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.

Acknowledgements

We would like to thank Tamaki Aoki, Naoko Ito and Masanori Ito (Jichi Medical University) for providing excellent technical assistance. This study was supported by Grants-in-Aid for Scientific Research (T.O., S.M., J.M.), the Funding Program for Next Generation World-Leading Researchers (S.N.), the Translational Systems Biology and Medicine Initiative from JST (S.N.), the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education and Science (Y.S.), and Health, Labour and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare (Y.S.).

Author details

[1]Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University School of Medicine, 3111-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. [2]Division of Cardiovascular Medicine, Department of Internal Medicine, Jichi Medical University School of Medicine, 3111-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. [3]Department of Cardiovascular Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan. [4]Translational Systems Biology and Medicine Initiative, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan. [5]Department of Morphological and Biomolecular Research, Nippon Medical School, 1-1-5 Sendagi, Bunkyo, Tokyo 113-8602, Japan.

Received: 26 November 2013 Accepted: 9 December 2013
Published: 2 January 2014

References

1. Coller BS: **Historical perspective and future directions in platelet research.** *J Thromb Haemost* 2011, **9**(Suppl 1):374–395.
2. Watson SP, Auger JM, McCarty OJ, Pearce AC: **GPVI and integrin alphaIIb beta3 signaling in platelets.** *J Thromb Haemost* 2005, **3**:1752–1762.
3. Varga-Szabo D, Pleines I, Nieswandt B: **Cell adhesion mechanisms in platelets.** *Arterioscler Thromb Vasc Biol* 2008, **28**:403–412.
4. Shattil SJ, Kim C, Ginsberg MH: **The final steps of integrin activation: the end game.** *Nat Rev Mol Cell Biol* 2010, **11**:288–300.
5. Siess W: **Molecular mechanisms of platelet activation.** *Physiol Rev* 1989, **69**:58–178.
6. Guidetti GF, Torti M: **The small GTPase rap1b: a bidirectional regulator of platelet adhesion receptors.** *J Signal Transduct* 2012, **2012**:412089.
7. Moser M, Legate KR, Zent R, Fassler R: **The tail of integrins, talin, and kindlins.** *Science* 2009, **324**:895–899.
8. Moser M, Nieswandt B, Ussar S, Pozgajova M, Fassler R: **Kindlin-3 is essential for integrin activation and platelet aggregation.** *Nat Med* 2008, **14**:325–330.
9. Nieswandt B, Moser M, Pleines I, Varga-Szabo D, Monkley S, Critchley D, Fassler R: **Loss of talin1 in platelets abrogates integrin activation, platelet aggregation, and thrombus formation in vitro and in vivo.** *J Exp Med* 2007, **204**:3113–3118.
10. Petrich BG, Fogelstrand P, Partridge AW, Yousefi N, Ablooglu AJ, Shattil SJ, Ginsberg MH: **The antithrombotic potential of selective blockade of talin-dependent integrin alpha IIb beta 3 (platelet GPIIb-IIIa) activation.** *J Clin Invest* 2007, **117**:2250–2259.
11. Cantor JM, Ginsberg MH, Rose DM: **Integrin-associated proteins as potential therapeutic targets.** *Immunol Rev* 2008, **223**:236–251.
12. Glenney JR Jr, Zokas L: **Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton.** *J Cell Biol* 1989, **108**:2401–2408.
13. Brown MC, Turner CE: **Paxillin: adapting to change.** *Physiol Rev* 2004, **84**:1315–1339.
14. Deakin NO, Turner CE: **Paxillin comes of age.** *J Cell Sci* 2008, **121**:2435–2444.
15. Hagmann J, Grob M, Welman A, Van Willigen G, Burger MM: **Recruitment of the LIM protein hic-5 to focal contacts of human platelets.** *J Cell Sci* 1998, **111**(Pt 15):2181–2188.
16. Osada M, Ohmori T, Yatomi Y, Satoh K, Hosogaya S, Ozaki Y: **Involvement of Hic-5 in platelet activation: integrin alphaIIbbeta3-dependent tyrosine phosphorylation and association with proline-rich tyrosine kinase 2.** *Biochem J* 2001, **355**:691–697.
17. Rathore VB, Okada M, Newman PJ, Newman DK: **Paxillin family members function as Csk-binding proteins that regulate Lyn activity in human and murine platelets.** *Biochem J* 2007, **403**:275–281.
18. Liu S, Slepak M, Ginsberg MH: **Binding of Paxillin to the alpha 9 integrin cytoplasmic domain inhibits cell spreading.** *J Biol Chem* 2001, **276**:37086–37092.
19. Liu S, Thomas SM, Woodside DG, Rose DM, Kiosses WB, Pfaff M, Ginsberg MH: **Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses.** *Nature* 1999, **402**:676–681.
20. Coller BS, Anderson K, Weisman HF: **New antiplatelet agents: platelet GPIIb/IIIa antagonists.** *Thromb Haemost* 1995, **74**:302–308.
21. Ohmori T, Kashiwakura Y, Ishiwata A, Madoiwa S, Mimuro J, Sakata Y: **Silencing of a targeted protein in in vivo platelets using a lentiviral vector delivering short hairpin RNA sequence.** *Arterioscler Thromb Vasc Biol* 2007, **27**:2266–2272.
22. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Ihrig MM, McManus MT, Gertler FB, *et al*: **A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference.** *Nat Genet* 2003, **33**:401–406.
23. Ohmori T, Kashiwakura Y, Ishiwata A, Madoiwa S, Mimuro J, Furukawa Y, Sakata Y: **Vinculin is indispensable for repopulation by hematopoietic stem cells, independent of integrin function.** *J Biol Chem* 2010, **285**:31763–31773.
24. Suzuki H, Okamura Y, Ikeda Y, Takeoka S, Handa M: **Ultrastructural analysis of thrombin-induced interaction between human platelets and liposomes carrying fibrinogen gamma-chain dodecapeptide as a synthetic platelet substitute.** *Thromb Res* 2011, **128**:552–559.
25. Bergmeier W, Schulte V, Brockhoff G, Bier U, Zirngibl H, Nieswandt B: **Flow cytometric detection of activated mouse integrin alphaIIbbeta3 with a novel monoclonal antibody.** *Cytometry* 2002, **48**:80–86.
26. Nishimura S, Manabe I, Nagasaki M, Kakuta S, Iwakura Y, Takayama N, Ooehara J, Otsu M, Kamiya A, Petrich BG, *et al*: **In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling.** *Blood* 2012, **119**:e45–e56.
27. Smolenski A: **Novel roles of cAMP/cGMP-dependent signaling in platelets.** *J Thromb Haemost* 2012, **10**:167–176.
28. Ming Z, Hu Y, Xiang J, Polewski P, Newman PJ, Newman DK: **Lyn and PECAM-1 function as interdependent inhibitors of platelet aggregation.** *Blood* 2011, **117**:3903–3906.
29. Wong C, Liu Y, Yip J, Chand R, Wee JL, Oates L, Nieswandt B, Reheman A, Ni H, Beauchemin N, Jackson DE: **CEACAM1 negatively regulates platelet-collagen interactions and thrombus growth in vitro and in vivo.** *Blood* 2009, **113**:1818–1828.
30. Signarvic RS, Cierniewska A, Stalker TJ, Fong KP, Chatterjee MS, Hess PR, Ma P, Diamond SL, Neubig RR, Brass LF: **RGS/Gi2alpha interactions modulate platelet accumulation and thrombus formation at sites of vascular injury.** *Blood* 2010, **116**:6092–6100.
31. Hagel M, George EL, Kim A, Tamimi R, Opitz SL, Turner CE, Imamoto A, Thomas SM: **The adaptor protein paxillin is essential for normal**

development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 2002, **22**:901–915.
32. Feral CC, Rose DM, Han J, Fox N, Silverman GJ, Kaushansky K, Ginsberg MH: **Blocking the alpha 4 integrin-paxillin interaction selectively impairs mononuclear leukocyte recruitment to an inflammatory site.** *J Clin Invest* 2006, **116**:715–723.
33. Dorsam RT, Kim S, Jin J, Kunapuli SP: **Coordinated signaling through both G12/13 and G (i) pathways is sufficient to activate GPIIb/IIIa in human platelets.** *J Biol Chem* 2002, **277**:47588–47595.
34. Nieswandt B, Schulte V, Zywietz A, Gratacap MP, Offermanns S: **Costimulation of Gi- and G12/G13-mediated signaling pathways induces integrin alpha IIbbeta 3 activation in platelets.** *J Biol Chem* 2002, **277**:39493–39498.
35. Gupta S, Braun A, Morowski M, Premsler T, Bender M, Nagy Z, Sickmann A, Hermanns HM, Bosl M, Nieswandt B: **CLP36 Is a Negative Regulator of Glycoprotein VI Signaling in Platelets.** *Circ Res* 2012, **111**:1410–1420.
36. Kim-Kaneyama JR, Miyauchi A, Lei XF, Arita S, Mino T, Takeda N, Kou K, Eto K, Yoshida T, Miyazaki T, *et al*: **Identification of Hic-5 as a novel regulatory factor for integrin alphaIIbbeta3 activation and platelet aggregation in mice.** *J Thromb Haemost* 2012, **10**:1867–1874.
37. Chew V, Lam KP: **Leupaxin negatively regulates B cell receptor signaling.** *J Biol Chem* 2007, **282**:27181–27191.

doi:10.1186/1477-9560-12-1
Cite this article as: Sakata *et al.*: Paxillin is an intrinsic negative regulator of platelet activation in mice. *Thrombosis Journal* 2014 **12**:1.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

Koyama et al. Critical Care 2014, 18:R13
http://ccforum.com/content/18/1/R13

CRITICAL CARE

RESEARCH

Open Access

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

Kansuke Koyama[1,2*], Seiji Madoiwa[1,2*], Shin Nunomiya[1], Toshitaka Koinuma[1], Masahiko Wada[1], Asuka Sakata[2], Tsukasa Ohmori[2], Jun Mimuro[2] and Yoichi Sakata[2]

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).

(Continued on next page)

* Correspondence: k_koyama@jichi.ac.jp; madochan@ms2.jichi.ac.jp
[1]Division of Intensive Care, Department of Anesthesiology & Intensive Care Medicine, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
[2]Research Divisions of Cell and Molecular Medicine, Center of Molecular Medicine, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan

© 2014 Koyama et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

(Continued from previous page)

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 (× 10³/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

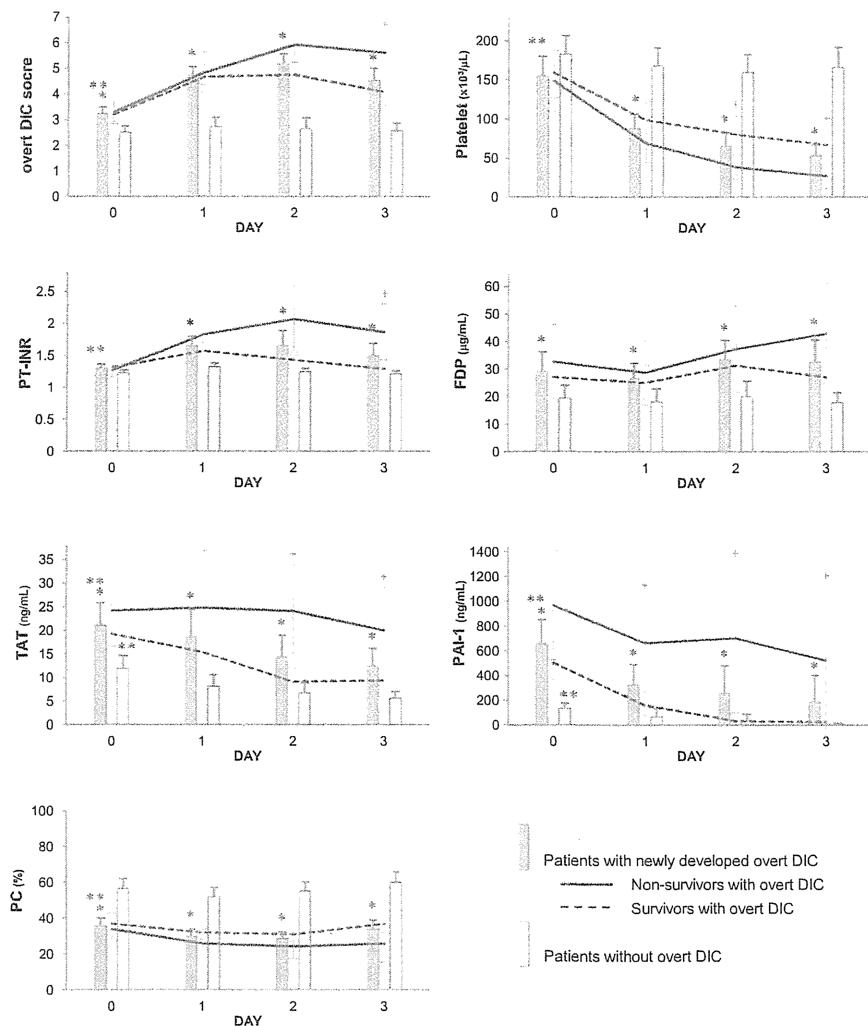


Figure 1 Time course of overt DIC scores and hemostatic biomarkers from baseline to Day 3. Overt disseminated intravascular coagulation (DIC) scores, platelet count, prothrombin time-international normalized ratio (PT-INR), fibrin degradation product (FDP), thrombin-antithrombin complex (TAT), plasminogen 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 ($\times 10^3/\mu L$)	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.

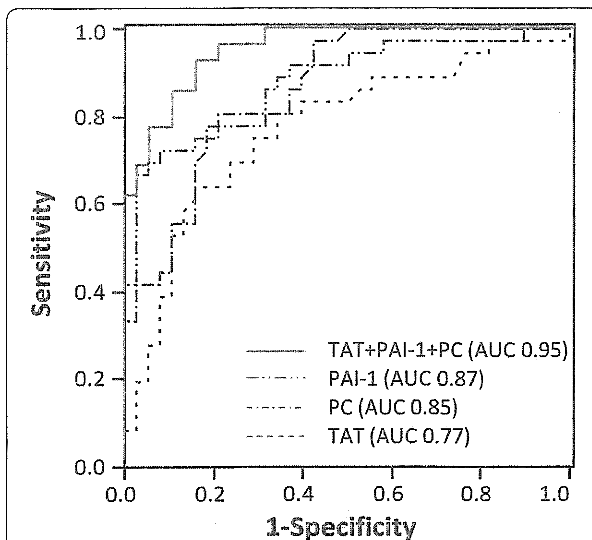


Figure 2 ROC curves of TAT, PAI-1 and PC activity for prediction of overt DIC. Area under the receiver operating characteristic curve (AUROC) for thrombin-antithrombin complex (TAT), 0.77 (95% CI, 0.64 to 0.86), plasminogen activator inhibitor-1 (PAI-1), 0.87 (0.78 to 0.92), protein C (PC), 0.85 (0.76 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 (× 10^3/μL)	0.41	0.79	0.32	0.84
	2	0.81 (0.64 to 0.91)	66 (× 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.

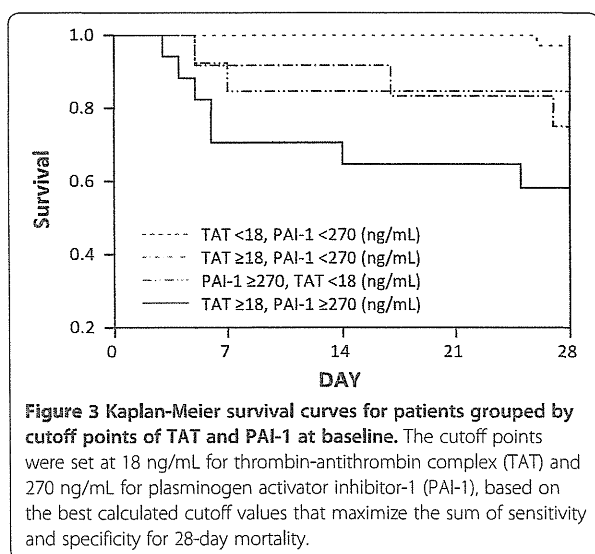


Figure 3 Kaplan-Meier survival curves for patients grouped by cutoff points of TAT and PAI-1 at baseline. The cutoff points were set at 18 ng/mL for thrombin-antithrombin complex (TAT) and 270 ng/mL for plasminogen activator inhibitor-1 (PAI-1), based on the best calculated cutoff values that maximize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

Acknowledgements

The authors thank the research staff of the Divisions of Cell and Molecular Medicine and the nursing staff of the Intensive Care Unit at Jichi Medical University Hospital for their assistance.

Received: 30 September 2013 Accepted: 6 January 2014
Published: 13 January 2014

References

1. Kinasewitz GT, Yan SB, Basson B, Comp P, Russell JA, Cariou A, Um SL, Utterback B, Laterre PF, Dhainaut JF, PROWESS Sepsis Study Group: **Universal changes in biomarkers of coagulation and inflammation occur in patients with severe sepsis, regardless of causative micro-organism [ISRCTN74215569].** *Crit Care* 2004, **8:**R82–R90.
2. Levi M, Ten Cate H: **Disseminated intravascular coagulation.** *N Engl J Med* 1999, **341:**586–592.
3. Zeerleder S, Hack CE, Wuillemin WA: **Disseminated intravascular coagulation in sepsis.** *Chest* 2005, **128:**2864–2875.
4. Taylor FB Jr, Toh CH, Hoots WK, Wada H, Levi M, Scientific Subcommittee on Disseminated Intravascular Coagulation (DIC) of the International Society on Thrombosis and Haemostasis (ISTH): **Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation.** *Thromb Haemost* 2001, **86:**1327–1330.
5. Dhainaut JF, Yan SB, Joyce DE, Pettila V, Basson B, Brandt JT, Sundin DP, Levi M: **Treatment effects of drotrecogin alfa (activated) in patients with severe sepsis with or without overt disseminated intravascular coagulation.** *J Thromb Haemost* 2004, **2:**1924–1933.
6. Oh D, Jang MJ, Lee SJ, Chong SY, Kang MS, Wada H: **Evaluation of modified non-overt DIC criteria on the prediction of poor outcome in patients with sepsis.** *Thromb Res* 2010, **126:**18–23.
7. Angstwurm MW, Dempfle CE, Spannagl M: **New disseminated intravascular coagulation score: a useful tool to predict mortality in comparison with Acute Physiology and Chronic Health Evaluation II and Logistic Organ Dysfunction scores.** *Crit Care Med* 2006, **34:**314–320. quiz 328.
8. Voves C, Wuillemin WA, Zeerleder S: **International Society on Thrombosis and Haemostasis score for overt disseminated intravascular coagulation predicts organ dysfunction and fatality in sepsis patients.** *Blood Coagul Fibrinolysis* 2006, **17:**445–451.
9. Ten Cate H: **Trombocytopenia: one of the markers of disseminated intravascular coagulation.** *Pathophysiol Haemost Thromb* 2003, **33:**413–416.
10. Song J, Hu D, He C, Wang T, Liu X, Ma L, Lin Z, Chen Z: **Novel biomarkers for early prediction of sepsis-induced disseminated intravascular coagulation in a mouse cecal ligation and puncture model.** *J Inflamm (Lond)* 2013, **10:**7.
11. Toh CH, Downey C: **Performance and prognostic importance of a new clinical and laboratory scoring system for identifying non-overt disseminated intravascular coagulation.** *Blood Coagul Fibrinolysis* 2005, **16:**69–74.
12. Levi M, van der Poll T: **Inflammation and coagulation.** *Crit Care Med* 2010, **38:**S26–S34.
13. O'Brien M: **The reciprocal relationship between inflammation and coagulation.** *Top Companion Anim Med* 2012, **27:**46–52.
14. Koyama K, Madoiwa S, Tanaka S, Koinuma T, Wada M, Sakata A, Ohmori T, Mimuro J, Nunomiya S, Sakata Y: **Evaluation of hemostatic biomarker abnormalities that precede platelet count decline in critically ill patients with sepsis.** *J Crit Care* 2013, **28:**556–563.
15. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G, International Sepsis Definitions Conference: **SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.** *Intensive Care Med* 2001, **2003(29):**530–538.
16. Knaus WA, Draper EA, Wagner DP, Zimmerman JE: **APACHE II: a severity of disease classification system.** *Crit Care Med* 1985, **13:**818–829.
17. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonca A, Bruining H, Reinhart CK, Suter PM, Thijs LG: **The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine.** *Intensive Care Med* 1996, **22:**707–710.
18. Gando S, Iba T, Eguchi Y, Ohtomo Y, Okamoto K, Koseki K, Mayumi T, Murata A, Ikeda T, Ishikura H, Ueyama M, Ogura H, Kushimoto S, Saitoh D, Endo S, Shimazaki S, Japanese Association for Acute Medicine Disseminated Intravascular Coagulation (JAAM DIC) Study Group: **A multicenter, prospective validation of disseminated intravascular coagulation diagnostic criteria for critically ill patients: comparing current criteria.** *Crit Care Med* 2006, **34:**625–631.
19. Dellinger RP, Levy MM, Carlet JM, Bion J, Parker MM, Jaeschke R, Reinhart K, Angus DC, Brun-Buisson C, Beale R, Calandra T, Dhainaut JF, Gerlach H, Harvey M, Marini JJ, Marshall J, Ranieri M, Ramsay G, Sevransky J, Thompson BT, Townsend S, Vender JS, Zimmerman JL, Vincent JL: **Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008.** *Intensive Care Med* 2008, **34:**17–60. Erratum in: *Intensive Care Med* 2008, **34:**783–785.
20. Amaral A, Opal SM, Vincent JL: **Coagulation in sepsis.** *Intensive Care Med* 2004, **30:**1032–1040.
21. Faust SN, Levin M, Harrison OB, Goldin RD, Lockhart MS, Kondaveeti S, Laszik Z, Esmon CT, Heyderman RS: **Dysfunction of endothelial protein C activation in severe meningococcal sepsis.** *N Engl J Med* 2001, **345:**408–416.
22. Levi M, van der Poll T: **The role of natural anticoagulants in the pathogenesis and management of systemic activation of coagulation and inflammation in critically ill patients.** *Semin Thromb Hemost* 2008, **34:**459–468.
23. Bergmann S, Hammerschmidt S: **Fibrinolysis and host response in bacterial infections.** *Thromb Haemost* 2007, **98:**512–520.
24. Madoiwa S, Nunomiya S, Ono T, Shintani Y, Ohmori T, Mimuro J, Sakata Y: **Plasminogen activator inhibitor 1 promotes a poor prognosis in sepsis-induced disseminated intravascular coagulation.** *Int J Hematol* 2006, **84:**398–405.
25. Lorente JA, Garcia-Frade LJ, Landin L, de Pablo R, Torrado C, Renes E, Garcia-Avello A: **Time course of hemostatic abnormalities in sepsis and its relation to outcome.** *Chest* 1993, **103:**1536–1542.
26. Saetre T, Lindgaard AK, Lyberg T: **Systemic activation of coagulation and fibrynolysis in a porcine model of serogroup A streptococcal shock.** *Blood Coagul Fibrinolysis* 2000, **11:**433–438.
27. Kinasewitz GT, Zein JG, Lee GL, Nazir SA, Taylor FB Jr: **Prognostic value of a simple evolving disseminated intravascular coagulation score in patients with severe sepsis.** *Crit Care Med* 2005, **33:**2214–2221.
28. Dhainaut JF, Shorr AF, Macias WL, Kollef MJ, Levi M, Reinhart K, Nelson DR: **Dynamic evolution of coagulopathy in the first day of severe sepsis: relationship with mortality and organ failure.** *Crit Care Med* 2005, **33:**341–348.
29. Iba T, Gando S, Murata A, Kushimoto S, Saitoh D, Eguchi Y, Ohtomo Y, Okamoto K, Koseki K, Mayumi T, Ikeda T, Ishhikura H, Ueyama M, Ogura Y, Endo S, Shimazaki S, Japanese Association for Acute Medicine Disseminated Intravascular Coagulation Study Group: **Predicting the severity of systemic inflammatory response syndrome (SIRS)-associated coagulopathy with hemostatic molecular markers and vascular endothelial injury markers.** *J Trauma* 2007, **63:**1093–1098.

30. Iba T, Kidokoro A, Fukunaga M, Sugiyama K, Sawada T, Kato H: Association between the severity of sepsis and the changes in hemostatic molecular markers and vascular endothelial damage markers. *Shock* 2005, 23:25–29.
31. Egi M, Morimatsu H, Wiedermann CJ, Tani M, Kanazawa T, Suzuki S, Matsusaki T, Shimizu K, Toda Y, Iwasaki T, Morita K: Non-overt disseminated intravascular coagulation scoring for critically ill patients: the impact of antithrombin levels. *Thromb Haemost* 2009, 101:696–705.
32. Kienast J, Juers M, Wiedermann CJ, Hoffmann JN, Ostermann H, Strauss R, Keinecke HO, Warren BL, Opal SM, KyberSept Investigators: Treatment effects of high-dose antithrombin without concomitant heparin in patients with severe sepsis with or without disseminated intravascular coagulation. *J Thromb Haemost* 2006, 4:90–97.
33. Mavrommatis AC, Theodoridis T, Economou M, Kotanidou A, El Ali M, Christopoulou-Kokkinou V, Zakynthinos SG: Activation of the fibrinolytic system and utilization of the coagulation inhibitors in sepsis: comparison with severe sepsis and septic shock. *Intensive Care Med* 2001, 27:1853–1859.

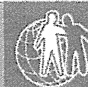
doi:10.1186/cc13190
Cite this article as: Koyama *et al.*: Combination of thrombin-antithrombin complex, plasminogen activator inhibitor-1, and protein C activity for early identification of severe coagulopathy in initial phase of sepsis: a prospective observational study. *Critical Care* 2014 18:R13.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

Haemophilia The Official Journal of the World Federation of Hemophilia, European Association for Haemophilia and Allied Disorders and the Hemostasis & Thrombosis Research Society



Haemophilia (2014), 20, e40–e44 DOI: 10.1111/hae.12311

ORIGINAL ARTICLE *Laboratory science*

Production of functional coagulation factor VIII from iPSCs using a lentiviral vector

Y. KASHIWAKURA,*† T. OHMORI,* J. MIMURO,* S. MADOIWA,* M. INOUE,‡ M. HASEGAWA,‡ K. OZAWA§ and Y. SAKATA*
**Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan; †Department of Immunology, Dokkyo Medical University School of Medicine, Tochigi, Japan; ‡DNAVEC Corporation, Ibaraki, Japan; and §Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan*

Summary. The use of induced pluripotent stem cells (iPSCs) as an autologous cell source has shed new light on cell replacement therapy with respect to the treatment of numerous hereditary disorders. We focused on the use of iPSCs for cell-based therapy of haemophilia. We generated iPSCs from mesenchymal stem cells that had been isolated from C57BL/6 mice. The mouse iPSCs were generated through the induction of four transcription factor genes Oct3/4, Klf-4, Sox-2 and c-Myc. The derived iPSCs released functional coagulation factor VIII (FVIII) following transduction with a simian immunodeficiency virus vector. The subcutaneous transplantation of iPSCs expressing FVIII into nude mice resulted in teratoma formation, and significantly increased plasma levels of FVIII. The plasma concentration of FVIII was at levels appropriate for human therapy at 2–4 weeks post transplantation. Our data suggest that iPSCs could be an attractive and prospective autologous cell source for the production of coagulation factor, and that engineered iPSCs expressing coagulation factor might provide a cell-based therapeutic strategy appropriate for haemophilia.

Keywords: haemophilia, induced pluripotent stem cells, lentiviral vector, cell therapy, gene therapy, blood coagulation

Introduction

Haemophilia is an X-linked inherited bleeding disorder, caused by mutations within the *coagulation factor VIII* (*F8*) or *coagulation factor IX* (*F9*) genes. This results in a longer than average time for blood to clot, which can lead to significant bleeding. Haemophilia is considered suitable for gene therapy, as it is caused by a single gene abnormality, and therapeutic coagulation factor levels can vary across a broad range [1]. Recently, therapeutic levels of coagulation factor have been achieved in haemophilia B patients through the direct administration of adeno-associated virus vectors in clinical trials [2]. Another gene therapy strategy for haemophilia is the application of cells transduced *ex vivo*, as a delivery vehicle for coagulation factor [3]. Cell-based therapy reduces the risk of unwanted virus dissemination, and ensures the selection of highly expressing clones prior to commencement of the procedure. However, cell-based therapies for haemophilia have been hampered in animals and during human clinical trials, because of the short life span of transplanted cells, difficulties in obtaining therapeutic plasma levels, and elimination of the transduced cells by immune reactions [4].

Induced pluripotent stem cells (iPSCs) are artificially generated stem cells, made by reprogramming somatic cells through the expression of defined transcription factors [5,6]. These iPSCs are pluripotent, with the ability to differentiate into cells of the three germ lineages *in vitro*. Mouse-derived iPSCs can be passaged through the germ line, as is the case with embryonic stem cells (ESCs). Using iPSCs has a distinct advantage over ESCs, as cells differentiated from iPSCs exhibit limited immunogenicity, and are therefore more easily tolerated by a recipient following transplantation. Accordingly, iPSC technology offers the possibility of patient-specific cell therapy for haemophilia, in which the use of genetically identical cells would prevent immune rejection. In this study, we examined whether iPSCs could release

Correspondence: Tsukasa Ohmori, MD, PhD, Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, 3111-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan.
Tel: +81-285-58-7397; fax: +81-285-44-7817;
e-mail: tohmori@jichi.ac.jp

Accepted after revision 7 October 2013

 © 2013 John Wiley & Sons Ltd

functional coagulation factor using lentiviral transduction. We then focused on the possible clinical application of the engineered iPSCs to cell-based gene therapy for haemophilia.

Materials and methods

Mice

C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). BALB/cAJcl-*nu/nu* mice (nude mice) were obtained from CLEA Japan, Inc. (Shizuoka, Japan). All animal procedures were approved by the Institutional Animal Care and Concern Committee at Jichi Medical University, and animal care was in accordance with the committee's guidelines.

Generation of iPSCs and cell culture

Murine mesenchymal stem cells (MSCs) were isolated and maintained as described previously [7]. We selected MSCs for the establishment of iPSCs because MSCs have a higher reprogramming efficiency compared with fibroblasts [8,9]. Two plasmid vectors to generate iPSCs (pCX-OKS-2A and pCX-cMyc) were obtained from Addgene (Cambridge, MA). MSCs were transduced with the plasmid vectors by nucleofection as described previously [10]. Colonies that were ES-like were cloned at 30 days post transduction, and MSC-derived iPSCs were maintained on mouse embryonic fibroblasts with knockout Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 15% foetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.5 mM monothioglycerol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1000 U mL^{-1} ESGRO (Merck Millipore, Billerica, MA).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared using an RNeasy Mini Kit (QIAGEN, Montgomery, MD). The RT-PCR assays were conducted using a SuperScript One-Step RT-PCR System (Invitrogen). Primer pairs for the RT-PCR assays used in this study have been previously reported [5].

Lentiviral vector construct and production

The cDNA for human B-domain-deleted FVIII (hBDD-FVIII) was generated as previously described [11]. The *hBDD-FVIII* gene, under the control of a chicken β-actin promoter coupled with cytomegalovirus promoter early enhancer element (CAGp), was cloned into a self-inactivating simian immunodeficiency virus (SIV) lentiviral vector [12]. The SIV lentiviral vectors were generated as previously described [13].

Measurement of FVIII activity and antigen

The hFVIII antigens (FVIII:Ag) were measured using an anti-hFVIII-specific enzyme-linked immunosorbent assay (ELISA) kit (ASSERACHROM VIII:Ag; Diagnostica Stago, Seine, France). The functional activity of FVIII (FVIII:C) was measured using a one-stage clotting time assay on an automated coagulation analyser (Sysmex CA-500 analyser; Sysmex Corp., Kobe, Japan). We used pooled normal human plasma as a reference to measure both FVIII:C and FVIII:Ag.

Results and discussion

We first attempted to establish iPSCs from C57BL/6 mice. Bone marrow-derived MSCs were transduced with plasmid vector expressing the defined transcription factors. We cloned ESC-like cell colonies after transduction, and SSEA-1 positive cells were sorted by flow cytometry (Fig. 1a). The sorted cell colonies exhibited typical ESC morphology and alkaline phosphatase activity (Fig. 1b). The mRNA expression patterns of endogenous pluripotent-specific genes (*Sox2, Oct3/4, Nanog* and *c-Myc*) in the cells were similar to those in E14tg2a mouse ESCs (Fig. 1c). Furthermore, subcutaneous transplantation of these cells (1×10^6 cells) into nude mice resulted in the formation of teratomas containing tissues derived from the ectoderm, mesoderm, and endoderm (Fig. 1d and e). This would suggest that the cells possess the potential to differentiate into cells and tissues of the three germ layers. Therefore, we used these iPSCs for further experiments in this study.

We next examined whether iPSCs could produce functional coagulation factor after transduction by a lentiviral vector. The iPSCs were transduced with the SIV vector expressing hBDD-FVIII under the control of CAGp (SIV-CAG-hFVIII) (Fig. 2a). We cloned iPSC colonies from the cells transduced with SIV-CAG-hFVIII at a multiplicity of infection (MOI) of 30, and selected three iPSC clones that stably produced hFVIII in the supernatant (Fig. 2b). The iPSC clones expressing hFVIII were subcutaneously transplanted into nude mice. Following transplantation, plasma levels of hFVIII:Ag in nude mice gradually increased according to teratoma formation derived from the transplanted iPSCs (Fig. 2c and d). Although the plasma level of hFVIII:Ag in the nude mice was at 20%, we could not measure hFVIII:C in nude mice because of the existence of mouse FVIII. We simultaneously measured hFVIII:C and hFVIII:Ag produced from transduced

© 2013 John Wiley & Sons Ltd

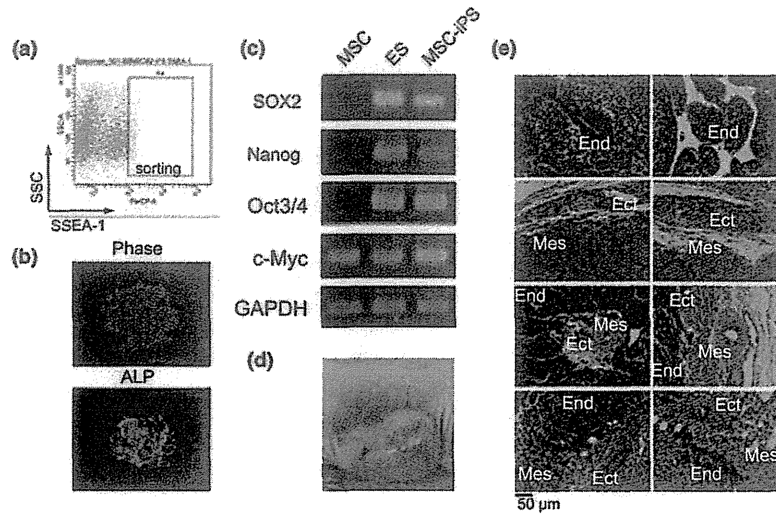


Fig. 1. Characterization of induced pluripotent stem cells (iPSCs) derived from mesenchymal stem cells (MSCs). MSCs isolated from C57BL/6 mice were transduced with plasmid vectors expressing Oc3/4, Klf4, Sox2 and c-Myc. (a) Stage-specific embryonic antigen 1 (SSEA-1) expression was examined by flow cytometry following transduction. SSEA-1-positive cells (shown in the square) were sorted as MSC-derived iPSCs. (b) Morphology of iPSC colonies derived from SSEA-1-positive cells. Alkaline phosphatase activity in iPSC colonies was detected using HNP/Fast Red TR. (c) The mRNA expression levels of the pluripotency markers SOX2, Nanog, Oct3/4 and c-Myc were determined by reverse transcription polymerase chain reaction. (d) Teratoma formation in nude mice after subcutaneous transplantation of iPSCs. (e) Differentiation into cells and tissues of the three germ lineages were confirmed by histological analysis. End, endoderm; Ect, ectoderm; Mes, mesoderm.

iPSCs, and calculated the ratio of hFVIII:C to hFVIII:Ag (0.30 ± 0.041). Accordingly, hFVIII:C levels seemed to reach the therapeutic level of FVIII required for haemophilia A (6%) in transplanted mice.

Recent studies have reported the potential of iPSCs for the treatment of many human diseases; iPSCs possess the ability to differentiate into cardiovascular [14–16], haematopoietic [16,17], neural [18], and hepatic [19,20] progenitor cells. Treatment of inherited disorders using iPSCs has been proposed for animal models of sickle cell anaemia [17]. The haemophilic mouse model could also be used to examine the potential of iPSC therapy. Xu *et al.* reported that transplantation of endothelial progenitor cells derived from iPSCs into the liver increased FVIII levels, resulting in a corrected bleeding phenotype for haemophilia A mice [21,22]. The results reported by Xu *et al.* were surprising because the differentiated endothelial progenitor cells that were transplanted only contained one copy of *F8*, yet they were able to increase plasma levels of FVIII. In contrast, lentiviral transduction using our procedure resulted in 5–10 proviral integration sites in the diploid genome at an MOI of 30, suggesting that the same procedure using engineered iPSCs expressing coagulation factor would result in a more potent therapeutic effect.

In our preliminary experiments, we used nude mice to verify the following: the net production of FVIII from iPSCs by excluding the role of the immune system; and pluripotency of iPSCs by teratoma formation. However, tumourigenesis by iPSCs, should be

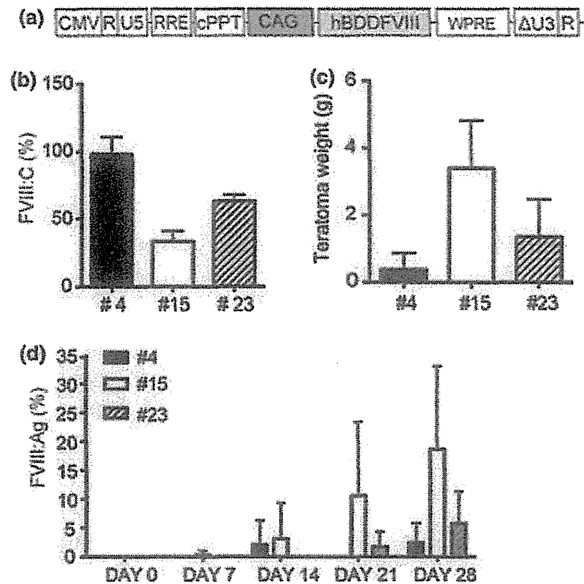


Fig. 2. Increase in FVIII levels after transduction with the simian immunodeficiency virus (SIV) lentiviral vector. The induced pluripotent stem cells (iPSCs) were transduced with SIV vector expressing human B-domain deleted coagulation factor VIII (hBDD-FVIII) under the control of a CAG promoter (SIV-CAG-hFVIII) at an MOI of 30. (a) Schematic of the SIV vectors used in our experiments. (B) FVIII activities in the supernatants derived from cloned iPSCs (#4, #15, and #23) transduced with SIV-CAG-hFVIII were measured using a one-stage clotting time assay. Values are presented as means ±SD (n = 4 for each experiment). (c, d) iPSC clones expressing hFVIII were subcutaneously transplanted into nude mice. (c) Teratoma weight at 4 weeks after subcutaneous transplantation of cloned MSC-iPSCs transduced with SIV-CAG-hFVIII. Values are presented as means ±SD (n = 3–5). (d) Plasma FVIII antigen levels in nude mice at the indicated times after subcutaneous transplantation of the iPSC clones. Values are means ±SD (n = 3–5).

 © 2013 John Wiley & Sons Ltd

completely avoided in their application. To reduce tumourigenicity and to improve the safety of iPSCs, the use of non-integrative vectors and changes of defined factors has been widely examined [6,23–25]. We also should differentiate iPSCs into the appropriate cells before transplantation, and plan to establish a more realistic cell therapy approach using immunocompetent FVIII-deficient mice.

One strategy to increase the safety of iPSCs for cell therapy would be to administer anucleated cells, such as red blood cells and platelets, differentiated from iPSCs. Integration of transgenes into genomic DNA during iPSC induction and lentiviral transduction might be negligible in the transplantation of anucleate cells. We have previously reported, along with other researchers, that expression of coagulation factor in red blood cells [26] and platelets [13,27] using lentiviral vectors has corrected the phenotype of mouse models of haemophilia. The use of blood cells to deliver coagulation factor is attractive as it avoids interference from circulating inhibitors. Recent reports have suggested the production of functional platelets from human iPSCs [28]; the transfusion of these blood cells expressing coagulation factor produced from iPSCs *in vitro* is possibly the most efficient and effective method for treating haemophiliacs.

In conclusion, we have proposed a new cell-based treatment for haemophilia involving iPSCs. Our proposed approach appears to be feasible, as transplantation of iPSCs resulted in increased and therapeutically appropriate FVIII plasma levels. Further investigations are needed to explore the risks of tumourigenicity from iPSC-derived cells, and to efficiently increase plasma levels of coagulation factor following cell therapy.

Acknowledgements

Y. Kashiwakura and T. Ohmori designed and performed the experiments, analysed the data and wrote the manuscript; M. Inoue and M. Hasegawa provided vital reagents and critically reviewed the manuscript; and J. Mimuro, S. Madoiwa, K. Ozawa and Y. Sakata analysed the data and revised the manuscript. We thank Naoko Ito and Masanori Ito (Jichi Medical University) for their technical assistance. This study was supported by a Grant from the Japan Baxter Hemophilia Scientific Research & Education Fund; Grants-in-Aid for Scientific Research (23591427, 21591249 and 23591426); Special Project Award from Bayer Hemophilia Award Program 2011; the Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education and Science, and Health, Labour and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare.

Disclosures

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

References

1 Mannucci PM, Tuddenham EG. The hemophilias–from royal genes to gene therapy. *N Engl J Med* 2001; 344: 1773–9.
2 Nathwani AC, Tuddenham EG, Rangarajan S *et al.* Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* 2011; 365: 2357–65.
3 Lillicrap D, VandenDriessche T, High K. Cellular and genetic therapies for haemophilia. *Haemophilia* 2006; 12(Suppl 3): 36–41.
4 Roth DA, Tawa NE Jr., O'Brien JM, Treco DA, Selden RF. Nonviral transfer of the gene encoding coagulation factor VIII in patients with severe hemophilia A. *N Engl J Med* 2001; 344: 1735–42.
5 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126: 663–76.
6 Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007; 448: 313–7.
7 Kashiwakura Y, Ohmori T, Mimuro J *et al.* Intra-articular injection of mesenchymal stem cells expressing coagulation factor ameliorates hemophilic arthropathy in factor VIII-deficient mice. *J Thromb Haemost* 2012; 10: 1802–13.
8 Niibe K, Kawamura Y, Araki D *et al.* Purified mesenchymal stem cells are an efficient source for iPS cell induction. *PLoS ONE* 2011; 6: e17610.
9 Oda Y, Yoshimura Y, Ohnishi H *et al.* Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. *J Biol Chem* 2010; 285: 29270–8.
10 Gonzalez F, Barragan Monasterio M, Tiscornia G *et al.* Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. *Proc Natl Acad Sci USA* 2009; 106: 8918–22.
11 Ogata K, Mimuro J, Kikuchi J *et al.* Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for hemophilia A gene therapy. *Gene Ther* 2004; 11: 253–9.
12 Nakajima T, Nakamaru K, Ido E, Terao K, Hayami M, Hasegawa M. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther* 2000; 11: 1863–74.
13 Ohmori T, Mimuro J, Takano K *et al.* Efficient expression of a transgene in platelets using simian immunodeficiency virus-based vector harboring glycoprotein Ibalpha promoter: in vivo model for platelet-targeting gene therapy. *FASEB J* 2006; 20: 1522–4.
14 Kuzmenkin A, Liang H, Xu G *et al.* Functional characterization of cardiomyocytes derived from murine induced pluripotent stem cells in vitro. *FASEB J* 2009; 23: 4168–80.
15 Narazaki G, Uosaki H, Teranishi M *et al.* Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation* 2008; 118: 498–506.
16 Schenke-Layland K, Rhodes KE, Angelis E *et al.* Reprogrammed mouse fibroblasts differentiate into cells of the cardiovascular and hematopoietic lineages. *Stem Cells* 2008; 26: 1537–46.
17 Hanna J, Wernig M, Markoulaki S *et al.* Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science* 2007; 318: 1920–3.
18 Wernig M, Zhao JP, Pruszak J *et al.* Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci USA* 2008; 105: 5856–61.
19 Cantz T, Bleidissel M, Stehling M, Scholer HR. In vitro differentiation of reprogrammed murine somatic cells into hepatic precursor cells. *Biol Chem* 2008; 389: 889–96.
20 Kasuda S, Tatsumi K, Sakurai Y *et al.* Expression of coagulation factors from murine induced pluripotent stem cell-derived liver cells. *Blood Coagul Fibrinolysis* 2011; 22: 271–9.
21 Xu D, Alipio Z, Fink LM *et al.* Phenotypic correction of murine hemophilia A using an iPS cell-based therapy. *Proc Natl Acad Sci USA* 2009; 106: 808–13.
22 Alipio Z, Adcock DM, Waner M *et al.* Sustained factor VIII production in hemophiliac mice 1 year after engraftment with induced pluripotent stem cell-derived factor VIII producing endothelial cells. *Blood Coagul Fibrinol* 2010; 21: 502–4.
23 Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009; 458: 771–5.

© 2013 John Wiley & Sons Ltd

24 Zhou H, Wu S, Joo JY *et al.* Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009; 4: 381–4.
25 Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* 2009; 85: 348–62.
26 Chang AH, Stephan MT, Sadelain M. Stem cell-derived erythroid cells mediate long-term systemic protein delivery. *Nat Biotechnol* 2006; 24: 1017–21.
27 Shi Q, Wilcox DA, Fahs SA *et al.* Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. *J Thromb Haemost* 2007; 5: 352–61.
28 Takayama N, Nishimura S, Nakamura S *et al.* Transient activation of c-MYC expression is critical for efficient platelet generation from human induced pluripotent stem cells. *J Exp Med* 2010; 207: 2817–30.

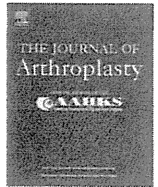
 © 2013 John Wiley & Sons Ltd

The Journal of Arthroplasty 29 (2014) 569–573

Contents lists available at ScienceDirect

The Journal of Arthroplasty

journal homepage: www.arthroplastyjournal.org



Changes in Blood Coagulation–Fibrinolysis Markers By Pneumatic Tourniquet During Total Knee Joint Arthroplasty With Venous Thromboembolism

Hideaki Watanabe, MD [a], Ichiro Kikkawa, MD [a], Seiji Madoiwa, MD [b], Hitoshi Sekiya, MD [a], Shinya Hayasaka, MD [c], Yoichi Sakata, MD [b]

[a] Department of Orthopedic Surgery, Jichi Medical University, Tochigi, Japan
[b] Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan
[c] Department of Health Science, Daito Bunka University, Higashimatsuyama, Japan

ARTICLE INFO

Article history:
Received 19 June 2013
Accepted 10 August 2013

Keywords:
cross-linked fibrin degradation products by leukocyte elastase (e-XDP)
D-dimer
soluble fibrin monomer complex
plasminogen activator inhibitor type 1
total knee arthroplasty
venous thromboembolism

ABSTRACT

This study investigated changes in blood coagulation–fibrinolysis markers during total knee arthroplasty (TKA). Preoperative 16-row multidetector row computed tomography (MDCT) revealed no asymptomatic venous thromboembolism (VTE) in the 42 patients recruited. Using MDCT postoperatively, patients were divided into thrombus (asymptomatic VTE, 19 patients) and no-thrombus (23 patients) groups. Blood taken at intervals before and after pneumatic tourniquet release revealed increased plasminogen activator inhibitor type-1 (PAI-1) at 30 s for both groups and at 90 s (both $P = 0.01$) in the thrombus group. D-dimer levels were highest at 30 and 90 s for both groups ($P = 0.01$). PAI-1 and D-dimer levels were strongly correlated at both time points in the thrombus group. Inactivating fibrinolysis due to PAI-1 may lead to asymptomatic VTE after TKA.

© 2014 Elsevier Inc. All rights reserved.

In orthopedic surgery, it is extremely important to prevent the development of postoperative venous thromboembolism (VTE), particularly symptomatic, fatal pulmonary embolism (PE), after total knee arthroplasty (TKA) [1]. Antithrombotic therapies using agents such as unfractionated or low-molecular-weight heparin have been administered to patients after surgery. Despite the implementation of aggressive antithrombotic protocols, however, the incidence of fatal PE remains at 0.15% [2] and that of symptomatic PE remains at 0.41% [3], with no changes in mortality rates since the 1990s [4]. Furthermore, in a cohort in Korea, the presence of asymptomatic VTE was 35.7% after TKA, as determined using multidetector row computed tomography (MDCT) [5]. Although it is thought that prophylactic antithrombotic treatments are necessary to prevent postoperative fatal and symptomatic PE, previous reports have found no difference in the incidence of these two entities or of asymptomatic VTE, regardless of whether prophylactic antithrombotic therapy was given [2–7]. In addition, reports indicate that the infection rate in prophylactically treated patients is increased owing to hematoma caused by hemorrhage [8–10] and coagulation abnormalities [11] associated with the therapy early after surgery. It is important for orthopedic surgeons to avoid these complications because such infections can last a lifetime. Even if patients achieve remission, they are prone to infection relapse. The routine administration of prophylactic antithrombotic treatment is not recommended in East Asia [12]. Based on these observations, to reduce postoperative infections associated with the overuse of antithrombotic treatment in low-risk patients, we have considered it clinically important to be able to detect early asymptomatic VTE that may cause fatal or symptomatic PE after surgery in patients who are not administered prophylactic antithrombotic treatments. Also, we start antithrombotic therapy only in those patients who need it [6,13]. There are currently no blood coagulation–fibrinolysis markers available for early detection of postoperative asymptomatic VTE following TKA.

Since 2005, some studies have indicated that VTE is affected by the use of the pneumatic tourniquet, causing particular postoperative changes in coagulation–fibrinolysis pathways [14–17]. Therefore, we hypothesized that detecting changes in blood coagulation–fibrinolysis markers in patients with asymptomatic VTE immediately after the pneumatic tourniquet is released might be used to indicate whether patients require antithrombotic therapy. This information could help prevent postoperative bleeding after administering antithrombotics to patients who were at low risk of developing VTE. The purpose of this study was to investigate the changes of blood coagulation–fibrinolysis markers in asymptomatic VTE immediately after release of the pneumatic tourniquet during surgery.

The Conflict of Interest statement associated with this article can be found at http://dx.doi.org/10.1016/j.arth.2013.08.011.

Reprint requests: Hideaki Watanabe, MD, Department of Orthopedic Surgery, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 3290498, Japan.

0883-5403/2903-0024$36.00/0 – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.arth.2013.08.011

Materials and Methods

Patients

The study protocol was approved by the Ethics Review Board of our university. This prospective, single-center study enrolled patients who underwent TKA at our institution between April 2007 and March 2009 and gave consent to participate in the study. As exclusion criteria, patients with a past history of symptomatic VTE, cerebral hemorrhage, cerebral infarction, cardiac infarction, or drug allergy to a contrast medium were excluded from the study. In addition, patients with liver disease, renal disease, and/or congenital clotting factor deficiencies and those undergoing antithrombotic therapy or hemodialysis were excluded from the study. Patients with asymptomatic VTE by preoperative MDCT were also excluded.

We enrolled 42 patients who underwent TKA for osteoarthritis (30 knees) or rheumatoid arthritis (12 knees). The cohort comprised 1 male and 41 female patients, with a mean age of 71 years (range 49–84 years). TKA was performed under general anesthesia in all patients, and a pneumatic tourniquet was used. Its pressure was raised before surgery while the leg was exsanguinated and lowered about 90 min later. The tourniquet was used only one time. The patients wore an elastic stocking on the unaffected leg during surgery. Later, they wore them on both affected and unaffected legs and used an intermittent pneumatic compression device until walking training was initiated, in accordance with the Japanese Guidelines for Prevention of Venous Thromboembolism [18]. No postoperative prophylactic antithrombotic therapy was administered. If the patients developed symptomatic VTE and/or if VTE was detected by MDCT, aggressive antithrombotic therapy was initiated.

MDCT

For diagnosis of VTE, 16-row MDCT was performed on the 4th day before surgery and then the 4th day after surgery. These time points mark the interval at which the incidences of PE and VTE are reported to be high [19]. The latter is the earliest point at which patients could comfortably undergo MDCT during the postoperative period.

The MDCT slice thicknesses were 2 mm in the thoracic region and 5 mm from the abdomen to the lower limbs. The window levels were 40–60 and 40–50, and the window widths were 400–500 and 200–400, respectively. A single radiologist (M.D.) evaluated the MDCT images in a blinded manner before and after the surgery. The incidence of postoperative new asymptomatic VTE was calculated.

Preoperative MDCT revealed no asymptomatic VTE in any of the 42 patients included in the study. The patients were classified postoperatively via MDCT into two groups. The thrombus group was defined as patients with a new asymptomatic VTE, and the no-thrombus group was defined as those without asymptomatic VTE.

Blood Coagulation–Fibrinolysis Markers

Blood samples were taken to measure the plasma levels of plasminogen activator inhibitor-1 (PAI-1), soluble fibrin monomer complex (SFMC), D-dimer, and cross-linked fibrin degradation products by leukocyte elastase (e-XDP) immediately before and after release of the pneumatic tourniquet and then at 30, 90, and 180 s after release of the pneumatic tourniquet (Fig. 1). Citrated plasma samples were stored at −80 °C until analysis. The plasma PAI-1 levels were measured by a latex photometric immunoassay (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) using the polyclonal antibody F(ab') fragment [20]. Plasma SFMC, D-dimer, and e-XDP levels were measured by latex immunoagglutination assays (Mitsubishi Chemical Medience Corporation) using the monoclonal antibodies IF-43 and JIF-23, respectively [21,22]. The plasma e-XDP levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody IF-123 [23].

Statistical Analysis

Statistical analyses were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, USA). PAI-1, SFMC, D-dimer, and e-XDP levels were analyzed by the Shapiro–Wilk test if they did not fit a normal distribution. The PAI-1, SFMC, D-dimer, and e-XDP levels were compared between the thrombus and no-thrombus groups before release using the Mann–Whitney U-test. The PAI-1, SFMC, D-dimer and e-XDP levels were compared between immediately, at 30, 90, 180 s after release, respectively, and immediately before release using the Friedman test. If a significant difference was noted, the data were compared using the Wilcoxon signed rank test and corrected using Bonferroni's inequality. Spearman's rank correlation was used to determine whether blood coagulation–fibrinolysis markers that differed significantly were affected by each other. The gender and disorder distributions were compared between the thrombus and no-thrombus groups using Fisher's exact test. Age, volume of intraoperative hemorrhage, and operation time were compared using an unpaired *t*-test. The level of statistical significance was set at $P < 0.05$ for all tests.

Results

No patients developed symptomatic VTE during or after TKA in this study. Postoperative MDCT revealed asymptomatic VTE in 19 (45.2%) patients (thrombus group) and no VTE in 23 patients (54.7%) (no-thrombus group). Aggressive antithrombotic therapy was initiated in the 19 patients in whom new asymptomatic VTE was detected following postoperative MDCT (Table).

Changes in Operative Blood Coagulation–Fibrinolysis Markers Before Release of the Pneumatic Tourniquet

There were no significant differences in the preoperative PAI-1, SFMC, D-dimer, or e-XDP levels between the thrombus and no-thrombus groups ($P = 0.23$, $P = 0.23$, $P = 0.39$, and $P = 0.89$, respectively) (Fig. 2).

Operative Blood Coagulation–Fibrinolysis Markers After Release of the Pneumatic Tourniquet

The PAI-1 level showed the most significant increases at 30 s (median 27.3 ng/ml, $P = 0.01$) and 90 s (median 28.5 ng/ml, $P = 0.01$) after release of the pneumatic tourniquet in the thrombus groups and at 30 s (median 38.7 ng/ml, $P = 0.01$) after release in the no-thrombus group (Fig. 2).

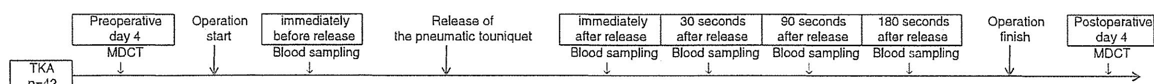


Fig. 1. Study protocol.