of the bleeding was 590 mL. No transfusion was administered during the operation. The donor's post-transplant predictive FIX activity was calculated as 52.9% (60.8% × 854/1001) for the left lateral segment graft (147 g).

Freeze-dried human blood coagulation FIX concentrate (Novact M, Kaketsuken, Kumamoto, Japan) was administered before the dissection of the liver and 12 h afterwards by bolus infusion (40 U/kg) and was continued on POD 1 to obtain a steady-state plasma level above 60.0%. Early discontinuation of FIX concentrate was possible because of good remnant liver function and an absence of bleeding episodes. On POD 1, 3, 5, and 13, the donor's FIX activity was 121.3%, 130.6%, 114.6%, and 50.2%, respectively (Fig. 1). The post-transplant course was uneventful, and the donor was discharged from the hospital on POD 18.

She is currently doing well at one yr after LDLT, and her FIX activity was 39.2% at the most recent examination (Fig. 1).

Recipient

A 12-month-old female infant with biliary atresia underwent LDLT because of intractable cholangitis and portal hypertension. Her body height and weight were 75.0 cm and 8.3 kg, and the standard liver volume was 288 mL. The blood test results were as follows: Hg 11.0 g/dL; Hct 35.5%; Plt 182 000/μL; Alb 3.4 g/dL; TB 1.25 mg/dL; AST 191 mU/mL; ALT 96 mU/mL; PT-INR 0.97; APTT 31.9 s; FVIII activity 179.8%; FIX activity 45.0%; von Willbrand factor >201.0%. Because the donor's predicted left lateral segment volume was 180 mL, the recipient's post-transplant predictive FIX activity was calculated as 38.0% (60.8% × 180/288), assuming that the graft liver would elaborate FIX.

The recipient underwent ABO-identical LDLT using a left lateral segment graft. The length of the operation was nine h 15 min, and the bleeding volume was 537 mL. A total of 292 mL of red blood cells concentrate and 128 mL of FFP were infused during the LDLT. The recipient's post-transplant predicted FIX activity was calculated as 31.0% (60.8% × 147/288) for the left lateral segment graft (147 g).

Freeze-dried human blood coagulation FIX concentrate was administered by a bolus infusion (100 U/kg) only at the time of the anesthesia induction to obtain a steady-state plasma level above 60%. The early post-transplant course was favorable, with a good allograft function and an

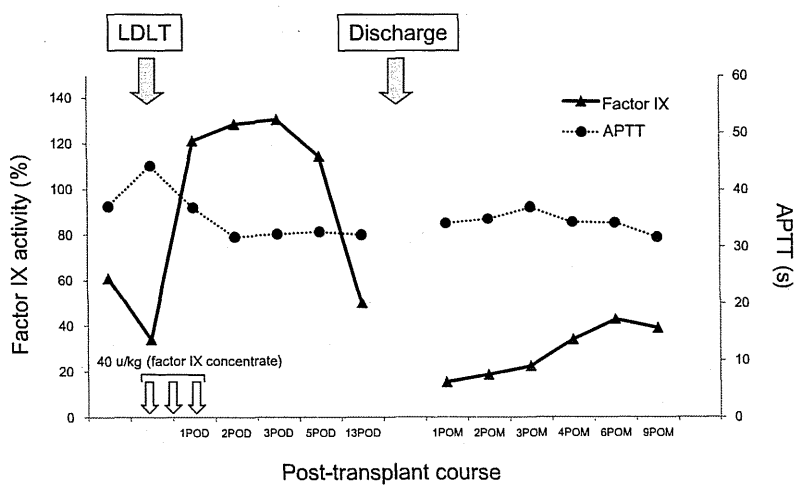

Fig. 1. The post-transplant course of a donor with mild coagulation FIX deficiency.

absence of bleeding episodes after single administration of FIX concentrate. On POD 1, 3, 5, 7, 13, 20, and 30, the recipient's FIX activity was 38.3%, 65.7%, 42.6%, 47.4%, 91.6%, 86.3%, and 59.6%, respectively (Fig. 2). The post-transplant course was uneventful, and the recipient was discharged from hospital on POD 34.

She is currently doing well at one yr after the LDLT, and her FIX activity was 58.0% at the most recent examination (Fig. 2).

Discussion

The anticipated problems associated with a hepatectomy for patients with hemophilia and coagulation factor deficiency include the peri-operative management of coagulopathy because the post-hepatectomy state exposes patients to greater risks of bleeding complications during the peri-operative period. Although the safety of factor replacement treatment during peri-operative surgical procedures has been reported (1), factor administration should be minimized as much as possible. Various regimens have been utilized, including bolus infusion and continuous infusion therapy, ranging from a few days to a few weeks to maintain a normal level of coagulation FIX activity (54–160%) (1–13). In this case, the bolus infusion of FIX concentrate might have been more appropriate and effective than a continuous infusion. Although normal levels of FIX activity were not achieved after the hepatectomy, we concluded that there was no further need for FIX replacement on POD 1 because there were no bleeding episodes. For donors with mild coagulation FIX deficiency, FIX concentrate should be administered at the time of anesthesia induction and at the end of the LDLT by bolus infusion (40 U/kg) and then continued for a few days after the LDLT by bolus infusion (40 U/kg), based on whether bleeding episodes occur during the monitoring of the plasma FIX level.

Regarding the suitability of a donor with coagulation FIX deficiency, we predicted the post-transplant FIX activity by pretransplant liver volumetry using Synapse Vincent. The pretransplant liver volumetry showed that the donor's whole liver volume was 1001 mL and the left lateral segment volume was 180 mL. The predicted post-transplant FIX activity of the donor and recipient was calculated as 49.9% (60.8% × 821/1001) and 38.0% (60.8% × 180/288), respectively. We considered the donor suitable because her predicted post-transplant FIX activity, as well as that of the recipient, would not fall within the category of moderate or severe coagulation factor deficiency (<5%). The post-transplant FIX activity could be predicted by pretransplant liver volumetry to some extent. We consider that pretransplant liver volumetry is important for an indication of donor suitability.

During the post-transplant course, the donor showed a gradual decrease in FIX activity until POM 1; thereafter, it increased gradually until POM 6 (Fig. 1). The recipient showed a gradual decrease in FIX activity until POM 2, which increased gradually until POM 3 (Fig. 2). The actual post-transplant FIX activity (43.0%) of the donor was lower than the predictive value (52.9%); it was 20% lower than the predictive FIX activity. The actual post-transplant FIX activity (61.6%) of the recipient was higher than the predicted value (31.0%) and >200% of the predictive FIX activity. The regeneration of the remnant liver and the graft liver might be associated with the recovery of FIX activity, and the

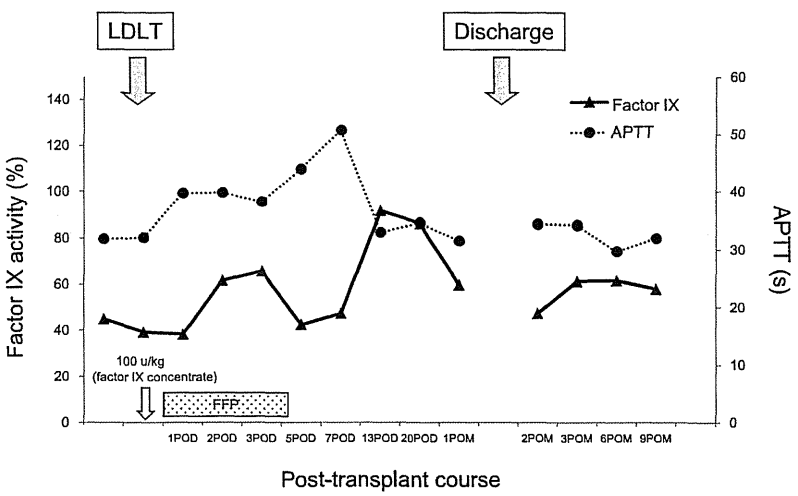

Fig. 2. The post-transplant course of the recipient transplanted with the graft with mild FIX deficiency.