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Shedding of procoagulant microparticles from unstimulated platelets by integrin-mediated destabilization of actin cytoskeleton

Sandra Cauwenberghs[a,b], Marion A.H. Feijge[a], Alan G.S. Harper[c], Stewart O. Sage[c], Joyce Curvers[b], Johan W.M. Heemskerk[a,*]

[a] *Department of Biochemistry, CARIM, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands*
[b] *Sanquin Blood Bank South-East, Maastricht, The Netherlands*
[c] *Department of Physiology, Development and Neuroscience, Cambridge University, Cambridge, UK*

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Abstract Platelet activation by potent, Ca^{2+}-mobilizing agonists results in shedding of microparticles that are active in coagulation. Here we show that platelets under storage produce procoagulant microparticles in the absence of agonist. Microparticle formation by resting platelets results from αIIbβ3 signaling to destabilization of the actin cytoskeleton in the absence of calpain activation. Integrin-mediated spreading of platelets over fibrinogen similarly results in microparticle formation. After transfusion of stored platelet preparations to thrombocytopenic patients, the microparticles contribute to coagulant activity in vivo.
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Keywords: Actin cytoskeleton; Calpain; Integrin αIIbβ3; Microparticles; Procoagulant activity

1. Introduction

Platelets contribute to hemostasis and thrombosis by assembling into aggregates and by stimulating blood coagulation. It is generally assumed that, to become procoagulant, platelets need to be stimulated with strong agonists, e.g. Ca^{2+} ionophore or the combination of collagen and thrombin [1,2]. In a Ca^{2+}-dependent response similar but non-identical to apoptosis, these agents cause surface exposure of negatively charged phospholipids, such as phosphatidylserine (PS). This process enhances the assembly of coagulation factor complexes, and leads to the formation of factor Xa and thrombin and, hence, to coagulation.

For over 20 years, it is known that the same strong agonists also stimulate platelets to shed microparticles (MP), via Ca^{2+} influx and activation of the Ca^{2+}-dependent protease calpain, which structures also express procoagulant PS at their outer surface [3–6]. This shedding of MP, also described for other cells of the cardiovascular system, is likely to be of physiological relevance, as elevated MP levels in plasma associate with cardiovascular disease and thrombotic complications [7,8].

The integrin αIIbβ3 (>80 000 copies per platelet) plays a well established role in platelet aggregate and plug formation. It signals in both directions over the plasma membrane. Following platelet stimulation, signaling from the inside results in an active integrin conformation, which is required for binding of fibrinogen and von Willebrand factor. In addition, once activated, the integrin transmits still poorly understood outside-in signals in platelets, which include Src kinase and phosphoinositide 3-kinase activity, and actin cytoskeleton modification via cofilin and filamin [9–12]. Integrin outside-in signaling is known to mediate actin rearrangements that, for instance, cause platelets to spread over an adhesive surface and to retract, when encapsulated within a clot [13].

Preparations of platelets that are stored under blood bank conditions and used for transfusion purposes appear to be enriched in MP with high coagulant activity [14]. How such MP are formed by stored platelets has not been investigated. In the present study, we show that resting platelets 'spontaneously' shed procoagulant MP via a mechanism that involves integrin αIIbβ3 signaling towards cytoskeleton remodeling, in the apparent absence of integrin activation and platelet aggregation. This pathway differs from that of agonist-induced MP formation in the way that it does not require Ca^{2+} elevation and calpain activity. The produced MP appear to be active in coagulation, when transfused together with stored platelets into thrombocytopenic patients.

2. Materials and Methods

2.1. Materials

Abciximab (reopro) was from Centocor (Leiden, NL), tirofiban (aggrastat) from Merck (Haarlem, NL), eptifibatide (integrilin) and lotrafiban from GlaxoSmithKline (Zeist, NL), (labeled) annexin A5 from Nexins Research (Hoeven, NL) and iloprost from Schering (Berlin, G). Fluorescein isothiocyanate (FITC)-labeled anti-P-selectin monoclonal antibody (mAb) was from Immunotech (Marseille, F), while FITC (PE)-anti-CD61 (anti-β3) mAb and isotype-matched IgG1 were from BD Biosciences (San Jose, CA). Fura-2/AM, DM-BAPTA1 AM and Alexa Fluor phalloidin were from Molecular Probes (Leiden, NL); jasplakinolide was from VWR (Amsterdam, NL). PP1-3 and calpeptin were from Calbiochem (San Diego, CA), cytochalasin D and latrunculin B from Sigma (St. Louis, MO). The fluorogenic calpain substrate, *t*Boc-Leu-Met chloroamino methylcoumarin (*t*Boc-LM-CMC), was from Invitrogen (Paisley, UK). Sources of other reagents are described elsewhere [15].

*Corresponding author. Fax: +31 43 3882159.
E-mail address: jwm.heemskerk@BIOCH.unimaas.nl (J.W.M. Heemskerk).

Abbreviations: FITC, Fluorescein isothiocyanate; mAb, monoclonal antibody; MP, microparticles; PFP, platelet-free plasma; PRP, platelet-rich plasma; PS, phosphatidylserine

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2.2. Blood collection and platelet preparation

Blood was drawn after full consent by venapuncture from healthy volunteers, free of medication [16]. Blood was also obtained from a thrombocytopenic patient with myeloproliferative disorder both before and shortly after platelet transfusion [17]. Experiments were approved by the local Medical Ethics Committee. Platelet-rich plasma (PRP) was collected by centrifuging blood at $240 \times g$ for 15 min; leukocyte count was $<1 \times 10^3$/ml. Washed platelets were prepared from PRP [16], and resuspended at 4×10^8 platelets/ml in HEPES buffer, pH 7.45 (136 mM NaCl, 10 mM HEPES, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% glucose).

2.3. Platelet incubation and aggregation

PRP or washed platelets, normalized at 4×10^8 platelets/ml, were slowly rotated (10 rpm) at room temperature in closed, air-transparent tubes for up to 24 h. Inhibiting or activating agents were added, as described. At indicated times, MP-containing platelet-free plasma (PFP) was obtained by centrifuging 0.5 ml samples of the PRP at $20000 \times g$ for 10 min. Platelet count in the PFP was below detection ($<1 \times 10^5$/ml), while loss of MP was about 20%.

Aggregation of platelets in PRP was determined under stirring at 37 °C by conventional aggregometry [18]. Citrate-anticoagulated PRP was recalcified with 16.6 mM $CaCl_2$ in the presence of Phe-Pro-Arg chloromethyl ketone (PPACK, 10 μM), to achieve mM concentrations of free Ca^{2+} and Mg^{2+} without coagulation [15].

2.4. Flow cytometry

Platelet activation was measured by flow cytometry, using FITC-labeled mAbs against P-selectin or activated αIIbβ3 (PAC1) [14]. FITC-annexin A5 was used to detect exposed PS. Platelet-derived MP in plasma were measured after dilution with anticoagulated, filtered HEPES buffer, pH 7.45. After 15 min labeling with PE-anti-β3 mAb, the MP were detected as fluorescent events with low forward and side scatter positively staining for αIIbβ3. Fluorescent beads were used for calibration.

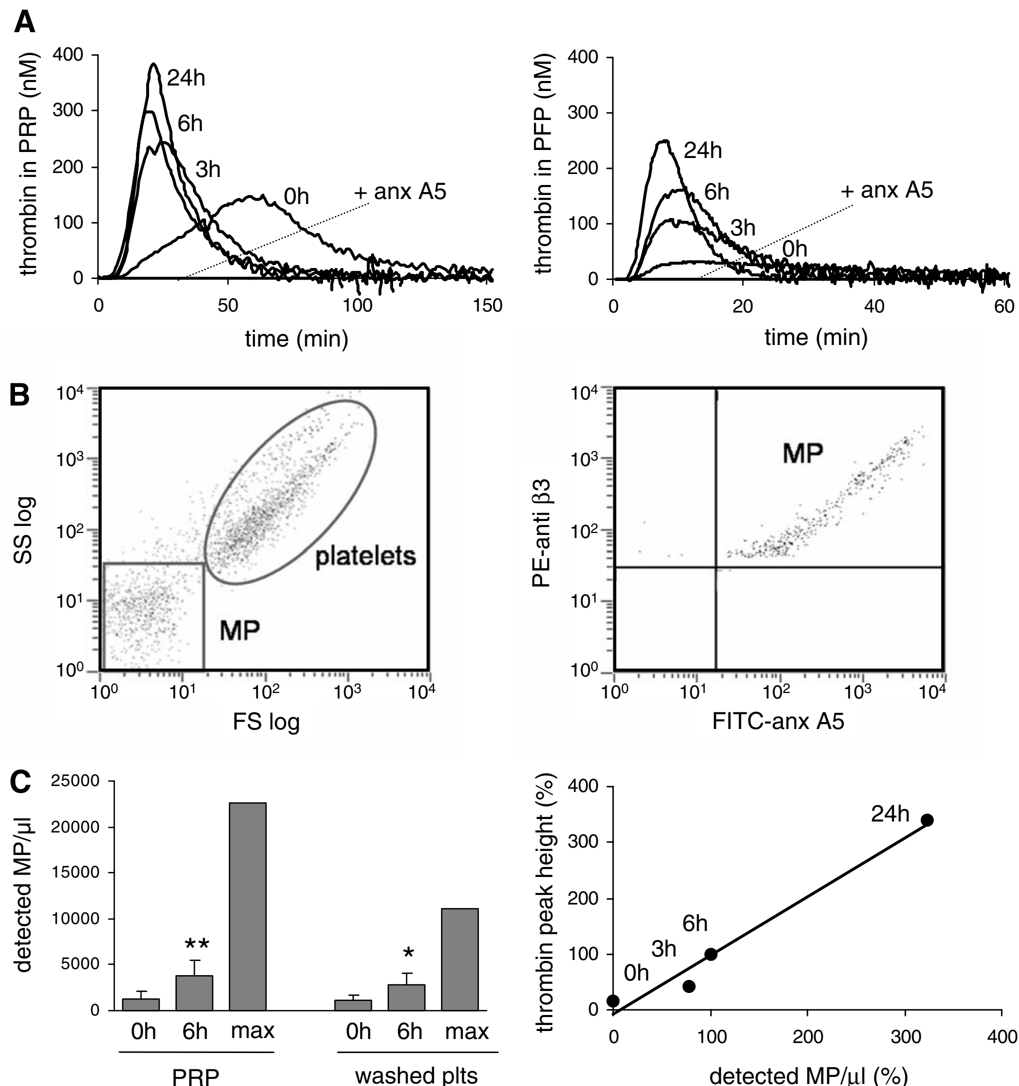

Fig. 1. Progressive shedding of procoagulant MP during platelet storage. (A) *Left:* Tissue factor-induced thrombin generation of PRP that was incubated at room temperature under rotation for 0-24 h. *Right:* Tissue factor-induced thrombin generation of PFP prepared by centrifuging of the rotated PRP. Annexin A5 (10 μg/ml) was added at 5 min before tissue factor, where indicated (6 h rotation, curves coincide with *x*-axis). (B) Flow cytometric detection of MP in 6-h rotated PRP. Co-labeling with FITC-annexin A5 (FL1) and PE-labeled anti-β3 mAb (FL2). (C) *Left:* Flow cytometric measurement of MP in PRP and in washed platelet suspension, incubated for 0–6 h. Maximal (max) amounts of MP were formed by stimulation with 50 ng/ml convulxin and 20 μM ADP (PRP), or with 10 μM ionomycin (washed platelets). *Right:* Correlation between MP detected by flow cytometry and MP-derived thrombin generation in plasma ($R^2 = 0.97$, $P = 0.013$). Data are means ± S.E.M. ($n = 3$–5), $^*P < 0.1$ and $^{**}P < 0.05$, compared to 0 h.

2.5. Measurement of thrombin generation in plasma

Platelet-dependent thrombin generation was measured in PRP [19]. Assay conditions were 1×10^8 platelets/ml, 67% citrate plasma and 33% buffer A (140 mM NaCl, 20 mM HEPES, 0.5% bovine serum albumin, pH 7.45). Reactions were started by addition of 1 pM (f.c.) tissue factor, 16.6 mM $CaCl_2$ and 415 μM fluorescent substrate Z-Gly-Gly-Arg aminomethyl coumarin. Similar reaction conditions were used to measure MP-dependent thrombin generation, but now using centrifuged (MP-containing) PFP. Assays were performed in triplicate at 37 °C using a Fluoroskan Ascent well plate reader [19]. Controls were always run to exclude interference of (ant)agonists with the coagulation process per se.

2.6. Real-time measurement of calpain activity

PRP, incubated for up to 24 h at room temperature, was used to prepare washed platelets in the presence of apyrase [15]. The washed cells were treated with vehicle of calpeptin (140 μM), and loaded with the fluorogenic calpain substrate, *t*Boc-LM-CMC, for 30 min at 37 °C, as described [20]. During the measurements, EGTA (1 mM) was added to prevent possible extracellular calpain activity. Intracellular calpain activity was measured from fluorescence increases due to substrate cleavage by stopped-flow fluorometry (agonists present as shown) [20]. Fluorescence increase in time was corrected for probe autofluorescence at baseline; averaged traces were produced from three repeated scans.

2.7. Microscopy

Phase-contrast and (confocal) fluorescence microscopy were performed as described [21].

2.8. Statistical analysis

Significance of differences was determined with the non-parametric Mann–Whitney *U* test.

3. Results

3.1. Platelets shed procoagulant MP at minimal activation

Triggering of freshly isolated PRP with tissue factor gives a delayed generation of thrombin [19,22]. Experiments indicated that, when platelets in plasma were incubated under slow rotation at room temperature for up to 24 h (conditions resembling those of platelet storage), the rate and extent of tissue factor-triggered thrombin generation greatly increased with the time of incubation (Fig. 1A). To determine the contribution of MP in the increased coagulant activity, incubated PRP samples were centrifuged at a high speed removing all platelets. The supernatant PFP was also increasingly active in thrombin formation (maximal levels 25–250 nM), after triggering with tissue factor (Fig. 1A). Control experiments showed that 10 μg/ml of PS-blocking annexin A5 completely inhibited thrombin generation, in both the incubated PRP and the centrifuged PFP samples (Fig. 1A). This indicated that all thrombin generation in plasma relied on the presence of procoagulant PS.

Flow cytometry was used to confirm the formation of MP in the rotated PRP. The MP were identified as small-size scatter events, staining positive for integrin β3. Double-staining indicated that most of the β3-positive events also stained with fluorescent-labeled annexin A5, pointing to exposed PS (Fig. 1B).

Prolonged, 6-h incubation of PRP resulted in a progressive accumulation of the MP, although their level remained lower than that of platelets stimulated with strong agonists (Fig. 1C, *left*). Interestingly, MP formation was also detected in suspensions of washed platelets that were long incubated in HEPES buffer under rotation. Thrombin generation measurements showed a high correlation between the MP levels, detected by flow cytometry, and the coagulant activity of the centrifuged PFP samples (Fig. 1C, *right*). As required, ultraspeed centrifugation of the PFP, to pellet all MP, resulted in completely abolished thrombin generation. Together, these data indicate that prolonged incubation of platelets in either plasma or buffer leads to shedding of MP with high thrombogenic activity.

To investigate how MP shedding influenced the function of platelets, we performed flow cytometric measurements of platelet activation markers. After 6-h incubation of PRP, only 5–8% of the platelets expressed P-selectin (a marker of exocytosis), activated αIIbβ3 integrin (detected with FITC-PAC1 mAb), or PS (Fig. 2A). This sharply contrasted the ~100% of platelets expressing these activation markers after stimulation with the strong agonists convulxin/ADP or ionomycin. Furthermore, the incubated PRP still had high aggregation responses with lower doses of ADP or collagen (Fig. 2B). It thus appears that the shedding of procoagulant MP is accompanied by minimal platelet activation, and is of little influence on agonist-induced platelet aggregation.

3.2. 'Spontaneous' MP formation is mostly independent of calpain but is controlled by αIIbβ3

Early reports indicate that strong platelet agonists activate the Ca^{2+}-dependent protease, calpain, which then plays a

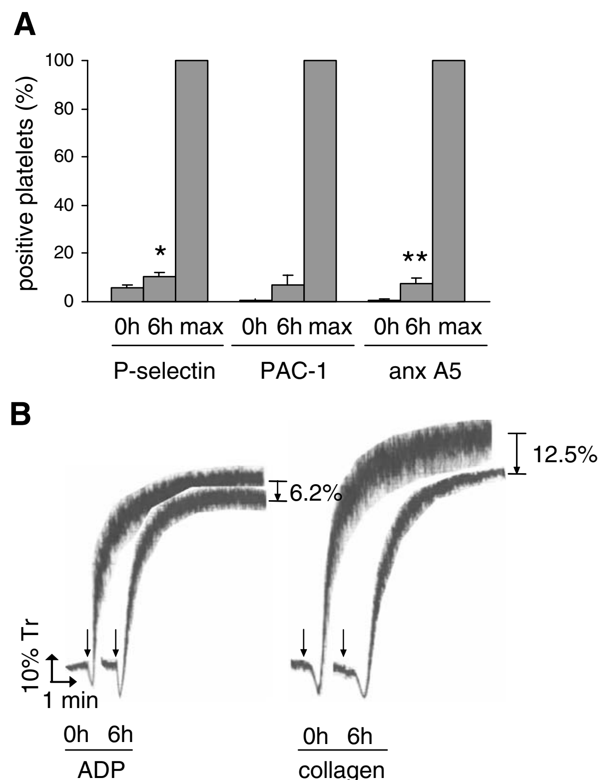

Fig. 2. Minimal activation state of MP-forming platelets. (A) Flow cytometric detection of activation markers on platelets, incubated in plasma for 6 h. Platelets expressing P-selectin, activated integrin αIIbβ3 (PAC1) or PS (Anx A5) after incubation in plasma for 0–6 h. Platelets were maximally activated (max), as described for Fig. 1. (B) Representative aggregation traces of PRP stimulated with ADP (10 μM) or collagen (2.5 μg/ml) after 6 h incubation. Data are means ± S.E.M. ($n = 3$). $^*P < 0.1$ and $^{**}P < 0.05$ compared to 0 h.

regulatory role in MP formation [6,23]. We determined that levels of cytosolic Ca^{2+} concentration in the platelets remained low during a 24-h incubation, i.e. changing from 26 ± 0.8 to only 29 ± 4 nM. Subsequently, we measured intracellular calpain activity before and after incubation of the platelets, using a newly developed procedure. Therefore, (incubated) platelets were loaded with the specific calpain substrate, *t*Boc-LM-CMC, and fluorescence development due to intracellular substrate cleavage was followed by stopped-flow fluorometry in the presence of EGTA to prevent contribution of possibly leaked calpain [20]. In agreement with earlier results [20], the Ca^{2+}-mobilizing agent, thapsigargin, caused an increase in basal calpain activity (Fig. 3A). In contrast, platelet incubation for 24 h resulted in a 33% decrease in calpain activity. As required, pretreatment of the platelets with the specific calpain inhibitor, calpeptin, abolished fluorescence development.

To determine whether the low calpain activity of resting platelets contributed to MP formation, PRP was incubated with calpeptin, and then evaluated on presence of coagulant MP. Calpeptin addition led to only a small, 35% reduction in the MP activity (Fig. 3B), indicating that the contribution of calpain-mediated protein cleavage in MP shedding is restricted.

Plasma contains ADP-degrading proteins that antagonize the effects of autocrine released ADP [15]. Yet, to determine the possible involvement of autocrine mediators, PRP was rotated in the presence of a cocktail of inhibitors of the ADP and thromboxane A_2 pathways [21]. This treatment had a small, about 30% reducing effect on the formation of coagulant MP (Fig. 3B). Treatment of PRP with the cAMP-elevating compound, iloprost, at concentrations giving a 3–4-fold increase in cytosolic cAMP, resulted in an about 65% suppression of MP formation (Fig. 3B). Control experiments further showed that this MP formation was not due to incomplete anti-coagulation, because the level of thrombin–antithrombin complexes remained far below threshold levels of coagulation initiation (<3 ng/ml) after 6 h or even 5 days of incubation of the PRP.

To study the involvement of αIIbβ3, the PRP was incubated with various clinically used blockers of this integrin: the high-affinity chimeric antibody, abciximab, a KGD-based peptide, eptifibatide, and the non-peptide antagonists, tirofiban and lotrafiban [24]. These antagonists potently decreased MP formation in 6-h incubated PRP with 65–80%, as determined from their coagulant activity (Fig. 4A). Also flow cytometry indicated that tirofiban reduced the number of MP in plasma with 87 ± 5% ($n = 7$). Further experiments showed that tirofiban inhibited thrombin generation, only when added to PRP before the 6-h incubation time (Fig. 4B). In contrast, tirofiban was without effect on coagulation when post-added to MP-containing PRP (Fig. 4C) or to PFP (not shown). These

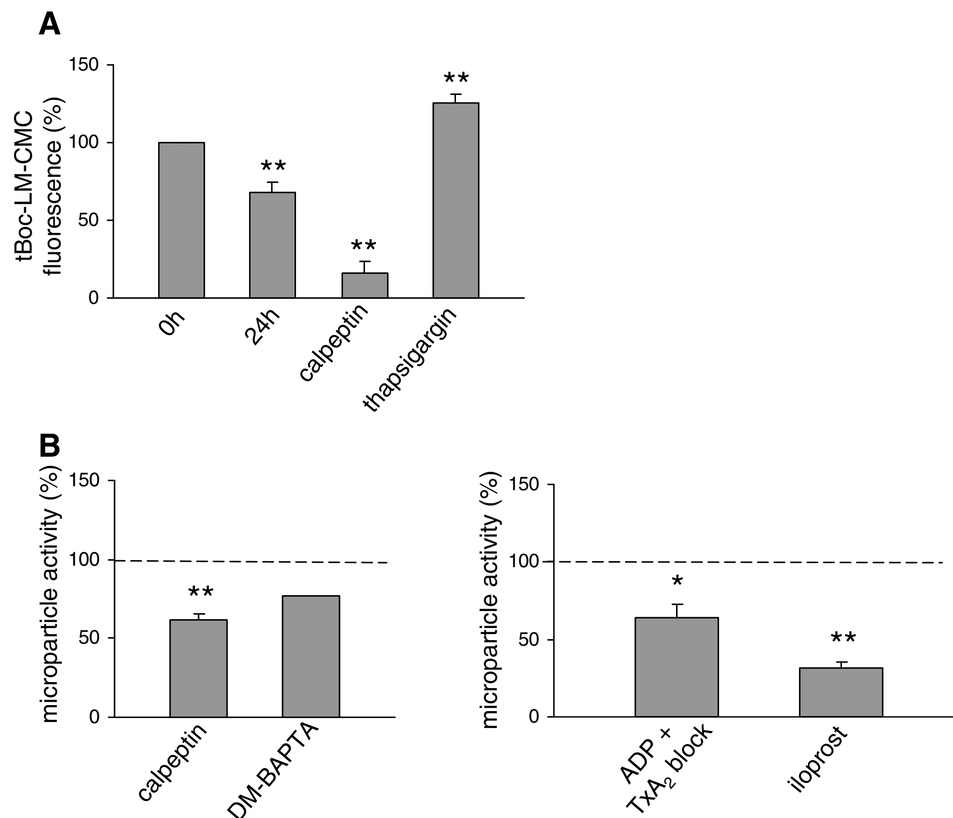

Fig. 3. Involvement of calpain and autocrine mediators in shedding of coagulant MP. (A) Calpain activity measurement. PRP, incubated for 0-24 h was loaded with *t*Boc-LM-CMC, and intracellular calpain activity was assessed in the presence of 1 mM EGTA by stopped-flow fluorometry. Samples were pre-treated with calpain inhibitor, calpeptin (140 μM), or activated with thapsigargin (0.2 μM), as indicated. Data are expressed as percentages of 0-h control. (B) MP coagulant activity. PRP was incubated for 6 h with vehicle or calpeptin (140 μM); alternatively, with iloprost (10 μM) or a mixture of MRS2179 (20 μM), AR-C66931MX (30 μM), apyrase (1 U/ml) and aspirin (100 μM) to block ADP and thromboxane A_2 effects. Platelets were preloaded with DM-BAPTA1, where indicated. Data are expressed as percentages of control (thrombin peak levels, Fig. 1A). Means ± S.E.M. ($n = 5$, $^{*}P < 0.1$ and $^{**}P < 0.05$ vs. control).

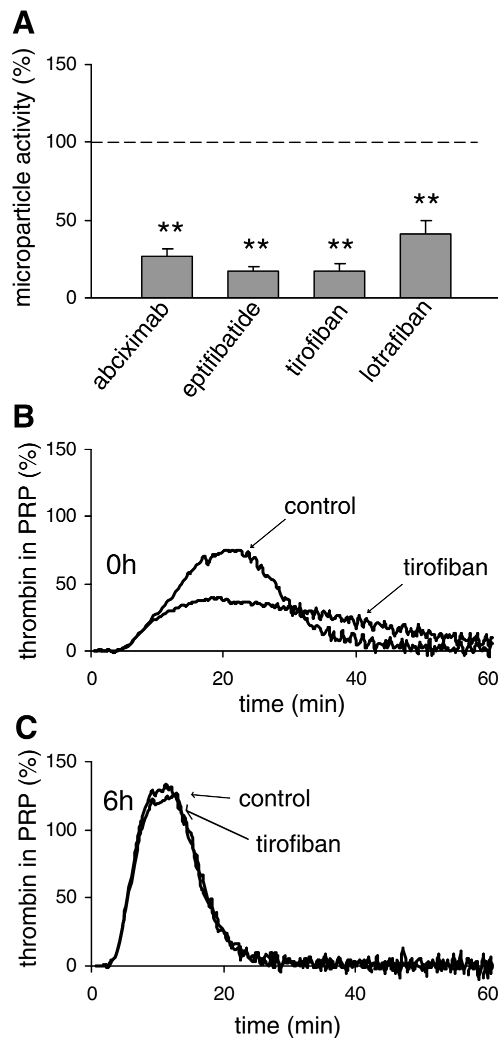

Fig. 4. Involvement of integrin αIIbβ3 in spontaneous MP formation. PRP was incubated for 6 h with or without αIIbβ3 inhibitors, abciximab (20 μg/ml), tirofiban (2 μM), lotrafiban (20 μM) or eptifibatide (6 μM). (A) Coagulant activity of MP in plasma (PFP) after 6 h of incubation. (B) Effect of tirofiban added to fresh PRP. (C) Effect of tirofiban added after incubation of PRP for 6 h. Shown are representative thrombin generation curves. Data are expressed as percentages of control PRP (6-h incubation without inhibitors), and are means ± S.E.M. ($n = 4$, $^{**}P < 0.05$).

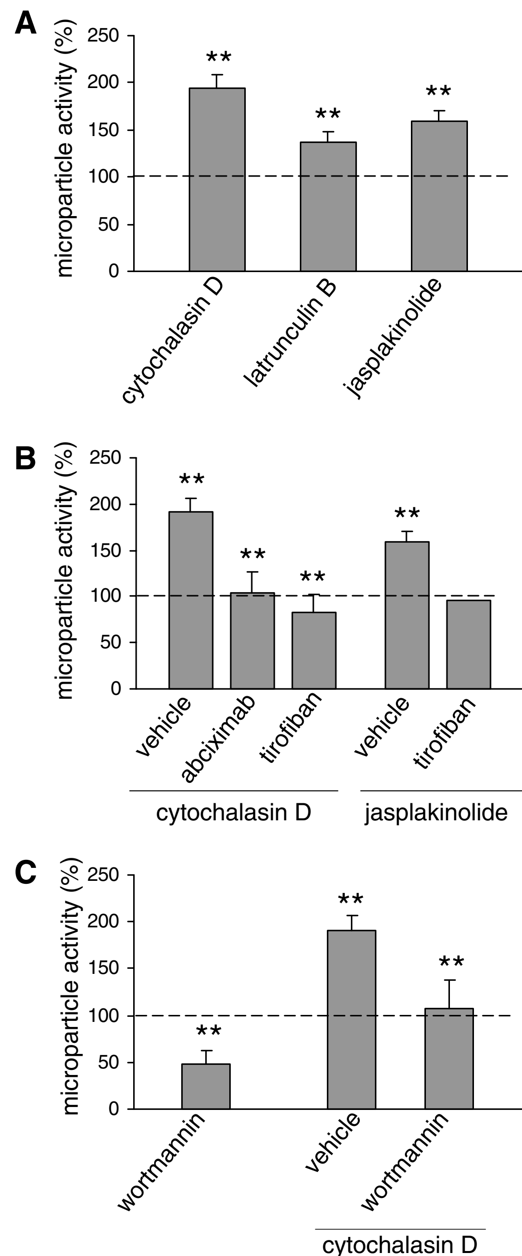

Fig. 5. Involvement of actin cytoskeleton in MP formation. (A) PRP was incubated for 6 h with/without cytochalasin D (10 μM), latrunculin B (0.5 μM) or jasplakinolide (10 μM). (B) PRP was incubated for 6 h with cytochalasin D in the presence of integrin blockers (fig. 4). (C) PRP was incubated with cytochalasin D plus wortmannin (1 μM). Data indicate MP coagulant activity as percentages of control PRP (incubation without drugs). $^{**}P < 0.05$ vs. control PRP,$^{**}P < 0.05$ vs. vehicle ($n = 3$–4).

findings suggest that the anticoagulant effect of integrin blockers is through inhibition of MP formation, and not by directly interfering with the thrombin generation process.

3.3. Involvement of αIIbβ3-mediated actin cytoskeleton turnover and outside-in signaling

As the actin cytoskeleton is of key importance for platelet structural integrity, we examined how compounds interfering with actin filament turnover influenced the shedding of MP. Platelets in plasma were incubated with cytochalasin D, an agent that caps actin filaments and prevents their polymerization [25]. Remarkably, this resulted in a potent, twofold increased formation of procoagulant MP (Fig. 5A). A similar, stimulating effect was obtained, when platelets were incubated with latrunculin B, which inhibits filament polymerization by binding to actin monomers; or with jasplakinolide, which freezes platelet actin filaments but does allow severing [26]. All compounds rearranged actin to a layer close to the plasma membrane, as was confirmed by staining of the platelets with fluorescent phalloidin.

Interestingly, the enhancing effects of cytochalasin D and jasplakinolide on MP shedding were reversed by integrin blockade with abciximab or tirofiban (Fig. 5B). This led to the suggestion that αIIbβ3 activity causes MP formation via destabilization of the actin cytoskeleton. The involvement of Src kinase and phosphoinositide 3-kinase was then considered,

which protein kinases have been implicated in signaling of αIIbβ3 to cytoskeletal changes [11]. Incubation of PRP with the 3-kinase inhibitor wortmannin halved MP formation, and also reversed the enhancing effect of cytochalasin D (Fig. 5C). Inhibition of Src kinases with 20 μM PP1 or PP2 (but not the control substance PP3) reduced microvesiculation by 39 ± 7% ($n = 5$, $P < 0.01$).

The cytochalasin D-enhanced and tirofiban-suppressed MP formation did not involve calpain activity, as calpeptin (140 μM) treatment was without influence on this process. Furthermore, platelet treatment with cytochalasin D or tirofiban changed the intracellular calpain activity insignificantly with 4% and 7 ± 3%.

Although the αIIbβ3 activation state of platelets in plasma in general is low (compare Fig. 2A), the above results suggest that a low, residual integrin activity is sufficient to cause MP formation. Support for this came from flow-cytometric measurements, showing that PAC1 binding to platelets stored in plasma was slightly above basal; the mean PAC1 binding reduced with 3 channel numbers by treatment of the platelets with cAMP-elevating iloprost.

The spreading of platelets over a fibrinogen surface is an event controlled by integrin outside-in signaling [27]. We investigated whether this signaling during spreading also leads to MP formation. High-resolution, phase-contrast microscopy showed that many platelets, in a late stage of spreading, often developed circular arrays of cell-attached MP after forming lamellipods (Fig. 6A, see also movie A). Confocal fluorescence microscopy indicated that these MP express PS and form at sites of disintegrated actin filaments (Fig. 6B). Dual labeling demonstrated PAC1 staining of the PS-positive MP (Fig. 6C). Control experiments confirmed that integrin blockers antagonized both the spreading and MP formation.

Finally, thrombin generation experiments were performed with PRP from thrombocytopenic patients, who were transfused with MP-containing platelet preparations stored at blood bank conditions. After transfusion, the thrombin peak level in both PRP and PFP was notably increased, pointing to the presence of circulating MP in the patient blood (Fig. 7). Thus, MP produced during platelet storage actively contribute to the coagulation process, when transfused into patients.

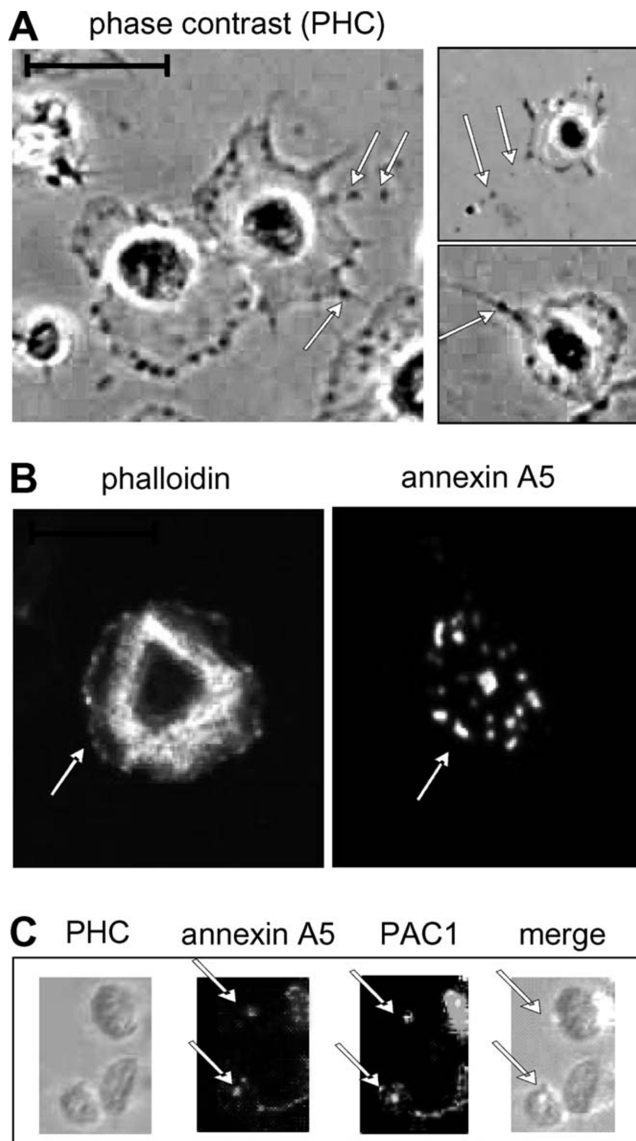

Fig. 6. MP formation of spreading platelets. Platelets in HEPES buffer were immobilized on a fibrinogen surface for 45 min at room temperature. (A) Representative phase-contrast image of spreading platelets with MP (arrows). (B) Microscopic images of actin staining with AF-phalloidin, and of PS exposure with OG-annexin A5. (C) Dual confocal staining of MP-forming platelets with AF-annexin A5 and FITC-anti β3. Bars represent 5 μm.

4. Discussion

This report describes the novel finding that human platelets during storage form procoagulant MP as a 'spontaneous' reaction, in a way dependent on integrin αIIbβ3 and cytoskeleton turnover, but at minimal platelet activation and calpain activity. This process is of clinical relevance, since MP-producing platelet preparations are routinely transfused to patients, and can enhance coagulation.

Flow cytometry was used to detect the gradual accumulation of MP in stored platelets. Complementary to this, thrombin generation measurements allowed quantification of the amounts of procoagulant MP in plasma. We find that the spontaneous MP formation is an ongoing process in platelets, which relies on integrin signaling even when platelet activation is minimal. Only a minority of the MP-producing platelets bound PAC1 mAb, or had signs of surface expression of P-selectin or PS. Furthermore, the aggregation tendency of MP-producing platelets was no more than slightly altered. The results do not indicate whether MP are produced by a small fraction of the platelets or by all platelets. In favor of the latter possibility is the overall decrease in mean platelet volume of about 10%, which is detectable after prolonged incubation.

The mechanism of MP shedding from resting platelets, typically, differs from the shedding mechanism that is described for platelets treated with potent agonists like Ca^{2+}-ionophore or collagen/thrombin. In these cases, it has been shown that basal calpain activity is insufficient for its protease activity to degrade cytoskeletal components, and that agonist-induced Ca^{2+} influx is required to reach the high, (sub)micromolar $[Ca^{2+}]_i$ levels triggering this protease activity. Similarly, with

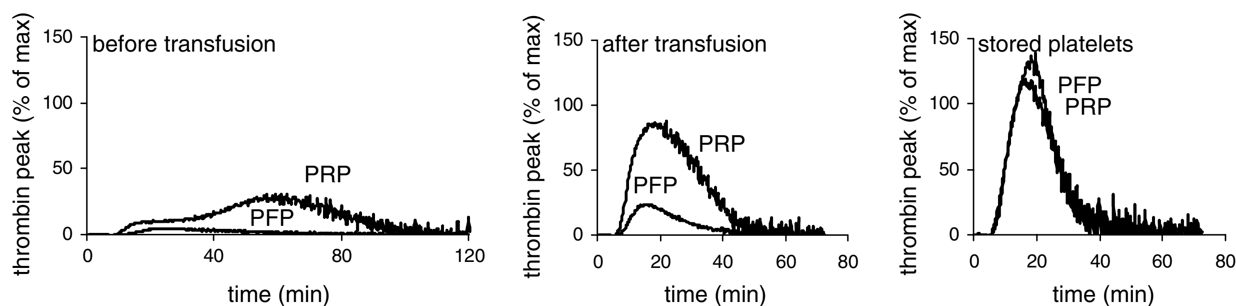

Fig. 7. Thrombin generation of PRP and PFP from a thrombocytopenic patient before and after transfusion with a platelet-plasma preparation. Platelet counts in patient PRP before and after transfusion were 16 and 64×10^9/l, respectively. PRP and PFP samples pre- and post-perfusion were triggered with tissue factor, as described for Fig. 1A. Also shown is thrombin generation of the PRP and plasma that was used for transfusion. Increased thrombin generation in post-transfusion PFP points to activity of coagulant MP. Representative thrombin generation curves are given (triplicate assays).

these potent agonists, Ca^{2+} influx was required to cause Ca^{2+}/calpain dependent MP formation [5,6,12,23,28]. In contrast, in the present setting, we find that Ca^{2+} levels remain low in the stored platelets (with low extracellular Ca^{2+} in the storage medium), while the basal calpain activity even decreases. Furthermore, we find that Ca^{2+} chelation and calpain inhibition have only minor effects on MP formation. Together, this indicates that the Ca^{2+}/calpain pathway plays only a minor role in MP shedding during platelet storage.

We find that integrin αIIbβ3 plays an important role in the shedding process of resting platelets. Some authors have reported that αIIbβ3 activation and aggregation are also involved in MP production by platelets stimulated with potent agonists [12,29], while others have not found such involvement of the integrin [30,31]. In activated platelets, αIIbβ3 can furthermore regulate calpain activation [32]. Based on these and other findings, Fox [6] and Wiedmer [28] and colleagues have suggested that there may be more than one mechanism of MP formation. Taken together with the present results, we conclude that there must be a separation between the Ca^{2+}/calpain and the αIIbβ3 pathways, although both may operate alongside each other or synergize. Mostly the αIIbβ3-mediated, calpain-independent pathway is then responsible for MP formation by stored platelets.

There is recent evidence to indicate that integrins on a cell surface are in constitutive equilibrium between inactive and active conformations [33]. This raises the possibility that patches of active (signaling) integrins on otherwise unstimulated platelets can trigger local shedding of MP. This would also explain why inhibition of Src or phosphoinositide 3-kinase signaling suppresses MP formation, likely via interference in outside-in signaling. However, it should be noted that, in stimulated platelets, these protein kinases also contribute to inside-out signaling [34].

A remarkable finding is that the αIIbβ3-mediated MP formation increases with compounds like cytochalasin D, interfering with the actin cytoskeleton turnover. It is known that actin-binding proteins, such as filamin and cofilin, provide physical interaction of the cytoskeleton with the β3 cytoplasmic tail of integrin αIIbβ3 [25]. Particularly, the phosphoinositide 3-kinase signaling pathway links between αIIbβ3 and actin dynamics [11]. This leads to the idea that integrin signaling via this pathway locally destabilizes the cytoskeleton (via cofilin), with as a result disconnection of the plasma membrane and shedding of MP. Support for such a mechanism comes from the hitherto unrecognized detection of MP at the edges of platelets spread on fibrinogen and the observed effect of Src kinase inhibitors. Thus, activated integrins may act by suppression of the (spontaneous) rearrangement of the actin cytoskeleton and, conversely, cytoskeleton turnover suppresses integrin activation and hence MP formation.

Integrin blockers have a clinically relevant, anticoagulant effect in the circulation by suppressing the availability of procoagulant lipid surface [35]. The present findings indicate that this anti-integrin effect particularly relies on inhibition of MP formation. We can thus conclude that integrin-mediated shedding of MP is a uniquely regulated cellular response that contributes to coagulant activity in a clinically relevant way.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.08.082.

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