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Roll, adhere, spread and contract: Structural mechanics of platelet function

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ABSTRACT

Platelets are involved in life-sustaining processes such as hemostasis, wound healing, atherothrombosis and angiogenesis. Mechanical trauma to blood vessels causes platelet activation resulting in their adherence and clot formation at the damaged site, culminating in clot retraction and tissue repair. Two of the major players underlying this process are the cytoskeleton, i.e., actin and microtubules, and the membrane integrin receptors. Rare congenital bleeding disorders such as Glanzmann thrombasthenia and Bernard–Soulier syndrome are associated with genetic alterations of platelet surface receptors, also affecting the platelet cytoskeletal structure. In this review, we summarize the current knowledge about platelet structure and adhesion, and delve into the mechanical aspects of platelet function. Platelets lack a nucleus, and can thus provide a minimal model of a biological cell. New biophysical tools may help to scrutinize platelets anew and to extend the existing knowledge on cell biology.

Introduction

The first observation of blood clotting dates back two millennia when the Greek philosopher Plato noticed that the blood forms "fibers" once it leaves the body warmth. In modern medical science, platelets (as colorless, little spherules of 2–3 μm diameter) were described for the first time by Schultze (1865), who observed them with a twin lens (compound) microscope. Their role in hemostasis was later identified by Bizzozzero (1881) who studied these spherules in circulating blood. Bizzozero named these spherules *piastrine* (little plates, hence the current term platelets), and showed that these blood elements did not possess a nucleus. He assessed their involvement in blood clotting, observing that platelets adhered to each other to form a thrombus together with a fibrous material (fibrinogen).

Although the principal function of platelets is in hemostasis (Machlus and Italiano, 2013), they have also been shown to be involved in inflammation, innate immunity and tumor metastasis. Upon injury to a blood vessel, the first events leading to the repair of the damage is the adhesion of the circulating platelets to the subendothelial matrix. Platelet adhesion is an intricate although well-orchestrated process: it begins with the agonist activation of the G-protein coupled receptors (GPCRs), ensuing a cascade of intracellular biochemical events leading to the so-called 'inside-out' signaling, which further causes integrin activation and binding to the extracellular matrix (ECM) ligands. This subsequently leads to 'outside-in' signaling, the process climaxing in platelet activation. Platelet activation is associated with distinct structural changes mainly in the form of extensive rearrangement of the actin cytoskeleton (Fig. 1). The dynamic remodeling of the actin network facilitates platelet metamorphosis from a free-floating disc to its rounding upon attachment to the ECM, spreading with the projection of pseudopodia and lamellipodia, and finally strong adhesion and contraction of the ECM resulting in the sealing of the injury (Allen et al., 1979; Escolar et al., 1986; Hartwig, 1992; Fig. 2). This review focuses on recent insights into the mechanical properties of platelets which have been made possible by advanced nanotechnological tools.

Platelet integrins

Integrins are the principal components of platelets that sense blood vessel damage. These heterodimeric, transmembrane

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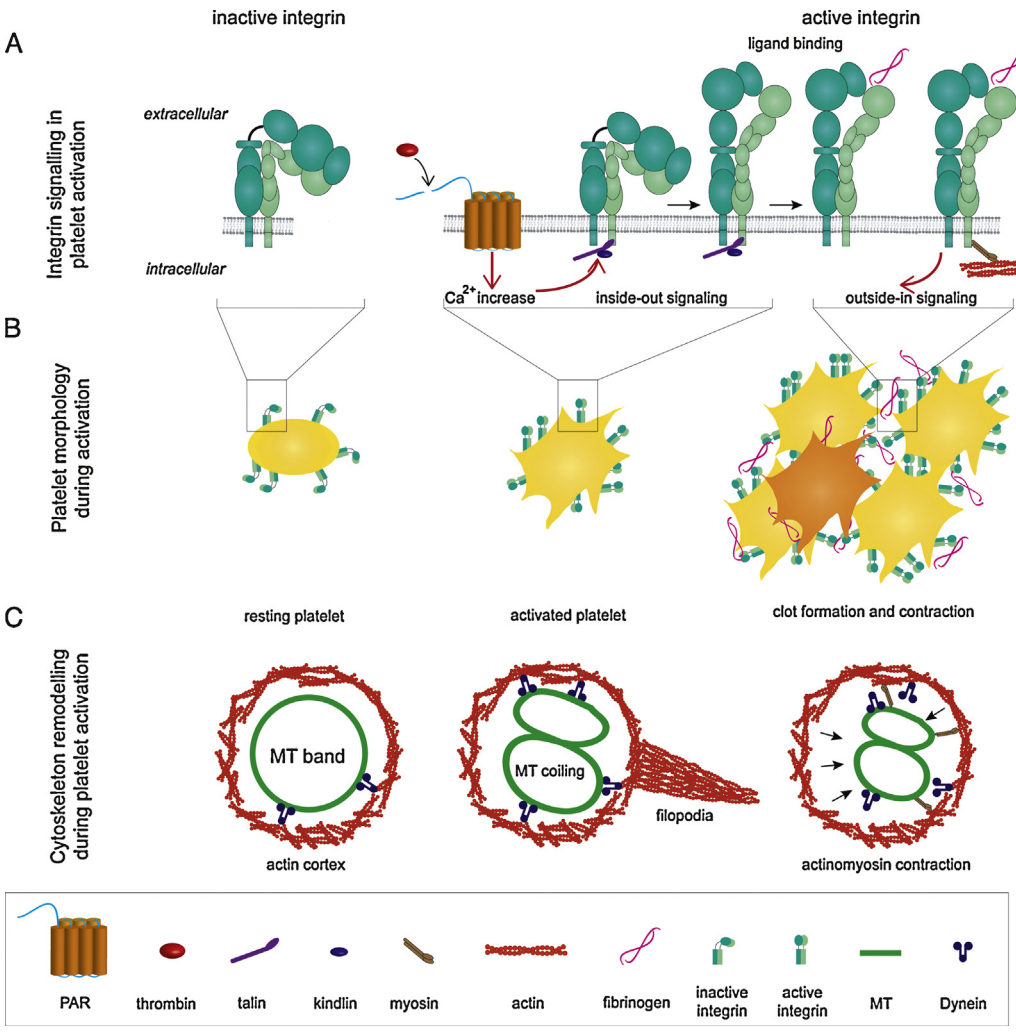

Fig. 1. *A simplified schematic overview of the molecular events during platelet activation and aggregation.* (A and B) In a resting platelet, the αIIbβ3 integrin is bent and in an inactive conformation. αIIbβ3 can be activated in two different ways; (1) the inside-out signaling events originate from agonist binding to platelet receptors inducing integrin activation. Shown is an example of thrombin-mediated activation of the protease-activated GPCRs (ADP binding to prurinergic receptors also leads to inside-out signaling), which triggers an increase in the cytosolic Ca^{2+} further promoting talin binding to the cytoplasmic tail of the β3 chain. This event converts the αIIbβ3 integrin from a bent state to an extended state. (2) In outside-in signaling, αIIbβ3 is activated upon binding of soluble ligands (*e.g.*, fibrinogen). The conformational switch of αIIbβ3 from an inactive, bent state to an active, extended state is of key importance in hemostasis because it enables the platelets recruited at an injury site to interact with other platelets through fibrinogen leading to clot formation. (B and C) The cytoskeleton of a resting platelet is composed of the actin cortex, formed of short and tightly packed actin filaments, and a MT band situated in the platelet center. Upon platelet activation, the dyneins slide on the actin causing the coiling of the MT band. At the same time, extensive actin rearrangement causes the protrusion of filopodia, where the actin forms long, parallel filaments. Finally, platelet contraction is driven by the actomyosin machinery, which uses the MT coil as a structural support.

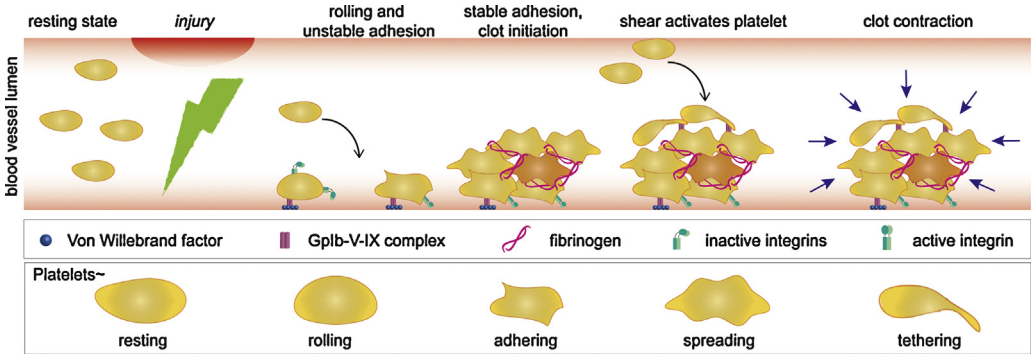

Fig. 2. *Physical biology of platelet function.* Normally, platelets circulate in the blood vessels in a resting state. Upon blood vessel injury, extracellular matrix components (not shown in the figure) are exposed, and von Willebrand factor (vWF) is released, enabling the platelets to roll at the injury site through low-affinity interactions (such as the one between vWF and GpIb-V–IX complex). This low affinity binding of the platelets gives time for integrin activation leading to stronger platelet adhesion (fibrinogen and integrin) at the injury site. Procoagulant factors are released (not shown in the figure), and more platelets are recruited at the injury site consequently initiating clot formation. Upon clot formation, the shear forces increase at the injury site causing the adhesion of tethering platelets to the clot. The clot subsequently contracts to close the vessel and heal the injury.

glycoprotein receptors comprise non-covalently interacting α and β subunits with large extracellular domains and short cytoplasmic tails connected through a single α-helical transmembrane domain (Hemler, 1990). αIIbβ3 is the most abundant integrin in the platelets with a population of ~80,000 copies per platelet (Wagner et al., 1996). It is the only integrin present exclusively in platelets, and at the molecular level is the major mediator of platelet adhesion and aggregation. The receptor binds to fibrinogen, fibronectin and von Willebrand factor (vWF) by recognizing the RGD (Arg-Gly-Asp) peptide sequence (Plow et al., 2000). The extracellular domain in the αIIb subunit consists of a β-propeller, a thigh domain and two calf domains (calf-1 and calf2). The β-subunit is composed of a βA domain (resembling the αA domain of several integrin subunits, *e.g.*, α1) that is connected through a hybrid domain (which resembles an I-set Ig domain) to a PSI domain (plexin, semaphorin, integrin), and 4 tandem EGF-like domains that, together with a βTD domain, form the "leg" of the β subunit (for a review see (Bennett et al., 2009)). The integrin β-subunit cytoplasmic tail connects the receptor to the actin cytoskeleton *via* linker protein complexes (Brown et al., 2002; Jenkins et al., 1998; Sharma et al., 1995). This connecting architecture provides the basis for platelet function during adhesion, spreading, pulling of the ECM and clot retraction by establishing an interface between the intracellular actin network and the extracellular matrix (Choquet et al., 1997; Jackson et al., 2009) (Fig. 1).

The second most abundant integrin α2β1 (2000–4000 copies per platelet) is one of the major collagen adhesion receptors (Santoro, 1986). Other integrins which are expressed in platelets are: the $\alpha_v\beta_3$, a vibronectin receptor identified in osteoclasts, activated endothelial cells and some cancer cell lines (*e.g.*, osteosarcoma, melanoma and glioblastoma; Pytela et al., 1985); the α5β1, a fibronectin receptor (Piotrowicz et al., 1988); the α6β1, a laminin receptor (Sonnenberg et al., 1988); and the αLβ2, that has been shown to be expressed only in activated platelets (Philippeaux et al., 1996).

Integrins as mechanical and biochemical relays

During platelet activation, when ligands bind to integrins, an intracellular signaling cascade is activated that, first, enhances the affinity of the integrin to its ligand, and subsequently promotes platelet adhesion and aggregation (Ruggeri and Mendolicchio, 2007). Integrins achieve a high-level of competence as mechanical transducers and biochemical modulators by aiding in the building of the focal adhesion complex which serves as a structural and functional link to the cytoskeleton (Coller and Shattil, 2008; Watson et al., 2005). The integrin "adhesome" defines more than 90 proteins, along with kinases, proteases and phosphatases to regulate their function (Zaidel-Bar et al., 2007); more than 40 of these proteins interact directly with the β tail. The adaptors can be divided into three main classes: structural adaptors, which connect integrins to the actin cytoskeleton (talin (Brown et al., 2002), α-actinin (Rajfur et al., 2002), filamin (Sharma et al., 1995) and myosin (Jenkins et al., 1998)); scaffolding adaptors, which provide binding sites for the other focal adhesion proteins, (kindlin 3 (Moser et al., 2008) and paxillin (Schaller et al., 1995)); and catalytic adaptors (FAK, focal adhesion kinase (Chen et al., 2000)). (For more details see the commentary (Legate and Fassler, 2009)).

The integrin functional states are associated with major conformational changes, which provide a trigger (and feedback) to the platelet for mechanical and biochemical signaling. Platelet function and the associated morphological changes are tightly correlated with the functional state of the integrins. Integrin receptors reside in a bent conformation in inactive platelets (Springer and Dustin, 2012); upon stimulation, the receptors switch to an erect conformation with a higher affinity for the ECM ligands (Takagi et al., 2002) (Fig. 1). The precise molecular mechanism underlying this conformational change is not well outlined; however two models, the deadbolt and the switchblade, have been proposed. The deadbolt model implies that, in the resting state the loop of the β3 extracellular domain restrains the headpiece domain close to the membrane; following platelet activation (inside-out signaling) this loop-enforced restrain is lost. As a result, the headpiece of the integrin changes its conformation with an ensuing increase in ligand affinity (Adair et al., 2005; Arnaout et al., 2007). The switchblade model suggests that the integrin receptor extends before binding the ligand. In particular, the headpiece of the transmembrane domain of the integrin complex drifts apart from the membrane and extends out, developing a high affinity to the ligands (Luo et al., 2007; Xiao et al., 2004; Zhu et al., 2007).

The transition from the low to the high ligand-affinity state is key to correct integrin and platelet function. This transition is regulated by intracellular adhesome proteins such as talin, vinculin and kindlin (Liu et al., 2000). The signal for the adhesome proteins to bind the cytoplasmic domain of the integrin β-subunit is triggered by inside-out signaling. The inside-out signaling is a cascade of intracellular signaling pathways originating after agonist-mediated activation of platelet receptors (for a review see (Springer and Dustin, 2012)). The thrombin induced activation of the protease-activated GPCRs (PAR receptors) (Kahn et al., 1998; Vu et al., 1991), and prurinergic receptor ($P2Y_1$, $P2Y_{12}$ and $P2X_1$) activation by ADP (Fabre et al., 1999; Hollopeter et al., 2001) increases the affinity of the integrin extracellular domain toward different ligands (Hughes and Pfaff, 1998; Fig. 1). The intracellular signaling preceding the integrin activation is a complex network of events (Banno et al., 1996; Jin et al., 1998; Nishizuka, 1992; Yacoub et al., 2006). The aforementioned receptors activate the phosphatidylinositol trisphosphate pathway and increase the calcium concentration in the cytosol (Irvine, 1990, 1991; Jin et al., 1998; Streb et al., 1983). The increase in cytosolic calcium induces talin recruitment to the cytoplasmic domain of the αIIbβ3 integrin, triggering a conformational change that increases integrin affinity to fibrinogen (Tadokoro et al., 2003), fibrin and vWF (Plow et al., 2000).

ECM ligand binding to activated integrins marks the onset of outside-in signaling (Giancotti and Ruoslahti, 1999). The biochemical events of the signaling pathway are preceded by integrin receptor clustering (Buensuceso et al., 2003), and finally manifested as macroscopic morphological changes of filopodia and pseudopodia formation which are key to platelet adhesion, cytoskeletal and ECM remodeling, and contraction.

Platelet structural elements in activation and contraction

Actin is the most abundant protein present in platelets (0.5 mM), with ~35–40% in the filamentous form (F-actin) in resting platelets (Boyles et al., 1985) increasing to ~70% upon activation (Fox and Phillips, 1981). The cytoskeletal proteins in platelets form a pliable structural framework, capable of amplifying biochemical signals and molecular mechanical cues into morphological changes during activation (Allen et al., 1979). The structural framework of platelet cytoskeleton has been widely studied by Hartwig and White using scanning electron microscopy (SEM) of critically dried platelets, and conventional transmission electron microscopy (TEM) of both thin sections of platelets (Fox et al., 1984) and whole mounts of membrane-stripped platelets (Hartwig and DeSisto, 1991; Fig. 3).

Cytokeleton in resting platelet

Analysis of membrane-stripped platelets showed that the cytoskeleton of resting platelets resembles a spoked wheel composed principally of actin filaments which form the hub of the

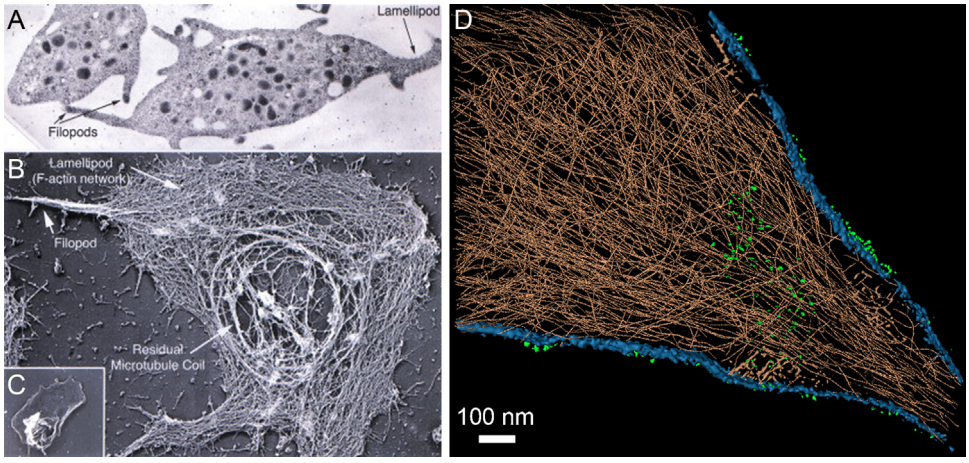

Fig. 3. *Structural biology of platelet function.* (A) Thin sections of whole activated platelets as observed in the electron microscope (EM). (B) Actin cytoskeleton reorganization in an active platelet pictured in the scanning electron microscope (SEM). The MT coil can be seen in the center of the spread platelet. (C) The surface of a whole active platelet. (D) Rendered view of a tomographic volume (cryo-ET) highlighting actin (reddish brown), membrane (blue) and integrins (green) in a filopodium of an active platelet. Whereas conventional EM approaches have been useful in defining the structural changes during platelet activation, techniques such as cryo-ET hold promise in providing mechanistic insights at high-resolution molecular details. Figures (A–C) are reproduced with permission from Hartwig (2012), Elsevier Limited, UK.

wheel. The membrane, acting as the rim of the wheel, is connected through ABP/Gp1b$_{\alpha\beta}$/IX complex to the hub *via* radial, long actin filaments originating from the center (Boyles et al., 1985; Hartwig and DeSisto, 1991; Karlsson et al., 1984; Okita et al., 1985). The cytoskeleton of resting platelets also consists of MTs and binding proteins such as spectrin tetramers (a cytoskeletal protein which connects the membrane to the actin filaments) and actin binding proteins (ABP) (Boyles et al., 1985; Fox et al., 1985; Hartwig and DeSisto, 1991; Okita et al., 1985). A dense, spectrin-rich shell (the membrane skeleton complex formed by the membrane and spectrin) covers the three-dimensional actin core (Hartwig and DeSisto, 1991). The center of the resting platelet presents a circumferential band of MTs running parallel to the plasma membrane and situated immediately beneath it (White, 1968b, 1982; White and Rao, 1998).

Cytokeleton in activated platelet

Upon activation, platelets undergo a morphological transformation from discoid to round to fried-egg shape; the shape change is dictated by both the disassembly of the MT coil (White and Rao, 1998) and the assembly of actin filaments (Fox et al., 1984; Oda et al., 1992; Pribluda and Rotman, 1982; White and Krumwiede, 1973; Winokur and Hartwig, 1995). The actin in resting platelets are irregular networks of small protrusions just outside the MT coil. The actin framework is extensively rearranged after platelet activation in order to create filopodia and lamellipodia (Fox et al., 1984; Hartwig and DeSisto, 1991; Pollard and Borisy, 2003; Fig. 3B). Platelet adhesion begins with spreading and concomitant assembly of actin filaments in two distinct zones. The first zone is found in the filopodia, where actin is organized in parallel bundles (White, 1984) that radiate from the platelet center and converge at the base of the filopodia (Hartwig, 1992). The second zone is present in the lamellae, where actin filaments are shorter and ordered in an orthogonal network (Hartwig, 1992). Actin reorganization starts with the severing of the filaments by gelsolin which is located under the plasma membrane and is close to the ABP/Gp1bαβ/IX complex. The barbed ends of the free actin filaments are then used as nucleation sites for new filament assembly. The barbed ends of the filaments are released by the spontaneous dissociation of gelsolin from the actin filaments (~50% of the gelsolin spontaneously dissociates from actin). Furthermore, activation of the Arp2/3 complex, which mimics the pointed ends of the actin filaments, creates additional nucleation sites (Falet et al., 2002). Electron micrographs of both thin sectioned and membrane-stripped platelets showed that after activation, the MT ring reduces in diameter and the MTs disappear in proximity to the edge (White and Gerrard, 1979). As the spreading of the platelets continues, MTs reorganize and radiate from the central area to the cell margin (Karlsson et al., 1984).

Microtubules and platelet structure–function

The widely accepted role of the marginal MT coil in resting platelets is to maintain their discoid shape. This hypothesis is supported by the observation that mice with β1-tubulin deficient megakaryocytes were affected by platelet spherocytosis (Italiano et al., 2003). Electron micrographs of platelets derived from mice with β1-tubulin deficient megakaryocytes showed that the MT band was either bent or broken, and that after platelet activation MTs were unusually assembling at the cell periphery (whereas normally they are present only in the center of the cell). Despite the structural defects observed in the β1-tubulin deficient platelets, platelets could still change shape and release the granular contents normally in response to activation with ADP or thrombin. Furthermore, the platelets could adhere normally on collagen and under physiological shear conditions generated in a flow chamber. These results suggest that intact MTs are important for platelet structure and shape maintenance even though they may not be essential for platelet activation and function (Italiano et al., 2003).

Also, the role of MTs in platelet contraction is open to discussion. For several years, it was generally accepted that the circumferential bundle of MTs was actively involved in the contraction mechanism. This conviction was derived from the observation that upon platelet activation MTs contract in tight coils in the platelet center (White, 1968a; White and Gerrard, 1979). Extensive studies using the MT-stabilizing drug Taxol proved that MTs were not necessary for any phase of platelet activation (White, 1982; White and Burris, 1984), and suggested that MT movement inside the platelet is due to actin filaments that interact with MTs through MT-associated proteins, thus suggesting a “passive” (or structural) role of MTs in platelet contraction (White and Burris, 1984).

Actin–myosin–microtubule in platelet contraction

The first evidence that myosin IIA is involved in platelet contraction was the observation that myosin regulatory light chain (MRLC) is phosphorylated before platelet shape change (Daniel et al., 1984).

Inhibition of platelet contraction in the presence of blebbistatin, a myosin inhibitor (Abe et al., 2003; Straight et al., 2003), provided support to the role of actin–myosin IIA in platelet contraction (Johnson et al., 2007). Electron micrograph of blebbistatin treated platelets showed that platelet "internal" contraction (observed as the contraction of the MT coil) is inhibited in the presence of 10 μM of the myosin inhibitor. This observation supports the hypothesis that actin–myosin IIA is responsible for the contraction of the MT coil in platelets (Johnson et al., 2007). The observation that MTs are associated with actin filaments through myosin IIa (Johnson et al., 2007), reinforcing the platelet cytoskeleton for stability, forwards further support to the structural role of MTs in platelets (Boyles et al., 1985). The current model of platelet contraction proposes that the myosin motor initiates the centripetal contraction, whereas MTs provide a structural support for the actin–myosin contraction (Johnson et al., 2007; Fig. 1C).

A recent study suggests an interplay between the MT motor proteins, dynein and kinesin, and actin–myosin in platelet contraction (Diagouraga et al., 2014). Platelets treated with the dynein inhibitor EHNA (erythro-9-[3-(2-hydroxynonyl)]adenine) lost their capacity to spread on glass. However, platelets treated with the kinesin inhibitor ATA (aurintricarboxylic acid) could still spread on glass but presenting a round morphology. A model was proposed where dynein is anchored to the actin cortex and, upon platelet activation, dyneins induce MTs to slide apart leading to their coiling. The coiling of the MTs would then rupture the interaction between the MTs and the actin cortex, triggering the actin–myosin to contract the MT band (Diagouraga et al., 2014).

The importance of actin–myosin IIA in platelet physiology has been confirmed *in vivo* in transgenic mice lacking MYH9 gene (encoding the non-muscle myosin heavy chain IIA) in the megakaryocyte cell lineage (Leon et al., 2007). These mice showed a decrease in the number of platelets, which were larger than normal and fragile. Furthermore, the contractile activity of platelets as well as clot retraction were impaired.

ECM–integrins–cytoskeleton

Integrins function as a bridge between the ECM and actin in the contraction process. Toward the extracellular side, integrins anchor platelets to the ECM providing a mechanical support, and on the cytoplasmic side they are linked to the actin cytoskeleton through several actin binding proteins (Brown et al., 2002; Sharma et al., 1995), among which are myosin (Jenkins et al., 1998) and the adhesome proteins (Fig. 1). Talin binds to the cytoplasmic domain of the integrin β-subunit (Tadokoro et al., 2003), F-actin (Lee et al., 2004) and actin binding proteins such as vinculin (Bass et al., 1999; Gilmore et al., 1993). Like talin, kindlin also binds directly to the cytoplasmic tail of the integrin β-subunit inducing conformational changes in the integrin receptor thereby increasing its affinity to the extracellular ligands (O'Toole et al., 1991, 1994; Shattil et al., 2010). Vinculin does not bind directly to integrin but acts as a bridge between talin (Burridge and Mangeat, 1984) and actin (Johnson and Craig, 1994) by binding to both, and thus functions as a mechanosensor (Ingber, 1993; Wang et al., 1993; Wang and Ingber, 1994).

The regulation of the interaction between αIIbβ3 and myosin is still not well understood; it has been shown that myosin binds to the phosphorylated β3-subunit cytoplasmic tail (Jenkins et al., 1998). Furthermore, the association between actin and myosin is stabilized by the phosphorylation of MLC (Fox and Phillips, 1982). MLC phosphorylation promotes actin–myosin contraction and platelet shape change with the subsequent release of pro-coagulant factors from granules sustaining clot formation (Nishikawa et al., 1980).

Platelet mechanics

In spite of its extreme importance in the process of clot formation, investigation of platelet mechanics in quantitative details has begun only recently (Henriques et al., 2012; Lam et al., 2011). With the recent advances in nanotechnology tools (*e.g.*, AFM, optical and magnetic tweezers, microfluidics, soft lithography), there are new opportunities to investigate the mechanics of platelet activation and thrombus formation (Ciciliano et al., 2014). Of direct relevance to platelet mechanics are the recent studies focused on the influence of shear rates on platelet activation, (Maxwell et al., 2007; Savage et al., 1996), the characterization of contraction forces of blood clot (Liang et al., 2010), and defining the mechanical dynamics of single platelets (Lam et al., 2011; Litvinov et al., 2002).

Platelet adhesion and aggregation

The events of platelet dynamics (activation and spreading) have been studied conventionally by fixing them on a glass surface (Allen et al., 1979). Although the 'static' experiments have increased our knowledge of the cytoskeletal structural rearrangement upon platelet activation, it is of fundamental importance to study platelets under shear flow as encountered in blood capillaries. To this end, microfluidics devices simulating flow rates akin to blood vessels have been employed to study clot formation under shear stress providing evidence that shear rates influence platelet activation. Savage et al. (1996) used a linear flow chamber capable of producing linearly increasing shear rates from the inlet to follow the activation of mepacrine-labeled platelets under flow on both vWF and fibrinogen substrates. Platelet adhesion on fibrinogen was observed only at low shear rates ($50\,s^{-1}$) but not at higher shear rates ($1500\,s^{-1}$); vWF induced thrombus formation only at high shear rates, whereas only minimal adhesion was observed at low shear rates. The αIIbβ3 antibody LJ-CP8 inhibited platelet adhesion on fibrinogen but not on vWF, implying that adhesion at low shear rates was mediated by a strong interaction between αIIbβ3 and fibrinogen. Further analysis of the vWF-mediated adhesion at high shear rates showed that the platelets established a transient interaction with the surface at the beginning, developing a "stop-and-go" motion reducing the platelet velocity, and only 10% of the platelets attached to the surface. The GP Ibα (GP1b/IX complex) antibody LJ-Ib1 completely blocked platelet adhesion on vWF surface, whereas LJ-CP8 prevented only platelet adhesion on the vWF surface but did not decelerate the platelets (Savage et al., 1996). The results suggest that platelet adhesion on vWF (under high shear rate) is a 2-step process: the first step is GP Ibα–vWF interaction which induces a reduction in platelet velocity followed by αIIbβ3 activation, probably by outside-in signaling; the second step of platelet adhesion is mediated by the activated αIIbβ3, which induces irreversible platelet adhesion on the surface (Savage et al., 1996).

Ruggeri and Dopheide further hypothesized that the initial stages in platelet adhesion resemble leukocyte extravasation in damaged tissues where there is rapid and transient receptor–ligand interactions (in platelets GP Ibα–vWF) supporting cell tethers on the vessel wall. The tethers promote both translocation of the cells into the injured vessel wall and a decrease in cell speed. The reduced speed enables a slow formation of strong ligand-receptor bonds, (*e.g.*, integrin–fibrinogen) (Dopheide et al., 2002; Ruggeri, 1997). Dopheide et al. analyzed the morphology of platelets perfused in vWF functionalized micro-capillary tubes with SEM showing that tether formation at high shear rates involves GP Ibα–vWF interaction. Tether formation was still observed in the presence of the αIIbβ3 integrin antibody 7E3 suggesting that αIIbβ3 is not involved in the process (Dopheide et al., 2002).

Platelet adhesion under high shear stress using microfluidics showed that platelets could adhere to surfaces coated with different ligands (collagen, vWF, and also extracellular matrix derived from mouse skin fibroblasts) without the need to be activated (Ruggeri et al., 2006). In the presence of platelet activation inhibitors and soluble vWF, rolling aggregates adhered to the substrate at high shear stress; SEM analysis of these aggregates revealed the presence of membrane protrusions (tethers) that interconnect platelets, and platelets to the surface. Tether formation was strictly dependent on the presence of soluble vWF, which initially induced tether-mediated platelet adhesion at a single platelet level, and then caused the recruitment of more platelets to the rolling aggregate without the need of activation as determined in the presence of a platelet activation inhibitor (Ruggeri et al., 2006).

Such a mechanism maybe relevant *in vivo* and responsible for the allocation of a large number of platelets at an injury site, *e.g.*, in vascular lesions. The formation of tethers *in vivo* was confirmed in injured mesenteric arterioles; clot formation at the injury site recorded with differential interference contrast revealed that the majority of the platelets translocated at the injury site in a rolling motion, and retained the discoid shape (Maxwell et al., 2007). Furthermore platelets in the superficial layers of the thrombi were connected to each other through short membrane tethers suggesting that tethers might regulate the initial interaction between platelets. This observation was further investigated with micro-capillary experiments. In the presence of both vWF and fibrinogen the aggregation of discoid platelets at mid-shear conditions ($1000\,s^{-1}$–$10{,}000\,s^{-1}$) is dependent on both αIIbβ3 and GPIb, and involves tether formation. SEM analysis of the aggregates showed that discoid platelets cluster around activated platelets, and are connected through membrane tethers (Maxwell et al., 2007).

On the basis of the studies described above different shear-dependent mechanistic models of clot initiation have been proposed. At low shear rates (100–$1000\,s^{-1}$) the adhesion of spherical platelets (with projecting filopodia) is mediated by αIIbβ3, requires platelet activation and does not involve tether formation (Savage et al., 1996). At high shear rates (1000–$10{,}000\,s^{-1}$) both αIIbβ3 and GPIb are involved, and they induce membrane tethers between co-adhering platelets, which present prevalently a discoid morphology (Maxwell et al., 2007). At shear stress $>10{,}000\,s^{-1}$ smooth, spherical platelets can aggregate in an activation-independent fashion, but in a GPIb-dependent mechanism (Ruggeri et al., 2006).

The relevance of these models *in vivo* was proved by studies on localized stenosis in mesenteric arterioles showing that localized shear micro-gradients accelerate the rate and extent of platelet aggregation (Nesbitt et al., 2009). In a nut-shell, platelets were recruited initially at the stenosis apex, discoid platelet aggregates were then formed downstream of the apex, within 10 min the discoid platelet aggregates would consolidate and lose their discoid morphology, culminating into tight packing of the platelets present at the thrombus base. Platelet inhibitors (such as aspirin and hirudin) did not affect the initial platelet aggregation, whereas they prevented the consolidation phase of the discoid aggregate in the downstream, low shear zone, indicating that shear micro-gradients are sufficient to initiate platelet aggregation with no need of platelet activation (Nesbitt et al., 2009).

Clot contraction

Another very important aspect of the platelet functional mechanics in hemostasis is clot contraction, which leads to full wound closure after clot formation. The initial studies measuring platelet contractile forces in blood clots were performed with a device consisting of two flat surfaces, one fixed and the other capable of moving, separated by a gap of ~800 μm (Carr and Zekert, 1991; Jen and McIntire, 1982). A clot was placed in between the two surfaces, and the change in gap distance between the two surfaces monitored in response to clot contraction. The platelet contraction force was measured using a transducer, where the displacement of the freely moving surface generated a voltage proportional to the force applied. A contraction force of 0.3–0.4 nN was measured from a human blood clot after 15 min of clot initiation (Brophy et al., 2007; Carr and Zekert, 1991). Jen et al. instead applied a small amplitude forced sinusoidal oscillation with a specific frequency to the fixed surface, and this amplitude was transmitted to the moving surface through the clot. They monitored the applied force (used to induce the sinusoidal oscillation) with a torque displacement transducer, and the clot contraction force was measured through a normal force transducer. The contraction force of the platelets was found to be 0.5 nN after 80 min of clot initiation (Jen and McIntire, 1982).

More recently, flexible posts (diameter 2 μm, height 6–7 μm, elasticity 2.9 MPa) made of silicon elastomer and printed with either fibrinogen or fibronectin to facilitate platelet adhesion were used to measure the contractile force of a thrombin-activated micro-clot of human platelets (Liang et al., 2010). The deflecting pillars measured an increase in the total contraction force of the clot from 20 nN to 150 nN within 1 h, and then determined the force of single platelets, which increased from 7 nN after 20 min to 15 nN after 50 min from the activation, and then decreased to 0.5 nN after 80 min (Liang et al., 2010).

Albeit the overall contraction force of a clot is the result of the forces exerted by its constituent platelets, probably not all the platelets in the clot contribute equally as assumed by the studies described above. Moreover, activated platelets release clotting agents (thrombin, vWF) present in their granules, thus changing the microenvironment of the platelets and probably influencing platelet properties in a clot. Clot contraction is probably not isotropic (platelets in the center of the clot may contract differently than those at the clot rim), and determining the contraction force of single platelets from measuring the contraction force of an entire clot may give misleading numbers. Because of these reasons, the mechanical properties of single platelets need to be characterized and included into the clot model.

The atomic force microscope (AFM) was used to characterize the contraction mechanics of single platelets (Lam et al., 2011). A single platelet was captured between a fibrinogen-coated cantilever and a fibrinogen-coated glass surface, and the contraction response of the platelet measured by monitoring the cantilever deflection. The average contraction force of an activated platelet was determined to be 29 nN (Lam et al., 2011). However, the contraction force exerted by a single platelet increased with cantilever stiffness (18 and 43 pN/nm) signifying the role of environment stiffness on platelet contraction. The elasticity of a contracted platelet was determined to be 9.8 kPa. The adhesion strength between a platelet (after contraction) and fibrinogen was measured to be 69 nN. Both elasticity and adhesion strength were directly proportional to the contraction force exerted by the platelet. As a consequence, a platelet exerting a higher contraction force is stiffer and adheres strongly to fibrinogen to hold that contraction force. The results can be extrapolated to physiological conditions where fibrinogen is non-uniformly distributed with regions of sparse density and a few places of high concentration. Owing to the high stiffness of regions (analogous to high stiffness cantilevers) composed of high fibrinogen concentrations, platelets at those spots would probably contract strongly and would be able to sustain the contraction force (because of strong adhesion to fibrinogen). It was proposed that the ability of platelets to adjust their contraction force in response to the environment stiffness would result in a homogeneous contraction in a non-homogeneous fibrinogen network in the clot. The

force exerted by a platelet on the fibrinogen would further lead to an increase in the stress stiffening of the adjacent fibrinogen fibers, thus contributing to an overall increase in clot stiffening.

Traction force microscopy (TFM) has also been employed to investigate platelet contraction forces (Henriques et al., 2012). In TFM, cells are placed on a polyacrylamide (PAA) substrate, incorporating 100 μm diameter marker beads that deform in response to cellular contraction. The traction force map of single platelets showed that platelet contraction reached a steady state where forces are stronger at the edge of the platelet body, and that the pulling force seems to be focused at the platelet center. Nevertheless the contraction forces are nearly isotropic (Henriques et al., 2012). The maximum contractile force of a platelet in the steady state was 34 nN, which is comparable to the force of 29 nN measured with the AFM (Lam et al., 2011). The contraction force of single platelets measured by AFM and TFM is approximately an order of magnitude higher than the forces estimated from clot contraction measurements described above (Brophy et al., 2007; Carr and Zekert, 1991; Jen and McIntire, 1982; Liang et al., 2010) suggesting that the contraction forces exerted by single platelets in a clot are not uniform, and probably these forces are influenced by the clot geometry, dimensions, and the physical properties of the protein matrix to which it is attached (Qiu et al., 2014).

In a recent work Lam and co-workers showed that platelet spreading and adhesion on polyacrylamide matrices of varying stiffness (0.25–100 kPa) is regulated by substrate stiffness (Qiu et al., 2014). It was observed that the number of adherent platelets and their spreading area increased with the substrate stiffness, reaching a plateau on the 50 kPa substrate. Using a pharmacological approach they identified the main players involved in the mechanosensation of platelets during activation, showing that two different signaling pathways drive mechanosensation during adhesion and spreading. In particular platelet mechanosensation during adhesion is driven by GTPase Rac-1 (adhesion on stiff substrates is inhibited by the treatment with a GTPase rac-1 inhibitor), and the mechanosensation during spreading is mediated by myosin activity and actin polymerization (spreading on stiff substrates is inhibited by both latrunculin A, actin polymerization inhibitor, and ML-7, an MLCK inhibitor. It was also shown that the Src kinase family (which activates actin polymerization and is involved in integrin αIIbβ3 outside-in signaling) is involved in platelet spreading on a stiff substrate (the use of Src kinases inhibitor PP2 prevents the spreading on stiff substrates). Rac-1, which is involved in lamellipodia formation, has been shown to be necessary for this mechanism. The involvement of the actomyosin machinery is also proposed to balance the higher resistance force of the stiff substrate.

Macromolecular organization of platelets

The macromolecular cytoskeletal organization of both resting and active platelets has been studied using conventional TEM (Fox et al., 1984; Hartwig and DeSisto, 1991). The classical sample preparation for TEM involves aldehyde fixation, post fixation with osmium tetroxide, dehydration and embedding in a methoxy or methacrylic resin for sectioning. These sample preparation steps can lead to artifacts, *e.g.*, aldehyde fixation can cause cell blebbing and vesiculation of tubular structures (actin and MTs) (Murk et al., 2003), and sample fixation may distort protein assemblies due to sample dehydration (Sosinsky et al., 2008). Cryo-electron tomography (cryo-ET) is a technique that relies on flash freezing of biological samples immobilized on specialized grids by rapidly plunging the sample in liquid N_2 cooled liquid ethane (−186 °C). Cryo-ET bypasses drastic treatment of biological samples and preserves sample integrity close to the native state (Dobro et al., 2010) owing to the use of rapid freezing techniques (Dubochet et al., 1988). The frozen sample is imaged at −180 °C by tilting the sample grid from −60° to +60° with angle increments of 2°. The images of the tilt series are then aligned and back-projected to reconstruct the 3D volume of the sample at nanometer resolution (Baumeister et al., 1999; Koning and Koster, 2009; Lucic et al., 2005).

Cryo-ET of frozen-hydrated preparations is currently the most promising tool for deciphering a detailed, high-resolution (1.5–3 nm) 3D structural construction of surface proteins, intracellular compartments (van Nispen tot Pannerden et al., 2010), cytoskeletal networks (Medalia et al., 2007) and macromolecules in their native environment (Harapin et al., 2013; Medalia et al., 2002). Cryo-ET has been used to image eukaryotic cells at a high resolution enabling the reconstruction of the cytoskeleton (Kurner et al., 2004; Medalia et al., 2002), and the proteasome of *D. discoideum* (Medalia et al., 2002). It was also possible to image in high details the Golgi apparatus, nuclear pores and intracellular vesicles of *O. tauri* (Henderson et al., 2007).

Due to their reduced dimensions (2–3 μm diameter compared to 10–100 μm of a eukaryotic cell), platelets are ideal for imaging with cryo-ET. Although a highly detailed 3D perspective of platelet organelles has been obtained (van Nispen tot Pannerden et al., 2010), the cytoskeletal framework has not been analyzed in detail. Vitrification of samples in liquid ethane maintains cytoskeletal networks close to native states (Dobro et al., 2010; Fig. 2). Cryo-ET procedures have been utilized to structurally characterize focal adhesions in fibroblasts (Patla et al., 2010), revealing that actin filaments are orientated parallel to the long axis in the core of the adhesion sites, whereas the parallel orientation is not preserved at the periphery of the adhesion sites. It was also possible to determine the geometry of focal adhesion associated-particles presenting a characteristic doughnut-shaped structure localized below the actin network of the cell. Immuno-gold labeling of vinculin proved the presence of vinculin in the proximity of these structures. Furthermore, treating the cells with the contractility inhibitor Y-27632 affected the doughnut-shaped structures, reducing their diameter from approximately 25 nm to 15 nm. Although the individual components of the macromolecular assembly were not identified, the macromolecular structure of a complex which is involved in contraction and is specific to the focal adhesion sites was elucidated. Similar studies can be performed on platelets to analyze the organization of actin in platelet pseudopodia and lamellipodia (Medalia et al., 2007) (Fig. 2).

Platelet-related disorders

The importance of integrin activation, cytoskeleton reorganization and the associated signaling pathways in platelet activation is widely recognized. In case, one of these mechanisms is impaired, bleeding diseases ensue. For example, in Glanzmann thrombasthenia (GT) platelets, αIIbβ3 is either not present or dysfunctional leading to impaired platelet aggregation (Patel et al., 2003). Biochemical analysis of the actin content of thrombin-activated GT platelets suggest a defective actin association with the membrane (George and Morgan, 1981). Bernard–Soulier syndrome (BSS) is caused by mutations in the GPIb-IX–V complex, leading to either quantitative or qualitative defect of the complex (Bernard and Soulier, 1948). Congenital macrothrombocytopenia (CMPT) is a heterogeneous group of rare platelet disorder characterized by a reduction in the platelet count and an abnormally large size. This class includes MYH9-related platelet disorders, which are caused by mutations in the myosin heavy chain 9 (MYH9) (Kelley et al., 2000; Seri et al., 2000). Another mutation responsible for a CMPT disease in the ACTN1 gene (α-actinin-1, a member of the actin-crosslinking protein superfamily) has only been recently identified (Kunishima et al., 2013).

The pathological platelets present both impaired activation (due to the reduced binding to vWF) and cytoskeletal defects; a decreased expression of or dysfunctional surface integrins leads to a disordered cytoskeletal framework owing to the break in contact links between the membrane receptors and actin filaments *via* adaptor proteins (Bernard and Soulier, 1948). This break of anchor points destabilizes the internal scaffolding network resulting in a more extensible and, in some cases, a fragile membrane envelope, causing the platelets to swell.

Wiskott–Aldrich syndrome (WAS) is characterized by immunodeficiency, eczema and microthrombocytopenia (less and smaller platelets). Platelets of WAS patients have hyperstable MTs, and the organization of the MT band is not preserved. Interestingly, transgenic mice lacking Profilin1 (Pfn1; binds G-actin and promotes actin filament elongation by accelerating ADP-ATP exchange) in megakaryocytes cell lineage exhibit similar molecular defects in platelets and are affected by microthrombocytopenia, an effect that is not reproduced in WASp- or WIP (WASp interacting protein)-deficient mice (Bender et al., 2014). Pfn1-deficient platelets show misarranged and hyperstable microtubules but the platelets contain more tubulin and a higher number of MT coils. Pfn1, which strongly co-localizes with α- tubulin and is dependent on MT assembly both in mice and human platelets, is mislocalized in WAS patients. These results suggest an interaction between WASp and Pfn1, and that WAS platelets may have an altered Pfn1 function and/or localization (Bender et al., 2014).

Physical, structural and molecular characterization of platelets in diseases is crucial not only to understand the pathophysiology of platelet-related disorders but also to stress their role as unique model systems in cell biology. In the coming years, biophysical, nanotechnological and structural tools (*e.g.*, AFM, soft-lithography, cryo-ET) will be key to our understanding of the structural and mechanical components in platelet function (Ciciliano et al., 2014).

Future perspectives

Because of their general medical importance, platelets have been studied for decades with a focus on integrin signaling and cytoskeleton rearrangement. However, several aspects of platelet function still need to be elucidated. For example, very less is known about the mechanical function of platelets and its coordination with intracellular structural remodeling.

Owing to their relatively simple make-up (mainly the lack of a nucleus) but at the same time maintaining the complex protein machinery needed for adhesion, spreading and contraction, platelets have served as a good model for elucidating mechanisms related to the mechanical and structural aspects of integrin signaling and cytoskeletal remodeling. The interplay between ECM stiffness and integrin–cytoskeleton linkages was investigated in mice fibroblasts (Choquet et al., 1997). Choquet and co-workers used optical gradient trap to apply a force on the mice fibroblast α5β1 using fibronectin-functionalized latex beads. In particular, they applied restraining forces by pulling away the fibronectin-functionalized bead away from the nucleus toward the leading edge of the cell, and observed a strengthening of the cytoskeletal linkages, thus allowing more force to be exerted on the integrins. Although the reinforcement of the cytoskeletal linkages in response to the ECM force applied on an integrin may hold as a common mechanism for all integrins and cell types, it is worth investigating if this mechanism is conserved in platelets.

Cryo-ET analyses of the 3D organization of platelet cytoskeleton would help to identify the structures associated with the adhesion complex, thus allowing to investigate the complex interplay between talin, kindlin-3 and αIIbβ3 *in vivo*. Cryo-ET of fibroblasts allowed to identify a previously unseen structure involved in the formation of focal adhesion complex (Patla et al., 2010). Taking advantage of the reduced size of platelets, and with the use of immuno-gold labeling, it would be possible to identify the molecules needed for the formation of the focal adhesion complex and the consequent actin remodeling. Platelet spreading was recently studied on micro-patterned fibrinogen and collagen surfaces, showing that the adhesion process is precisely regulated by the spatial information of the environment (Kita et al., 2011). The results suggest that platelet activation and adhesion may be regulated by the physical features of its environment. The structural organization of focal adhesions, and the adhesion mechanism on surfaces of different geometries and physical properties may therefore be worth investigating. Super-resolution optical microscopy techniques are especially promising in scrutinizing platelet structural dynamics (Godin et al., 2014). Historically (and for good reason), investigation of platelet morphology and cytoskeletal rearrangement has been performed post chemical fixation. The introduction of new dyes may change this and enable live monitoring of platelet dynamics (Lukinavičius et al., 2014).

Both single cell mechanical tools and structural techniques should be employed to characterize the physical and structural differences in platelets isolated from patients; the approach will provide new insights into platelet function.

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