conjugated JON/A and 1 μL of biotin-conjugated anti-mouse P-selectin mAb for 5 min, and then supplemented with 1 μL of allophycocyanin (APC)-conjugated streptavidin. After 15 min of incubation, JON/A binding and P-selectin expression were determined by flow cytometry using a FACSAria Cell Sorter (Becton Dickinson, Mountain View, CA). Antibody binding was quantified as the mean fluorescence intensity (MFI) of GFP-positive platelets.

Platelet aggregation

Washed platelets were prepared as described above. The final suspensions were adjusted to 2×10^8 platelets/mL and supplemented with 1 mmol/L $CaCl_2$ and 200 μg/mL fibrinogen. The aggregation response to agonist stimulation was measured based on light transmission measured using a PA-200 platelet aggregation analyzer (Kowa, Tokyo, Japan).

Measurement of platelet products

Washed platelets (2×10^8/mL) were stimulated with the indicated agonists for 15 min, and then the supernatants were recovered by centrifugation. The levels of platelet factor 4 (PF4) and serotonin in the supernatants were measured using a mouse PF4 enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems) and an anti-serotonin ELISA kit (GenWay Biotech, San Diego, CA), respectively. The levels of TxB_2 in the supernatants were measured using an enzyme immunoassay (Cayman Chemical).

Platelet adhesion

Platelet adhesion to fibrinogen was assessed as described previously [21]. Briefly, eight-well dishes (Lab-Tek® Chamber Slide™) were coated with 400 μg/mL fibrinogen and then blocked with 1 mg/mL bovine serum albumin (BSA). Platelets were then added to the fibrinogen-coated dishes and incubated for 30 min at 37°C. Adherent platelets were fixed with 3% paraformaldehyde and then permeabilized with phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 5% donkey serum. After washing with PBS, the platelets were incubated with an anti-GFP polyclonal antibody (MBL, Aichi, Japan). Bound antibodies were detected by Alexa Fluor 488-conjugated anti-rabbit IgG. Actin filaments were detected by staining with 1 μg/mL rhodamine-conjugated phalloidin. Immunofluorescence staining was observed and photographed under a confocal microscope (FV1000; Olympus, Tokyo, Japan). The spread area of GFP-positive platelets was quantified using ImageJ software. Because Pxn-KD platelets were slightly larger than control platelets (Figure 1), the mean platelet size determined by BSA staining was subtracted from the total area on fibrinogen to calculate the actual increase in platelet spreading.

Clot retraction

Human platelet-poor plasma was mixed with the same volume of Hepes/Tyrode buffer containing washed mouse platelets (final concentration: 3×10^8 platelets/ml). Plasma coagulation was initiated by addition of 0.1 U/mL thrombin. The clots were photographed at various time points after thrombin addition. When indicated, 0.5 mmol/L manganese was added to exclude the role of inside-out signaling. The two-dimensional area of serum formation extruded by clot retraction was quantified using ImageJ software and expressed as the progression of clot retraction.

Calcium mobilization

Platelets were incubated with GFP-Certified™ FluoForte™ dye (Enzo Life Sciences, Farmingdale, NY). The fluorophore-loaded platelets (2×10^8/mL) were resuspended in Hepes-Tyrode buffer containing 1 mmol/L EDTA, 5 U/mL apyrase, and 10 μmol/L SQ29548 to exclude the effects of aggregation, extracellular calcium, released ADP, and TxA_2. After stimulation, the intracellular calcium concentration was determined by monitoring the fluorescence (excitation, 530 nm; emission, 570 nm) using a microplate spectrofluorometer (Gemini EM; Molecular Devices, Sunnyvale, CA).

Intravital microscopy and thrombus formation

Intravital microscopy was performed to analyze thrombus formation in vivo as reported previously [26]. Briefly, Texas Red-dextran (100 mg/kg body weight [BW], molecular weight: 70 kDa; Invitrogen), Hoechst 33342 (10 mg/kg BW; Invitrogen), Dylight 488-conjugated anti-CD42b antibody (200 μg/kg BW; Emfret), and hematoporphyrin (5 mg/kg BW; Sigma) were injected into anesthetized mice to produce reactive oxygen species (ROS) following laser irradiation. Blood cell dynamics were visualized during laser excitation (wavelengths 405, 488, and 561 nm; 1.5 mW total power at 100× objective lens). After laser irradiation, sequential images of the mesentery were obtained using a resonance scanning confocal microscope (Nikon A1R; Nikon, Tokyo, Japan). The areas of thrombus (shown by anti-CD42b antibody signals) before and after laser irradiation were calculated using NIS-Elements AR 3.2 (Nikon). When indicated, thrombus formation in the femoral artery was triggered by topical application of a filter paper tip saturated with 10% $FeCl_3$. After injection of Texas Red-dextran, Hoechst 33342, and Dylight 488-conjugated anti-CD42b antibody, thrombus formation was visualized and monitored by confocal microscopy using two photon microscopy (excitation wavelength 840 nm) by NikonA1R MP (Nikon).

Bleeding time

The distal tail tip (5 mm) of an anesthetized mouse was clipped, and the tail was immediately immersed in PBS

at 37°C. Tail bleeding times were defined as the time required for the bleeding to stop.

Results

Generation of paxillin knockdown (Pxn-KD) platelets

To address the function of paxillin in mouse platelets, we used a lentiviral vector carrying shRNA sequences and GFP [22]. We synthesized three shRNA sequences for mouse paxillin, and cloned them into a LentiLox vector plasmid (Additional files 1 and 2). We selected one sequence that significantly inhibited paxillin expression in embryonic fibroblasts after transduction (Pxn-1 sequence; Additional files 1 and 2). After transplantation of bone marrow cells transduced with either the control or Pxn-KD sequence, about 50% of the platelets expressed GFP, and the absolute numbers of GFP-positive platelets did not differ between experiments using control and Pxn-KD sequences (Figure 1A–B). Furthermore, there was no effect on the total number of platelets (control: $6.8 \pm 1.72 \times 10^8$/mL; Pxn-KD: $7.7 \pm 0.65 \times 10^8$/mL, $P = 0.18$). We compared the platelet aggregation response and release reaction in platelets from wild-type C57BL/6 J and control mice, and confirmed that platelet aggregation as well as the release reaction did not differ (data not shown). To confirm knockdown of paxillin in GFP-positive platelets, we selected mice in which more than 80% of platelets expressed GFP after transplantation. Immunoblotting of platelet lysates with an anti-paxillin mAb (clone 349) showed a marked reduction in paxillin expression following transplantation of bone marrow cells transduced with the Pxn-KD sequence (Figure 1C). This antibody also recognizes other members of the paxillin family, including Hic-5 and leupaxin [17]. However, Hic-5 and leupaxilin were not affected by expression of the Pxn-KD sequence (Figure 1C). Transmission electron microscopy of resting platelets revealed that the Pxn-KD platelets were slightly larger than control platelets (Figure 1D–E). This change was largely dependent on an increase of the cytoplasm volume, but not the granule volume (Additional file 3). Pxn-KD platelets showed marginally elevated expression levels of GPIb and integrin αIIbβ3, even though GPVI expression was normal (Additional file 4). These changes in Pxn-KD platelets were supposed to result from the increase in platelet size.

Augmentation of integrin αIIbβ3 activation in Pxn-KD platelets

We first focused on the role of paxillin in integrin αIIbβ3 activation that is critical for platelet aggregation. We performed flow cytometric analysis of integrin αIIbβ3 activation using an anti-JON/A mAb [25]. GFP-positive Pxn-KD platelets (Figure 2A, lower panel) showed significantly enhanced αIIbβ3 activation following stimulation compared with that of control platelets (Figure 2A, upper panel). Enhanced JON/A binding of Pxn-KD platelets was observed following stimulation with the GPVI agonist convulxin and G protein-coupled receptor agonists including a protease-activated receptor 4 agonist (AYPGKF), ADP, and U46619 (Figure 2A–B). However, JON/A binding was not enhanced in unstimulated or epinephrine-stimulated platelets, suggesting that Pxn-KD alone does not induce activation of integrin αIIbβ3. We next used light transmission aggregometry to assess platelet aggregation in vitro. We found that platelet aggregation was significantly augmented in Pxn-KD platelets, and this effect was evident at low agonist concentrations that induce platelet aggregation (Figure 2C–D).

Enhanced release reactions and Tx biosynthesis in Pxn-KD platelets

We next assessed the release reactions in response to stimulation. To address the role of paxillin in α-granule secretion, P-selectin expression was determined in GFP-positive platelets by flow cytometry. As shown in Figure 3A–B, P-selectin expression in Pxn-KD platelets was significantly increased following stimulation with convulxin, AYPGKF, and U46619. In contrast, P-selectin expression was not increased by stimulation with ADP or epinephrine. We observed negligible increases in P-selectin expression of Pxn-KD platelets under the resting condition and after incubation with the fibronectin peptide Gly-Arg-Gly-Asp-Ser (GRGDS) (Figure 3B). To examine whether Pxn-KD platelets are already activated during circulation, we compared P-selectin expression in washed platelets and whole blood platelets before the preparation. An increase of P-selectin expression after washing the platelet preparation was observed in Pxn-KD platelets (30.0 ± 9.71 to 37.2 ± 5.72 in the control vs. 27.8 ± 2.56 to 44.8 ± 7.87, $P < 0.05$), suggesting that the susceptibility of Pxn-KD platelets caused marginal activation during washing. Although PF4 and serotonin content in resting platelets did not differ between control and Pxn-KD platelets (Additional file 3), the actual release of PF4 and serotonin into the supernatant in response to platelet activation was also enhanced in Pxn-KD platelets (Figure 3C–D). Of note, a marked increase in TxB_2 biosynthesis was observed in Pxn-KD platelets (Figure 3E). Pretreatment with the ADP scavenger apyrase and thromboxane A_2 receptor antagonist SQ29548 somewhat corrected the increase of JON/A binding in Pxn-KD platelets. This result suggests that the extent of the increase of integrin activation is partially dependent on the release reaction (Additional file 5). Collectively, these data suggest that paxillin negatively regulates platelet activation signaling pathways leading to integrin activation, release reactions, and Tx synthesis. It is possible that general pathway (s) involved in platelet activation were enhanced by Pxn-KD, because platelet activation was increased in

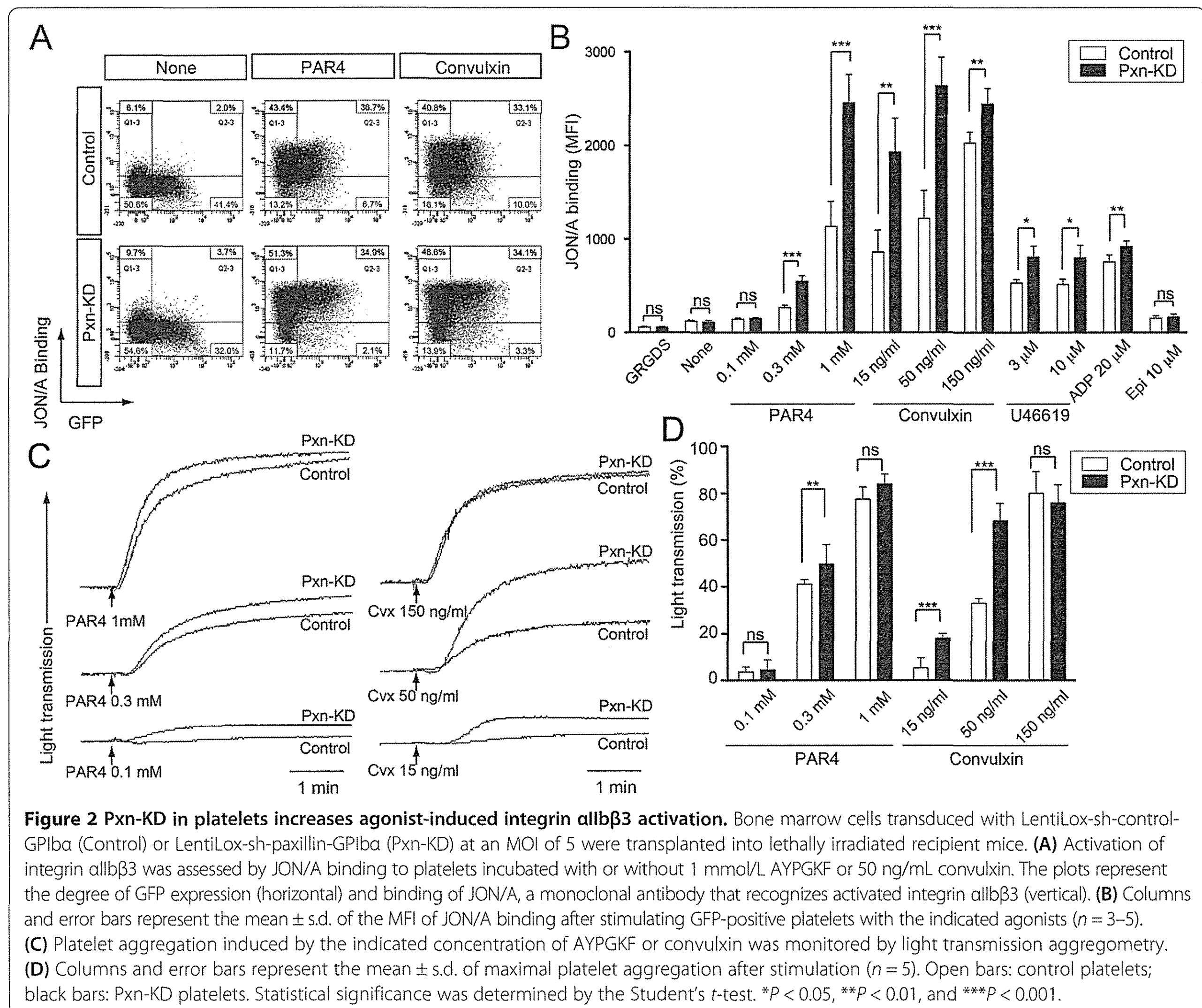

Figure 2 Pxn-KD in platelets increases agonist-induced integrin αIIbβ3 activation. Bone marrow cells transduced with LentiLox-sh-control-GPIbα (Control) or LentiLox-sh-paxillin-GPIbα (Pxn-KD) at an MOI of 5 were transplanted into lethally irradiated recipient mice. **(A)** Activation of integrin αIIbβ3 was assessed by JON/A binding to platelets incubated with or without 1 mmol/L AYPGKF or 50 ng/mL convulxin. The plots represent the degree of GFP expression (horizontal) and binding of JON/A, a monoclonal antibody that recognizes activated integrin αIIbβ3 (vertical). **(B)** Columns and error bars represent the mean ± s.d. of the MFI of JON/A binding after stimulating GFP-positive platelets with the indicated agonists ($n = 3$–5). **(C)** Platelet aggregation induced by the indicated concentration of AYPGKF or convulxin was monitored by light transmission aggregometry. **(D)** Columns and error bars represent the mean ± s.d. of maximal platelet aggregation after stimulation ($n = 5$). Open bars: control platelets; black bars: Pxn-KD platelets. Statistical significance was determined by the Student's *t*-test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

response to several classes of activators including GPVI and G protein-coupled receptors.

Assessment of outside-in signaling pathways in Pxn-KD platelets

To address the role of paxillin in outside-in signaling of integrin αIIbβ3, we assessed platelet spreading on fibrinogen and clot retraction. The cell area independent of integrin outside-in signaling (i.e., adherent to the BSA control) was slightly increased in Pxn-KD platelets compared with that in control platelets (data not shown), because the Pxn-KD platelets were marginally larger than control platelets (Figure 1). To quantify the increase in platelet spreading, the mean platelet size on BSA was subtracted from the total spreading area on fibrinogen. As shown in Figure 4A, the increase in platelet spreading on fibrinogen without or with convulxin stimulation was significantly greater for Pxn-KD platelets than that for control platelets (Figure 4A–B). In addition, clot retraction induced by thrombin was significantly enhanced in Pxn-KD platelets compared with that in control platelets (Figure 4C–D). Acceleration of clot retraction in Pxn-KD platelets was also observed in the presence of manganese at 15 min (6.98 ± 0.130 vs. 7.56 ± 0.072, $P < 0.05$). These observations suggest that paxillin is an important regulator of integrin outside-in signaling via integrin αIIbβ3.

The role of paxillin in calcium mobilization in platelets

Because GPVI initiates signaling cascades by activation of non-receptor tyrosine kinases, we assessed tyrosine phosphorylation elicited by the GPVI signaling pathway. As a result, tyrosine phosphorylation events induced by convulxin were not affected by Pxn-KD (Figure 5A). The agonist-induced increase in intracellular calcium mobilization is an important common and proximal signaling event controlling platelet activation. Therefore, we next examined whether Pxn-KD enhanced intracellular calcium mobilization following stimulation. To exclude

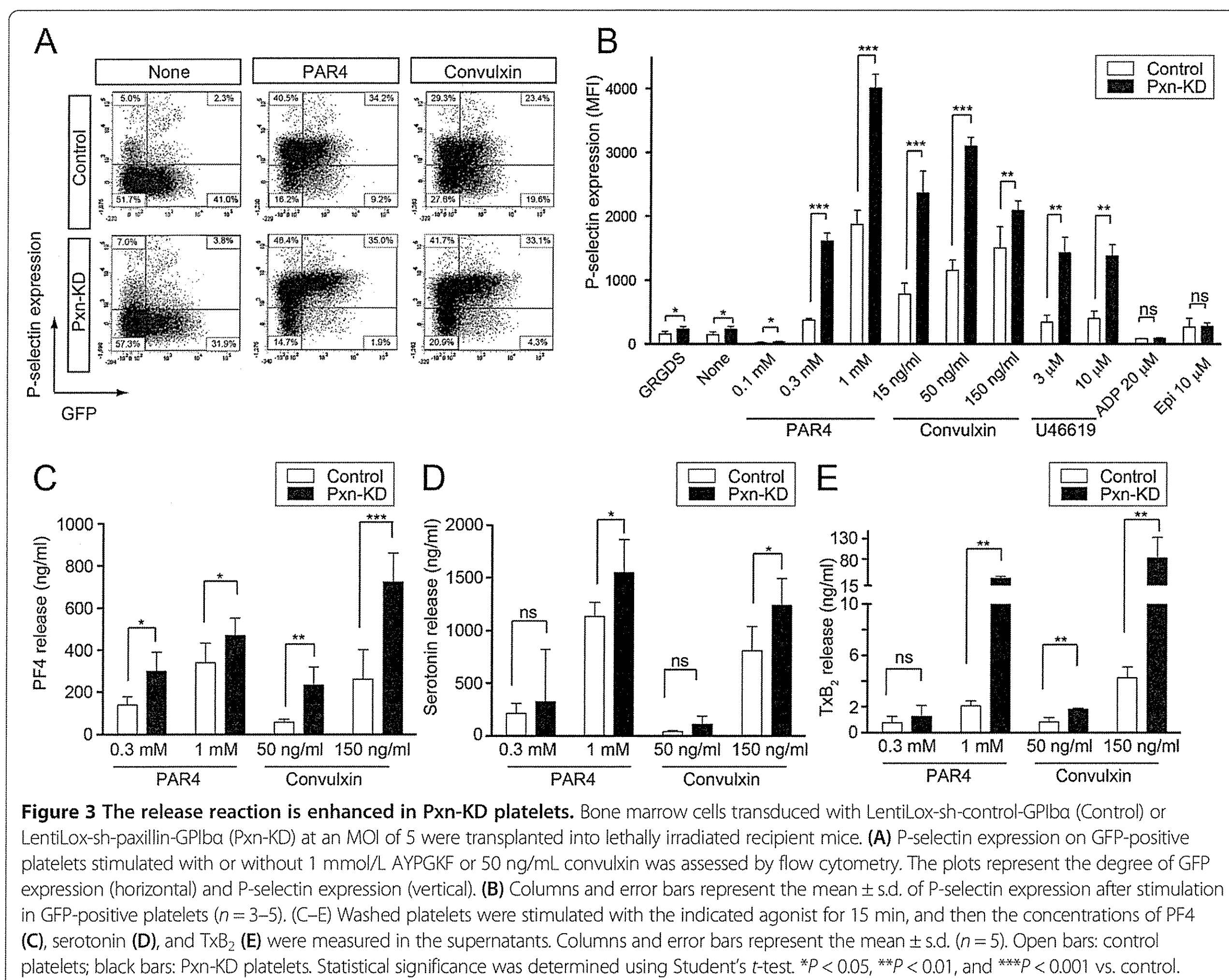

Figure 3 The release reaction is enhanced in Pxn-KD platelets. Bone marrow cells transduced with LentiLox-sh-control-GPIbα (Control) or LentiLox-sh-paxillin-GPIbα (Pxn-KD) at an MOI of 5 were transplanted into lethally irradiated recipient mice. **(A)** P-selectin expression on GFP-positive platelets stimulated with or without 1 mmol/L AYPGKF or 50 ng/mL convulxin was assessed by flow cytometry. The plots represent the degree of GFP expression (horizontal) and P-selectin expression (vertical). **(B)** Columns and error bars represent the mean ± s.d. of P-selectin expression after stimulation in GFP-positive platelets (n = 3–5). (C–E) Washed platelets were stimulated with the indicated agonist for 15 min, and then the concentrations of PF4 **(C)**, serotonin **(D)**, and TxB_2 **(E)** were measured in the supernatants. Columns and error bars represent the mean ± s.d. (n = 5). Open bars: control platelets; black bars: Pxn-KD platelets. Statistical significance was determined using Student's t-test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control.

secondary effects of platelet aggregation, influx of extracellular calcium, and release reactions, we preincubated the platelets with EDTA, apyrase, and SQ29548. Intracellular calcium mobilization induced by the GPVI agonist convulxin and G protein-coupled receptor stimulation with AYPGKF was rather decreased by Pxn-KD (Figure 5B). These data suggest that paxillin targets downstream signaling of calcium mobilization or a calcium-independent signaling pathway.

To explore the importance of calcium-independent signaling pathways in Pxn-KD platelets, we employed BAPTA-AM, an intracellular calcium chelator, to exclude the effect of calcium mobilization. Because JON/A requires extracellular calcium for antibody binding, we assessed P-selectin expression induced by an agonist. Pretreatment with BAPTA-AM significantly suppressed P-selectin expression in both control and Pxn-KD platelets (Figure 5C). On the other hand, P-selectin expression elicited by an agonist was still observed in Pxn-KD platelets even in the presence of BAPTA-AM (Figure 5C). These data indicate that downstream signaling from intracellular calcium mobilization is amplified by Pxn-KD, and the calcium-independent pathway is activated by Pxn-KD to increase platelet activation.

Pxn-KD augments platelet adhesion and thrombus formation in vivo

Finally, we examined the contribution of paxillin to thrombus formation in vivo. To visualize thrombus formation in vivo, we used a direct visual technique based on confocal microscopy in mesenteric capillaries [26]. Thrombus formation in this system was initiated by the production of ROS following laser irradiation [26]. Laser irradiation-induced thrombus formation was significantly enhanced in Pxn-KD platelets (Figure 6A and 6B and Additional files 6 and 7). In addition, there was an enhancement of thrombus formation initiated by $FeCl_3$ in large femoral arteries (Additional file 8). Moreover, bleeding times after tail clipping significantly shortened in Pxn-KD experiments (Figure 6C). These findings support

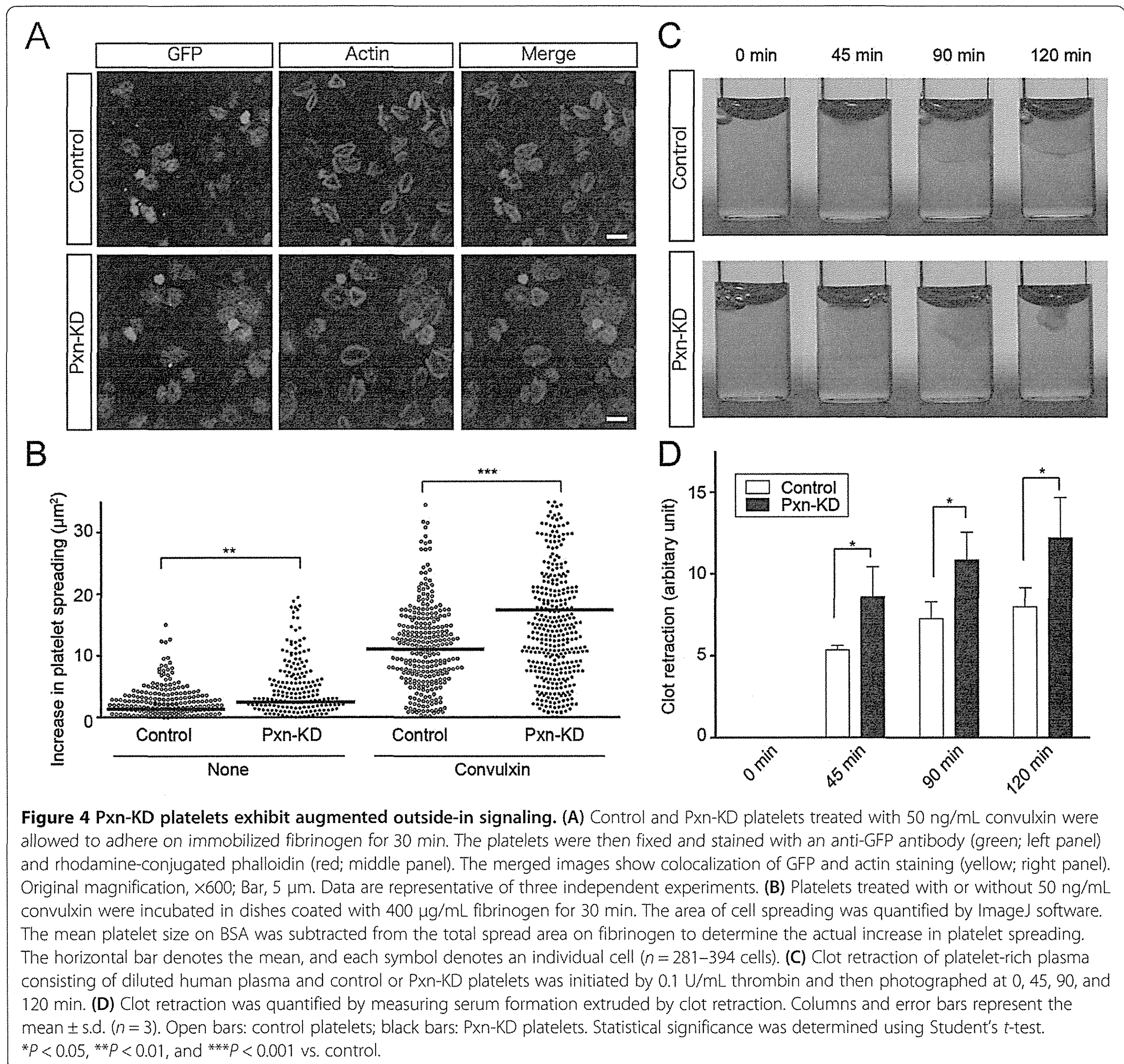

Figure 4 Pxn-KD platelets exhibit augmented outside-in signaling. (A) Control and Pxn-KD platelets treated with 50 ng/mL convulxin were allowed to adhere on immobilized fibrinogen for 30 min. The platelets were then fixed and stained with an anti-GFP antibody (green; left panel) and rhodamine-conjugated phalloidin (red; middle panel). The merged images show colocalization of GFP and actin staining (yellow; right panel). Original magnification, ×600; Bar, 5 μm. Data are representative of three independent experiments. **(B)** Platelets treated with or without 50 ng/mL convulxin were incubated in dishes coated with 400 μg/mL fibrinogen for 30 min. The area of cell spreading was quantified by ImageJ software. The mean platelet size on BSA was subtracted from the total spread area on fibrinogen to determine the actual increase in platelet spreading. The horizontal bar denotes the mean, and each symbol denotes an individual cell ($n = 281–394$ cells). **(C)** Clot retraction of platelet-rich plasma consisting of diluted human plasma and control or Pxn-KD platelets was initiated by 0.1 U/mL thrombin and then photographed at 0, 45, 90, and 120 min. **(D)** Clot retraction was quantified by measuring serum formation extruded by clot retraction. Columns and error bars represent the mean ± s.d. ($n = 3$). Open bars: control platelets; black bars: Pxn-KD platelets. Statistical significance was determined using Student's *t*-test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control.

our hypothesis that paxillin is an important negative regulator of platelet activation and thrombus formation in vivo.

Discussion

Here, we found that the LIM protein paxillin is a negative regulator of platelet activation in mice. The negative regulation of platelet activation by paxillin was not limited to a specific signaling pathway, because Pxn-KD enhanced platelet activation in response to a variety of agonists. We also confirmed that thrombus formation was augmented in Pxn-KD platelets in vivo. This finding is notable because several previous reports suggest that changes in paxillin function actually reduce integrin signaling [13,14]. Furthermore, a previous finding in platelets has demonstrated the possible role of paxillin as a negative feedback regulator after integrin ligation to regulate the activity of Lyn tyrosine kinase [17]. However, this mode of regulation cannot fully explain the phenotypes of Pxn-KD platelets, because both outside-in and inside-out signaling were augmented by Pxn-KD. Our results reveal a new cellular function of paxillin and indicate new mechanisms that modulate platelet activation.

The most interesting result of this study was that Pxn-KD significantly enhanced the upstream signaling pathways that converge on platelet activation. Appropriate inhibition of the platelet response is essential to control pathological thrombus formation. It is well known that the mediators that enhance intracellular cAMP or cGMP

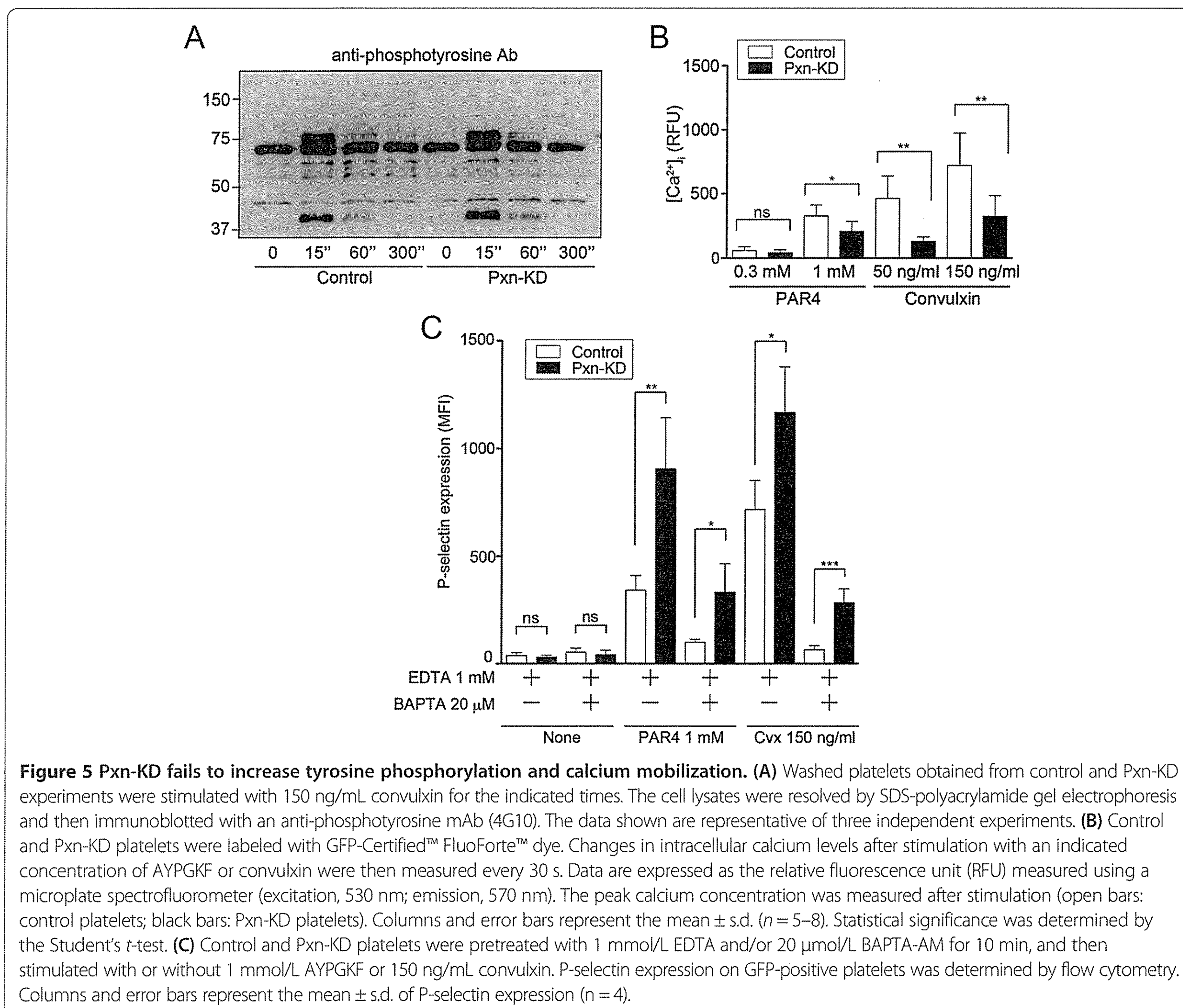

Figure 5 Pxn-KD fails to increase tyrosine phosphorylation and calcium mobilization. **(A)** Washed platelets obtained from control and Pxn-KD experiments were stimulated with 150 ng/mL convulxin for the indicated times. The cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and then immunoblotted with an anti-phosphotyrosine mAb (4G10). The data shown are representative of three independent experiments. **(B)** Control and Pxn-KD platelets were labeled with GFP-Certified™ FluoForte™ dye. Changes in intracellular calcium levels after stimulation with an indicated concentration of AYPGKF or convulxin were then measured every 30 s. Data are expressed as the relative fluorescence unit (RFU) measured using a microplate spectrofluorometer (excitation, 530 nm; emission, 570 nm). The peak calcium concentration was measured after stimulation (open bars: control platelets; black bars: Pxn-KD platelets). Columns and error bars represent the mean ± s.d. ($n = 5–8$). Statistical significance was determined by the Student's *t*-test. **(C)** Control and Pxn-KD platelets were pretreated with 1 mmol/L EDTA and/or 20 μmol/L BAPTA-AM for 10 min, and then stimulated with or without 1 mmol/L AYPGKF or 150 ng/mL convulxin. P-selectin expression on GFP-positive platelets was determined by flow cytometry. Columns and error bars represent the mean ± s.d. of P-selectin expression (n = 4).

levels, including prostacyclin, prostaglandin E_1, and nitric oxide, are strong extrinsic inhibitors of platelet activation [27]. These extrinsic mediators ameliorate the broad platelet activation elicited by various agonists [27]. Intrinsic negative regulators of platelet activation have been identified recently, but many of these proteins only control a specific receptor signaling pathway. GPVI-mediated immunoreceptor tyrosine-based activation motif (ITAM) signaling is regulated by immunotyrosine-based inhibitory motif (ITIM)-containing receptors including platelet endothelial cell adhesion molecule 1 and carcinoembryonic antigen-related cell adhesion molecule 1 [28,29]. Furthermore, Lyn tyrosine kinase has been reported to inhibit ITAM signaling by inducing tyrosine phosphorylation of ITIM [28]. It has also been reported that binding of a regulator of G-protein signaling to the Giα subunit limits platelet responsiveness to the receptor, which is independent of Rap1b [30]. Conversely, paxillin may downregulate platelet activity by modulating a common pathway, because Pxn-KD resulted in marked platelet hyperactivation in response to stimulation of tyrosine phosphorylation-based receptors and G protein-coupled receptors.

Although paxillin is reportedly involved in various integrin-mediated cellular functions, many of these functions are limited to outside-in signaling pathways. Paxillin-deficient embryos show embryonic lethality, and the phenotype closely resembles that of fibronectin-deficient mice [31]. Moreover, paxillin-deficient fibroblasts show reductions in cell migration and tyrosine phosphorylation following cell adhesion [31]. Chimeric integrin αIIbβ3 with a cytoplasmic tail substitution of α4β1 or α9β1, which facilitates paxillin binding, significantly inhibits cell spreading, but does not affect αIIbβ3-dependent cell adhesion [18,19]. Inhibition of paxillin binding to integrin α4 inhibits leukocyte recruitment to an inflammatory site [32]. These data suggest important roles of paxillin in outside-in signaling by direct interaction with the integrin α-subunit. However, in this study, inside-out and outside-

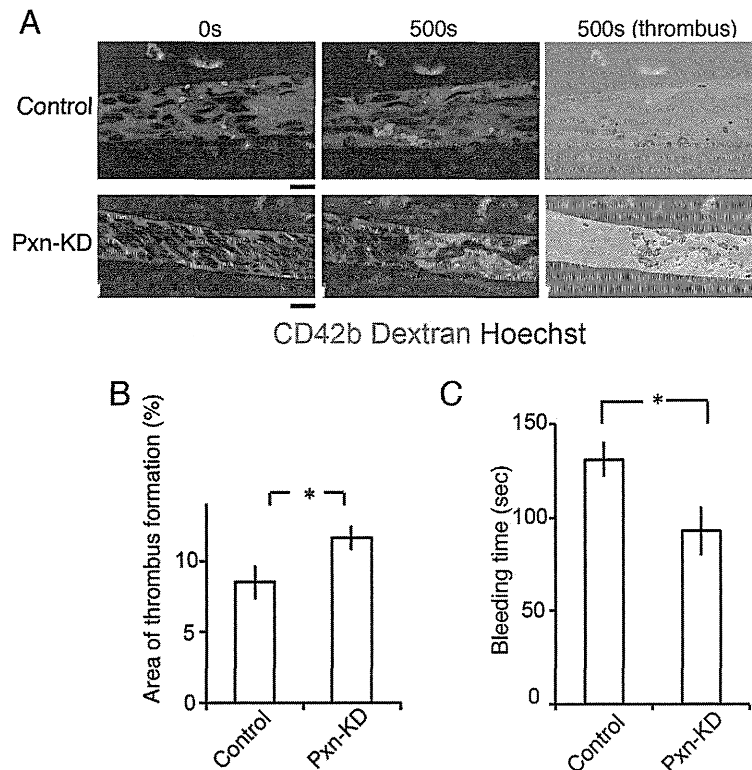

Figure 6 Pxn-KD in platelets expedites thrombus formation in vivo. (A) Intravital imaging of thrombus formation by laser irradiation of mesenteric arterioles in mice with control or Pxn-KD platelets. Thrombus formation was increased in mice with Pxn-KD platelets following laser irradiation. Bar, 10 μm.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

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

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

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

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

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

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

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

Additional files

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

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

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

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

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

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

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

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

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

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

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

Competing interests
The authors declare that they have no competing interests.

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

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CRITICAL CARE

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

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Abstract

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

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

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

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

Introduction

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

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

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

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

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

Material and methods

Study design and setting

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

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

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

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

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

Biomarker measurements

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

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

Patient management

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

Data analysis

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

Results

Patient characteristics and outcomes

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

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

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

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

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

Evidence of biomarker abnormalities at baseline and subsequent changes over time

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

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

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

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

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

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

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

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

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

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

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

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

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

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

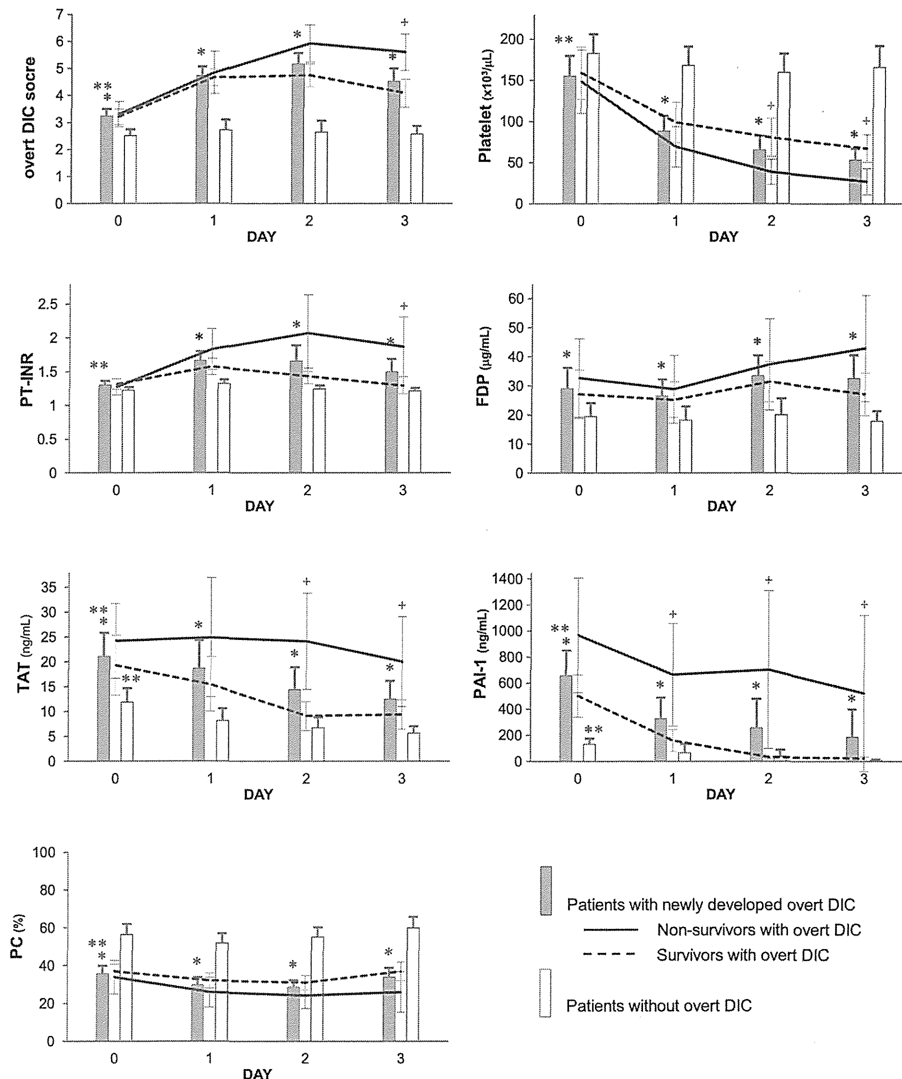

Figure 1 Time course of overt DIC scores and hemostatic biomarkers from baseline to Day 3. Overt disseminated intravascular coagulation (DIC) scores, platelet count, prothrombin time-international normalized ratio (PT-INR), fibrin degradation product (FDP), thrombin-antithrombin complex (TAT), plasminogen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

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

Discussion

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

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

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

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

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

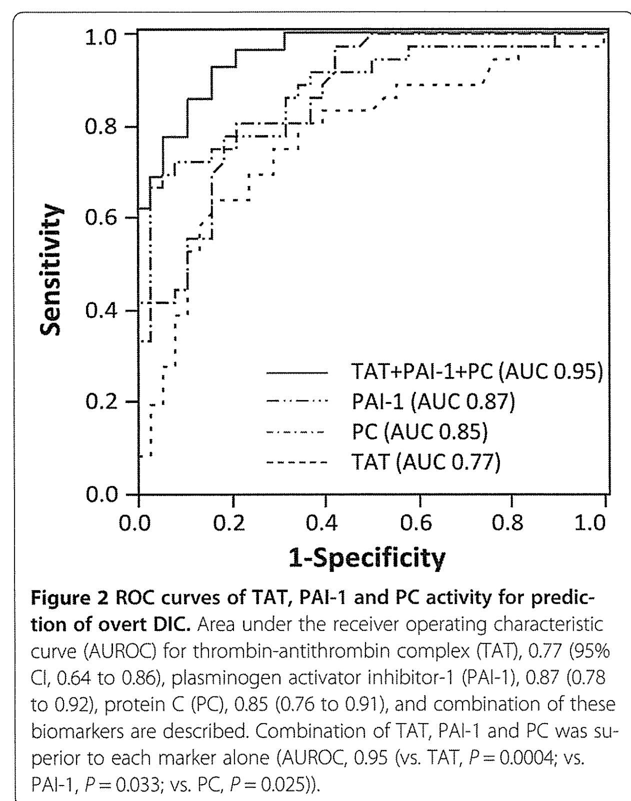

Figure 2 ROC curves of TAT, PAI-1 and PC activity for prediction of overt DIC. Area under the receiver operating characteristic curve (AUROC) for thrombin-antithrombin complex (TAT), 0.77 (95% CI, 0.64 to 0.86), plasminogen activator inhibitor-1 (PAI-1), 0.87 (0.78 to 0.92), protein C (PC), 0.85 (0.76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

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

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

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

Biomarkers	ICU day	AUC (95% CI)	Cutoff values*	Sensitivity	Specificity	PPV	NPV
Global markers							
Platelet	Day 0	0.58 (0.41 to 0.74)	117 (× 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.

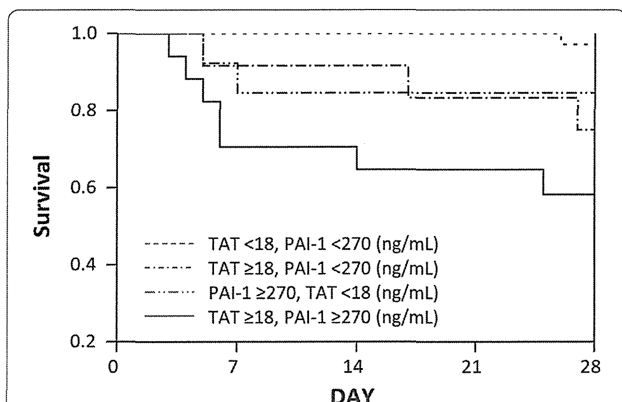

Figure 3 Kaplan-Meier survival curves for patients grouped by cutoff points of TAT and PAI-1 at baseline. The cutoff points were set at 18 ng/mL for thrombin-antithrombin complex (TAT) and 270 ng/mL for plasminogen activator inhibitor-1 (PAI-1), based on the best calculated cutoff values that maximize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

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