Platelets play a key role in hemostasis and thrombosis through aggregation, adhesion and procoagulant activation. However, their unnecessary activation is often linked to life-threatening thrombotic diseases including venous and arterial thrombosis, embolism and stroke (Coller, 2013). Numerous drugs modulating platelet activation have been developed and are hugely successful in the market for the prevention of thrombotic diseases and thrombotic complications in diverse cardiovascular diseases, exemplified by aspirin, Plavix (Bristol-Myers Squibb, NY, USA; clopidogrel), Efient (Lilly, Indianapolis, IN, USA; prasugrel) and Pletal (Otsuka, Tokyo, Japan; cilostazol) (Collins and Hollidge, 2003; Rao et al., 2006). In contrast to aggregation or adhesion, functions that are well-illustrated in the thrombus formation, role of the morphological changes of platelet, i.e., platelet shape change, is relatively less established. Platelets dynamically undergo remarkable morphological alterations when exposed to physical or biochemical stimuli such as high shear or endogenous thrombotic agonists (Hartwig, 2013). Platelet shape changes frequently occur even when the cells are exposed to minimal degrees of perturbation that cannot result in aggregation or adhesion, such as sub-threshold concentrations of platelet agonists or shear stress. In this review, we discuss the current knowledge concerning the mechanisms of platelet shape change and also pathological implications and therapeutic opportunities for regulating the associated cytoskeleton dynamics.

**Key Words:** Platelet shape changes, Cytoskeleton dynamics, Thrombosis, Aggregation, Adhesion

**INTRODUCTION**

Platelets play a key role in hemostasis and thrombosis through aggregation, adhesion and procoagulant activation. However, their unnecessary activation is often linked to life-threatening thrombotic diseases including venous and arterial thrombosis, embolism and stroke (Coller, 2013). Numerous drugs modulating platelet activation have been developed and are hugely successful in the prevention of thrombotic diseases and thrombotic complications in diverse cardiovascular diseases, exemplified by aspirin, Plavix (Bristol-Myers Squibb, NY, USA; clopidogrel), Efient (Lilly, Indianapolis, IN, USA; prasugrel) and Pletal (Otsuka, Tokyo, Japan; cilostazol) (Collins and Hollidge, 2003; Rao et al., 2006). In contrast to aggregation or adhesion, functions that are well-illustrated in the thrombus formation, role of the morphological changes of platelet, i.e., platelet shape change, is relatively less established. Platelets dynamically undergo remarkable morphological alterations when exposed to physical or biochemical stimuli such as high shear or endogenous thrombotic agonists (Hartwig, 2013). Platelet shape changes frequently occur even when the cells are exposed to minimal degrees of perturbation that cannot result in aggregation or adhesion, such as sub-threshold concentrations of platelet agonists or shear stress. In this review, we discuss the current knowledge concerning the mechanisms of platelet shape change and also pathological implications and therapeutic opportunities for regulating the associated cytoskeleton dynamics.

**PROCESS OF THE SHAPE CHANGES AND CYTOSKELETON DYNAMICS IN PLATELETS**

Resting platelets are small discoid shapes around 1.5-3 μm in size. The cytoskeleton of platelets mainly consists of actin, tubulin, spectrin and filamin. Spectrin, which has a two-dimensional web-like structure, laminates the cytoplasmic side of the plasma membrane, both ends of which are connected to actin filaments. Actin exists in polymer where filamin and α-actinin...
act as cross-linkers with approximately 1:10 ratios (Smyth et al., 2010). In resting platelets, microtubule coils, polymers of α-tubulin, are located beneath the plasma membrane, maintaining the discoid shape (Smyth et al., 2010). Upon exposure to various biological stimuli and agonists, shape change occurs via dismantling and reorganization of the cytoskeletons. There are generally two steps in agonist-induced platelet shape change. First, platelets become spherical as actin filaments are fragmented, which is followed by the dismantling and release of spectrin networks composed of filamin A, GP1b/IX and spectrin. Second, the membranes of the cells protrude due to actin filament assembly, which manifests as lamellipodia and filopodia (Sandmann and Koster, 2016). Filopodia protrudes from the core actin fibers while lamellipodia grows from the short actin filament templates (Hartwig, 1992).

Each step in platelet shape change involves the participation of a variety of actin filament-related proteins that are highly concentrated in platelets (Fig. 1). In resting human platelets, the actin filaments from the core to the membrane skeleton are tightly bound to the plasma membrane by GP1b/IX-filamin A complexes (Kovacsovics and Hartwig, 1996). When an agonist binds to its receptor, the activation of phospholipase C (pl) induces inositol-1,4,5-triphosphate (IP3) generation and increases intracellular calcium up to 5-10 μM (Berridge et al., 2000; Zheng et al., 2015). Gelsolin is an actin binding protein that severs and caps the barbed-end actin filaments to prevent actin monomer exchange upon intracellular calcium increase in the initial step. Cofilin also binds to actin and contributes to the disassembly of actin filaments and the subsequent release of actin monomers. The actin cross-linking complex, GP1b/IX-filamin A complex, translocates from the plasma membrane to the cytoskeleton during this step. Therefore, severance of actin filaments unlocks the membrane skeleton and induces cell deformation with marginal short actin filaments. This leads to the swelling of the membrane, probably in collaboration with the open canalicular system (OCS), which is considered a reservoir of membrane substances like GP1b/IX and some portion of integrin αIIbβ3 (Smyth et al., 2010) for platelet shape change owing to its invaginated membranous structure connected to the surface. Polyphosphoinositides (PPI) such as PI-3,4-P2, PI-4,5-P2, and PI-3,4,5-P3 are known to deactivate the capping proteins in actin filaments and expose barbed-end nucleation sites. Also, the Arp2/3 complex stimulates the assembly of actin filaments. There are many actin sequestering proteins such as profilin and thymosin-β4 in platelets that carry an actin monomer to the barbed end and protrude filopodia. Profilin binds to both formin and actin monomers to increase the addition of actin monomers to the barbed end of the filament.

Meanwhile, intracellular organelles and granules as well as fragmented microtubules are concentrated around the cell center. Finally, the capZ protein recaps the barbed filament ends to complete the assembly and these actin cytoskeletons can be used for cellular contraction, resulting in the final activated platelet shape: spiny spheres with long, thin filopodia extending several micrometers from the platelets.

**PLATELET SHAPE CHANGES PRECEDING AGGREGATION**

Platelet shape change is regarded as a prerequisite for platelet aggregation. Shape changes of platelets facilitate their adhesion to the site of vascular injury and cohesion with other platelets or erythrocytes, ultimately contributing to the formation of stable aggregates (Jackson, 2007). The degree of platelet aggregation can be easily measured by light transmission aggregometry (LTA) since platelet aggregates consume and precipitate floating platelets, thereby, reducing
the turbidity of platelet suspension. In LTA, upon the addition of agonists or exposure to stimuli, light transmission through platelet suspension decreases initially and then increases thereafter. This initial phenomenon observed in LTA, i.e., the decrease of light transmission, is thought to be attributable to platelet shape changes (Maurer-Spurej and Devine, 2001), while some ascribe it to micro-aggregation (Born et al., 1978).

It is unclear why platelets change their shapes prior to aggregation, but one explanation is that platelet shape change reduces electrostatic repulsion between two negatively surface-charged platelets.

Many studies have been conducted to understand the mechanisms and interaction of shape change and aggregation. $G_{q}/PLC \gamma$ and PLC $\gamma$ pathways are important for platelet aggregations activated by thrombin, ADP and collagen, mediated through G-protein coupled receptors, PAR1, PAR4, P2Y1 and GPVI receptor, respectively. The activation of the $G_{q}/PLC \gamma$ pathway results in platelet shape changes through activating IP$_3$-calcium signaling (Offermanns et al., 1997). In $G_{q}$-/- mice, however, while thrombin and collagen induce typical platelet shape changes, aggregation is not triggered, suggesting that the morphological change does not initiate platelet aggregation and the pathways for the two phenomena do not fully coincide. There are some examples of platelet aggregation without preceding shape changes. Again, in the $G_{q}$-/- mice model, platelets did not undergo platelet shape change or aggregation by ADP at the effective dose (10 $\mu$M). Interestingly, high concentrations (~100 $\mu$M) of ADP prompted platelet aggregation, presumably due to the $G_{i}$-mediated adenylyl cyclase inhibition (Ohlmann et al., 2000). This suggests that the shape changes are accomplished by the specific interaction between G proteins, $G_{q}$ and $G_{i}$, which is distinct from that for the platelet aggregation.

**PLATELET SHAPE CHANGES OBSERVED DURING ADHESION**

Another distinctive pattern of platelet shape change, spreading to the adhesion molecules, is different from that observed prior to platelet aggregation. While rolling ball-shaped platelets or hemisphere-shaped platelets with filopodia observed before aggregation are reversible, platelets that exhibit a spreading shape, which is flat with a large adhesive surface (platelet diameter >2.5 $\mu$m), are terminal. In this case, the cells make more lamellipodia than filopodia, which consists of ac-
tin networks cross-linked orthogonally by filamin A (Hartwig et al., 1999). Platelet spreading can further recruit other platelets or neutrophils to increase procoagulant activity. Kuwahara et al. (2002) tried to explain the molecular mechanism occurring during these adherence steps by testing the effects of the PI3K inhibitor wortmannin, αIIbβ3 blocker c7E3 and an intracellular calcium chelator, dimethyl BAPTA. They reported that while GP1b-αWF (von Willebrand factor) interaction is essential to initiate the platelet shape change with filopodia formation, both αIIbβ3 activation and intracellular calcium increase are necessary for platelet spreading.

**SIGNAL TRANSDUCTION FOR PLATELET SHAPE CHANGES**

**Rho GTPases, Formins, WASp and WAVE**

In the platelet, a family of small (~21 kDa) signaling G proteins, Rho GTPases, RhoA, Cdc42 and Rac1 play key roles in the dynamics of the actin cytoskeleton, platelet aggregation, secretion, and spreading as well as in thrombus formation. It has been reported that Rho GTPase-related actin remodeling, filopodia and lamellipodia formation are mediated through the activation of formins and the Arp2/3 complex (Fig. 2) (Heesman and Ridley, 2008). Cdc42 can induce Arp2/3-mediated filopodia formation through the activation of WASp (Wiskott-Aldrich syndrome proteins) and neuronal N-WASP (Rohatgi et al., 1999). Similarly, Rac1-enhanced lamellipodia formation is related to Arp2/3 activation by the WAVE (WASP-family verprolin-homologous) complex (Miki et al., 1998). Not only WASP and WAVE, but formin proteins (formins) have also emerged as vital effectors of Rho GTPases (Kuhn and Geyer, 2014). Among the 15 formins in mammalian cells, mDia and Daam are shown to be activated upon interaction with Rho GTPases (Kuhn and Geyer, 2014). The Arp2/3 complex provides a platform for new actin polymerization through activating the barbed-end where formin serves as an elongation factor.

**Myosin light chain phosphorylation, Calcium, small GTPase proteins**

Phosphorylation of the myosin light chain and moesin, an actomyosin filament/plasma membrane cross-linker molecule, is essential for the initiation of shape change in platelets (Daniel et al., 1984; Klages et al., 1999; Retzer and Essler, 2000). Phosphorylation of adhesion site proteins such as moesin facilitates their binding to actin filaments, ultimately generating contractile force on the actin framework. Previously, it has been reported that calcium might not change directly by phosphorylation. (ROCK), which induces shape changes directly by phosphorylating MLC or indirectly through inhibiting myosin phosphatase (PP1M).

**ACTIVATORS OF PLATELET SHAPE CHANGES**

**Physiological stimuli**

Prior to aggregation, many agonists such as ADP, platelet-activating factor (PAF), 5-hydroxytryptamine (5-HT, serotonin), thrombin and collagen induce remarkable morphological changes (Maurer-Spurej and Devine, 2001). Previously, Sanderson et al. (1996) determined the time and dose dependency forward/side-scatter profile of platelets following exposure to platelet activating agonists using a flow cytometer. Depending on the agonist expressed, the onset of platelet forward/side scatter changes was different. For instance, ADP, PAF and 5-HT initiated platelet shape changes rapidly and strongly, whereas other agonists like A23187 and collagen induced strong but slower responses. Interestingly, although each agonist could induce both platelet shape change and aggregation, it is not always mediated via a single receptor activation. For instance, a selective P2Y purinoceptor antagonist ARL66096 was effective against aggregation but had no effect on ADP-induced platelet shape changes, indicating that two different receptors and distinct signaling pathways could be involved in agonist-stimulated platelet activation. Epinephrine is well known for its potentiating effects on platelet shape change, aggregation and granule secretion induced by ADP or thrombin (Haaland and Holmsen, 2011). Epinephrine alone can also induce shape changes and platelet aggregation but with a much slower time-frame and to a lesser degree compared with that by ADP. It has been shown that the epinephrine-induced shape change results in pseudopods without changing the main cell-body size (Milton and Frojmovic, 1984).

In addition to platelet agonists, shape changes can occur without apparent aggregation by various stimuli. Angiotensin II, an important hormone for hypertension and vascular remodeling (Crowley et al., 2006), causes a significant increase in median platelet volume (MPV) in platelet rich plasma in vitro and potentiates platelet shape changes induced by ADP and serotonin, indicating that angiotensin II and the related signal transduction pathway may be linked with shape changes (Jagroup and Mikhalidis, 2000). This was further corroborated by the attenuation of angiotensin II-induced MPV increase and U46619 (a thromboxane A2 analogue)-induced platelet shape changes by losartan (a selective angiotensin II antagonist) at its therapeutic concentrations for antihypertensive effects. Immunological activation is also associated with platelet shape changes. Opsonized zymosan A, an immunological stimulus, can induce platelet shape changes that accompany reactive oxygen species (ROS) generation and mitochondrial membrane hyperpolarization (Matarrese et al., 2009). Immunoglobulin G (IgG) antiplatelet antibody can also induce morphological changes, as can be observed under microscopy, as a disk-to-sphere transformation associated with the formation of bulbous, short surface projections or pseudopodia (Colman et al., 1983).

Pathologically high shear stress, as observed in various disease states such as atherosclerosis, cancer or stenotic area, can induce platelet shape changes (Nesbitt et al., 2009). Shear rate can increase significantly up to 10,000 s⁻¹ in car-
diovascular diseases. The normal shear rate is around 200 s⁻¹. Platelets respond to blood vessel injury and start rolling through the interaction between the GP1b/IX complex and the von Willebrand factor (vWF). The shear stress induces the rolling platelets which tightly adhere to the vessel wall by interactions between integrin αIIβ3, and surface matrices.

**Exogenous stimuli**

Many environmental toxicants and xenobiotics can induce platelet activation and cardiovascular diseases (Savouret et al., 2003; Abrescia and Golino, 2005; Kim et al., 2014). Methymercury, a cardiovascular toxicant that can induce platelet aggregation and prime granular release by activating the prostaglandin synthesis pathway, also primes platelet shape changes (Macfarlane, 1981; Jennrich, 2013). Xenobiotics that can affect actin dynamics also induce morphological changes in platelets. Cytochalasin D, which interferes with actin polymerization, can induce platelet shape changes (Fox and Philipps, 1981). The microtuble disassembling drug, vincristine, induces the transformation of discoid platelets to rounded cells. Nanoparticles have gained a lot of attention due to their effects on platelets. Platelets tend to be more susceptible to nanoparticles compared to other cells with larger sizes. A variety of nanoparticles can induce cytoskeletal alterations and the aggregation of platelets. Carbon nanoparticles (Radomski et al., 2005), silver nanoparticles (Jun et al., 2011) and more recently, polystyrene latex nanoparticles (PLNPs) (Smyth et al., 2015) have been reported to induce platelet shape changes along with aggregation.

In cold environments, most eukaryotic cells either assume discoid shapes or do not change but platelets undergo deformation (Winokur and Hartwig, 1995). Chilling platelets (4 degree) in vitro results in an increase in volume and the formation of loose marginal microtubules as well as pseudopods. The shape changes are observed even at 0 degree or in ice water but are reversible. They can also be mediated by myosin phosphorylation, cytosolic calcium increase and actin filament elongation (Van Poucke et al., 2014).

**PATHOLOGICAL IMPLICATION OF PLATELET SHAPE CHANGES AND CYTOSKELETON DYNAMICS**

Platelets with morphological changes become hyper-reactive to agonists (Maurer-Spurej and Devine, 2001) and xenobiotics that can prime platelet shape changes can potentiate the platelet aggregation responses and ultimately thrombosis. Hyper-reactivity or prothrombotic traits in morphologically changed platelets can be explained by granule secretion. Platelet shape change can trigger granule secretion through actin-myosin contraction. After platelet shape change is completed, granules and organelles are centralized in the cells (Furman et al., 1993). Although it is not clearly defined whether granule secretion occurs in platelets through OCS or plasma membrane fusion, the movement of granules following platelet shape change is considered a hallmark of granule secretion. A couple of Rab proteins including Rab27 (Tolmachova et al., 2007), SNAREs (Ren et al., 2007) and Munc (Schraw et al., 2003) proteins are known to interact in the process of platelets. In the mice cremaster muscle arteriole thrombosis model, platelets are integrated and thrombi are formed upon laser-induced injury. Interestingly, platelets are disaggregated and easily washed off by blood flow with mild injury, whereas tight thrombi can form in accordance with P-selectin expression after severe injury (Stalker TJ et al., 2014). This suggests that stable thrombus formation is related to the irreversible platelet shape change and subsequent α-granule secretion.

Platelet shape change and concomitant actin filament turnover can release activating molecules that are bound to the cytoskeleton as inactive forms. Integrin αIIβ3 is retained by the actin cytoskeleton in resting platelets but is released to the cell surface during platelet activation. It provides a receptor site for fibrinogen as well as vWF and promotes further adhesion of platelets. Therefore, it could increase pathologic thrombosis if platelet αIIβ3 is expressed through thrombogenic agent-driven shape change. Down-regulation of αIIβ3 as witnessed in some inherent diseases like Glanzmann’s thrombasthenia (Nurden, 2006) or by antiplatelet agents or anticancer drugs can lead to bleeding, namely compromised thrombosis.

In order to understand the mechanism underlying the turnover of these functional receptors, cytoskeleton related regulation has to be discussed. OCS might play a key role in platelet shape change and spreading, as the OCS occupies 50% of the total plasma membrane (White and Clawson, 1980). Patients with the Budd-Chiari syndrome, a condition caused by the formation of a blood clot within the hepatic veins, display dilated OCS in their platelets (Dayal et al., 1995). In line with this, it has been reported that integrin αIIβ3 is highly expressed in the membrane of the OCS (Escolar et al., 1989). Recently, Mountford et al. (2015) showed that PI3KC2α knockdown significantly increases shear stress-induced platelet adhesion to fibrinogen and the collagen surface. Interestingly, the PI3KC2α disrupted platelets display enlarged OCS, suggesting that the pathologic platelet shape change may enhance thromboembolism (Mountford et al., 2015).

**THERAPEUTIC OPPORTUNITY FOR REGULATING CYTOSKELETON DYNAMICS AND PLATELET SHAPE CHANGES**

The polymerization of actin filaments and tubulin has been explored as therapeutic targets for a variety of diseases including cancer, cardiovascular diseases and thrombosis over the years. Since the morphological changes of platelets are closely associated with the pathophysiology states, modulation of the platelet cytoskeleton can be a good therapeutic approach to improve disease states. Semaphorins were originally identified to induce growth cone collapse in developing neurites. Many studies have revealed the involvement of semaphorins in angiogenesis, immune cell regulation, and organogenesis. Among 25 Semaphorin family members, Semaphorin 3A (Sema3A) was found to be produced and secreted by endothelial cells. To investigate its role in blood, Kashiwagi et al. (2005) applied Sema3A to platelets in various conditions and demonstrated the inhibitory effects of Sema3A in platelet aggregation, αIIβ3 expression, granular secretion, adhesion, and spreading through its receptors on the cells. In platelets, sema3A inhibited agonist-induced Rac1 activation and phosphorylation of actin-depolymerizing protein, coflin, and indeed, it reduced F-actin contents. Interestingly, they could not detect any effect on the major platelet inhibitory pathways including cAMP and cGMP contents or alterations in the intracellular Ca²⁺ concentration. Therefore, sema3A mediated
platelet inhibition appears to be related to the modulation of actin polymerization and morphological changes. The authors proposed that sema3A supports the maintenance of circulating platelets in a quiescent state and that the sema3A signaling pathway could be a novel target for antiplatelet therapy. Similarly, 3 semaphorins and 8 plexins, receptors of semaphorin, have been detected in platelets (Wannemacher et al., 2011). Their roles in platelet function and in cell to cell interaction remain to be elucidated, although studies on sema4D-/- mice have demonstrated that sema4D plays a positive role in thrombus formation in vivo and platelet aggregation in vitro (Zhu et al., 2007). Importantly, sema4D-/- mice did not show gross bleeding and therefore, the authors suggested that sema4D could be a promising drug target to limit excessive platelet activation without disrupting normal platelet function.

Recently, Estevez et al. (2013) investigated the role of LIM kinase 1 (LIMK1) in platelet activation and thrombosis. LIMK1 is one of the effectors of Rac1, which facilitates actin polymerization, mediated through the phosphorylation and deactivation of cofilin. In the FeCl3-induced arterial thrombosis model as well as the tail bleeding time analysis model, the LIMK1-null mice revealed increased thrombotic occlusion time, but no difference in bleeding time compared with the wild-type. GP1bIX signaling-induced actin polymerization was indeed reduced in platelets from KO mice and more importantly, cytosolic phospholipase A2 (cPLA2)-dependent thromboxane A2 (TXA2) generation was inhibited.

It should be clarified further whether the rearrangement of modified actin directly affects platelet function. Many attempts have also been made to answer the question by using inhibitors of actin polymerization such as cytochalasins (Lefebvre et al., 1993; Torti et al., 1996; Natarajan et al., 2000). The results are contradicting, however, depending on the concentration of drugs used. Cytochalasin treatment inhibited cofilin activation at high concentrations, but enhanced platelet activation at low concentrations (Bennett et al., 1999). Nevertheless, it has been reported that talin is essential for integrin activation in platelets by linking the integrin cytoplasmic tail to actin filaments, suggesting that the dynamics of actin filaments by agonists can be important for platelet activation (Tadokoro et al., 2003).

The generation of platelet microparticles (PMP) could be another important result of platelet cytoskeleton rearrangement and shape change. PMPs are found in both normal and disease states. PMPs are also proposed to play active roles in many diseases including rheumatoid arthritis (Kniitt-Dutmer et al., 2002), breast cancer (Janowska-Vieczorek et al., 2006), and cardiovascular diseases (Nomura et al., 2003). A recent study on the protein compositions of PMPs using a proteomics approach has revealed that the characteristics of PMPs are determined by the activating agonist, suggesting that PMPs are generated in specific pathophysiological states (Milioli et al., 2015).

Investigating platelet cytoskeleton regulation also represents an opportunity for the development of novel therapeutic targets. Gelsolin has been recently suggested as one of the targets (Liu et al., 2013), because platelet gelsolin level is positively correlated with cardiovascular diseases (CVD). The Xiongshao capsule, a Chinese herbal medicine used to improve blood circulation, reduced platelet gelsolin and platelet activation markers, similar to the antiplatelet drug aspirin (Liu et al., 2013).

Previous study has shown that filamin A (FnA) null platelets are rapidly removed from the circulation (Nakamura et al., 2011; Thon and Italiano, 2012). FnA-null megakaryocytes (MKs) did not show many differences in the expression of vWF receptor components such as GP1bα, ADAM17, metallocproteinase-9 (MMP-9) compared with wild-type MKs. However, the number of MKs was increased by 2.5-5 fold in the KO. The authors suggested that this is because of the fast removal of large and fragile platelets containing GP1bα degradation products, increased ADAM17 and MMP-9 (Jurak Begonja et al., 2011). Together with another report (Kanaji et al., 2012), the researchers have clarified that FnA and GP1bα play crucial roles in determining platelet size during thrombopoiesis.

CONCLUSIONS

Platelet shape changes have many important implications in pathophysiology and toxicology, therefore, understanding the underlying mechanism and the interaction with xenobiotics and thrombotic activators will illustrate the pathophysiology of thrombotic diseases and provide important insights into molecular drug targets. In the same vein, modulation of the targets related to platelet shape changes would provide a novel therapeutic opportunity that can satisfy unmet medical needs in thrombotic diseases and prevent toxicity from prothrombotic xenobiotics.

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