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Synergistic effect of signaling from receptors of soluble platelet agonists and outside-in signaling in formation of a stable fibrinogen-integrin αIIbβ3-actin cytoskeleton complex☆

Ivan Budnik [a,b,c], Boris Shenkman [d], Naphtali Savion [a,b,*]

[a] Goldschleger Eye Research Institute, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[b] Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[c] Department of Pathophysiology, I. M. Sechenov First Moscow State Medical University, Moscow, Russia
[d] National Hemophilia Center, Sheba Medical Center, Tel-Hashomer, Israel

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ABSTRACT

Introduction: Thrombus formation in the injured vessel wall is a highly complex process involving various blood-born components that go through specific temporal and spatial changes as observed by intravital videomicroscopy. Platelets bind transiently to the developing thrombus and may either become stably incorporated into or disengage from the thrombus. The aim of the present study was to reveal the processes involved in the formation of a stable thrombus.

Methods: Platelet-rich plasma and washed platelets were studied by the aggregometer. The aggregate stability was challenged by eptifibatide. Platelet Triton-insoluble fraction was prepared and the actin and αIIb content in the cytoskeleton was analyzed by western blot.

Results: Maximal actin polymerization is achieved 1 min after platelet activation while maximal αIIbβ3–actin cytoskeleton association requires 5 to 10 min of activation and fibrinogen-mediated platelet-to-platelet bridging. Thus, actin polymerization is dependent on platelet activation and requires neither αIIbβ3 integrin occupation nor platelet aggregation. Formation of a stable aggregate requires platelet activation for more than 1 min, complete increase in actin cytoskeleton fraction and partial association of αIIbβ3 with the actin cytoskeleton. However, direct αIIbβ3 activation is not sufficient for cytoskeleton complex formation. Thus, stable αIIbβ3–fibrinogen interaction, representing stable aggregate, is achieved after more than 1 min agonist activation, involving inside-out and outside-in signaling but not after direct integrin activation, involving only outside-in signaling.

Conclusions: Formation of a stable fibrinogen–αIIbβ3–actin cytoskeleton complex is the result of the combined effect of platelet stimulation by soluble agonists, activation of αIIbβ3, fibrinogen binding and platelet-to-platelet bridging.

Introduction

Formation of a platelet plug involves platelet stimulation through various activating receptors leading to a shift of integrin αIIbβ3 from a bent, non-active conformation to an extended, active conformation, a process referred to as "inside-out" signaling. Integrin activation allows ligand binding mediating platelet attachment to the thrombogenic surface and platelet-to-platelet bridging (aggregation). Ligand-bound integrins, in turn, initiate a series of intracellular events, collectively referred to as "outside-in" signaling, including interaction of αIIbβ3 with actin-based cytoskeleton and leading to platelet spreading, perpetuation of the platelet aggregates and contraction of the platelet plug [1,2]. All these events strongly depend on physical linking of fibrinogen, integrin αIIbβ3 and cytoplasmic actin filaments into a stable complex which serves for transmission of contractile force from platelet to platelet within aggregates and from platelet to the thrombogenic surface [3].

Despite numerous elegant studies showing the molecular mechanisms of αIIbβ3 conformational switches, interaction with its ligands, signaling molecules and membrane skeleton proteins [4–8], regulation of the fibrinogen–αIIbβ3–actin cytoskeleton complex formation at the cellular level has not been fully characterized. It was shown that upon activation platelet cytoskeleton undergoes significant rearrangement including severing of existing actin filaments and assembly of new

Abbreviations: ACD, acid-citrate-dextrose; ADP, adenosine diphosphate; ASA, acetylsalicylic acid; FFD, fibrinogen fragment D; LIBS6, ligand-induced binding site 6; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SD, standard deviation; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% (v/v) Tween 20; TRAP, thrombin receptor-activating peptide; TXA_2, thromboxane A_2.

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* Corresponding author at: Goldschleger Eye Research Institute, Sheba Medical Center, Tel Hashomer 52621, Israel. Tel.: +972 3 5302954; fax: +972 3 5351577.
E-mail address: eyeres@post.tau.ac.il (N. Savion).

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ones. These newly synthesized filaments drive filo- and lamellipodia protrusion and organize into stress-fiber like structures required for generation of contractile force [9]. Actin polymerization was also described in platelets stimulated in non-aggregating conditions [10,11] and in Glanzmann thrombasthenia platelets [12] suggesting that it is an αIIbβ3-independent event. It has been shown that actin filaments interact with numerous cytosolic and membrane proteins including αIIbβ3 which co-isolate with the Triton X-100-insoluble cytoskeleton core [13]. It was indicated that platelet aggregation is an essential event because ligand binding alone was not sufficient to induce interaction of αIIbβ3 with cytoskeleton. It was also demonstrated that cytoskeleton association of αIIbβ3 requires not only αIIbβ3 conformational switch to an active state allowing fibrinogen binding and aggregation but also platelet stimulation via activating receptors since aggregation induced by D3 monoclonal antibody (mAb), which directly activates αIIbβ3 without inducing general platelet activation, failed to show αIIbβ3 association with cytoskeleton [14]. Despite the importance of platelet stimulation via activating receptors for αIIbβ3 cytoskeleton association, the relative contribution of signaling from these platelet receptors to the efficient formation of fibrin(ogen)–αIIbβ3–actin complex has not been fully investigated. The recent studies using high-resolution intravital imaging techniques demonstrating the progressive process of aggregation-disaggregation occurring at the injured vessel wall till firm aggregate is formed [15] and the variables influencing the efficiency of conversion of reversible discoid platelet aggregates to stable aggregation [16] are all pointing the need to further explore the factors controlling the stability of the fibrinogen–αIIbβ3–actin cytoskeleton complex.

In this paper, we sought to determine the relative role of signaling initiated by platelet activating receptors leading to inside-out integrin activation, on one hand, and integrin-initiated outside-in signaling, on the other hand, in the induction of actin polymerization, integrin αIIbβ3 cytoskeleton association and ultimately the formation of a stable fibrinogen–αIIbβ3–actin cytoskeleton complex.

Materials and Methods

Antibodies

The following antibodies were used in the study: anti-αIIb mAb, clone SZ22 (Beckman Coulter, Indianapolis, IN, USA); anti-β-actin mAb, clone AC-15 and anti-mouse IgG, peroxidase-conjugated (both from Sigma-Aldrich, St. Louis, MO, USA); anti-ligand-induced binding site 6 (anti-LIBS6) mAb, kindly provided by Dr Mark Ginsberg (University of California, San Diego).

Platelet Agonists and Inhibitors

Thrombin receptor-activating peptide (TRAP; Sigma-Aldrich, Indianapolis, IN, USA) and anti-LIBS6 mAb were used as platelet agonists. Eptifibatide (Integrillin®, Schering-Plough, Kenilworth, NJ, USA), acetylsalicylic acid (ASA; Sigma-Aldrich, St. Louis, MO, USA), AR-C66096 and MRS2500 (both from Tocris Bioscience, Bristol, UK) were used as platelet function inhibitors.

Collection of Blood

Blood was obtained from adult healthy male and female volunteers (ranging in age from 18 to 65 years) who were free of any medication for at least two weeks prior to blood donation and signed an informed consent form in accordance with the Declaration of Helsinki. The study was approved by the Sheba Medical Center ethics committee. Whole blood was withdrawn by a puncture of the median cubital vein and collected into Vacuette blood collection tubes containing 3.2% trisodium citrate in a volume ratio of the anticoagulant to blood of 1:9 or into Vacuette tubes containing acid-citrate-dextrose (ACD) solution A in a volume ratio of the anticoagulant to blood of 1:5 (both from Greiner Bio-One, Frickenhausen, Germany). All blood samples were processed within 4 hours of collection. All manipulations were carried out at room temperature.

Preparation of Platelet-rich Plasma (PRP) and Platelet-poor Plasma (PPP)

Citrate-anticoagulated blood was allowed to stand for 30 min and then centrifuged at 134 ×*g* for 12 min. The upper fraction was collected as PRP. PPP was obtained by centrifugation of the remaining blood at 2288 ×*g* for 15 min. Platelet concentration was adjusted to 2.5×10^8 mL^{-1} with autologous PPP.

Preparation of Washed Platelets

ACD-anticoagulated blood was allowed to stand for 20 min, supplemented with 0.2 μg/mL PGE_1 and 0.6 U/mL apyrase (both from Sigma-Aldrich, St. Louis, MO, USA), further incubated for 10 min and then centrifuged at 134 ×*g* for 12 min to generate PRP (the supernatant). The PRP sample was diluted in equal volume of Tyrode's solution (140 mM NaCl, 2.9 mM KCl, 0.36 mM NaH_2PO_4, pH 6.5) supplemented with 5 mM dextrose, 0.2 μg/mL PGE_1 and 0.6 U/mL apyrase, incubated for 5 min and then centrifuged at 607 ×*g* for 10 min. Pelleted platelets were resuspended in Tyrode's solution with the supplements, incubated for 10 min, centrifuged at 607 ×*g* for 10 min and then the pelleted platelets were resuspended in Tyrode's-HEPES buffer (10 mM HEPES, pH 7.4) supplemented with 5 mM dextrose. Platelet concentration was adjusted to 3×10^8 mL^{-1}. Suspension of washed platelets was allowed to stand for 60 min before usage.

Platelet Aggregation

Platelet aggregation was evaluated by AggRAM analyzer (Helena Laboratories, Beaumont, TX, USA) at 37 °C. Aliquots of PRP or washed platelets were preincubated in the aggregometer cuvettes for 1 min then platelet aggregation was induced by TRAP or anti-LIBS6 mAb under continuous stirring at 600 rpm (unless otherwise specified) and monitored for at least 5 min. The aggregometer was calibrated for each sample before agonist addition by adjusting the light transmission to 0% for PRP and to 100% for PPP. Alternatively, the aggregometer was calibrated by adjusting the light transmission to 0% for suspension of washed platelets and to 100% for Tyrode's-HEPES buffer for each sample of washed platelets, which was supplemented with fibrinogen (Sigma-Aldrich) or fibrinogen fragment D (FFD; EMD Millipore, Billerica, MA, USA).

Fractionation of Platelets

Isolation of the platelet actin cytoskeleton complex was carried out using a modification of the method described by Fox [17]. Platelets were lyzed by addition of an equal volume (220 μL) of ice-cold 2X Triton X-100 lysis buffer [2% (v/v) Triton X-100, 5 mM EGTA (pH 7.8), 5 mM EDTA (pH 7.8), 100 mM Tris-HCl (pH 7.35), 4 mM sodium orthovanadate, 4% (v/v) protease inhibitor cocktail (Cat. P8340), 60 μM calpeptin, 2 mM PMSF (all from Sigma-Aldrich)] to the platelet suspension. The lysates were vortexed for a second, left on crushed ice for 30 min and then centrifuged at 10000 ×*g* for 10 min at 4 °C. The Triton X-100-insoluble pellets were washed once with 1X Triton X-100 lysis buffer and solubilized in 220 μL of 2X sodium dodecyl sulfate (SDS) sample buffer [4% (w/v) SDS, 20 mM EDTA (pH 7.8), 20% (v/v) glycerol, 125 mM Tris-HCl (pH 6.8), 4 mM sodium orthovanadate, 4% (v/v) protease inhibitor cocktail, 60 μM calpeptin, 2 mM PMSF]. The samples were supplemented with 5% (v/v) β-mercaptoethanol and promptly boiled at 100 °C with intermittent vigorous vortexing until complete dissolution of the Triton X-100-insoluble material and then stored at −20 °C until electrophoresis.