Platelets: context dependent vascular protectors or mediators of disease

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Platelets in atherogenesis and atherothrombosis

Atherosclerotic plaque rupture and subsequent arterial plaque-associated thrombus formation (atherothrombosis) leads to myocardial infarctions and ischemic strokes. While hyperlipidemia and abnormalities in hemostatic balance contribute to atherothrombosis, the sequence of events and mechanisms are largely unclear and remain of interest. Platelets are thought to play a major role in driving atherothrombosis by localizing to lesions, facilitating inflammatory cell infiltration into lesions, and locally delivering prothrombotic and proinflammatory molecules.

Mechanisms underlying platelet dysregulation in atherosclerosis may be due to exogenous factors, like diet. In fact, Fuller et al. investigated the genetic effects of combined scavenger receptor class B, type 1, (SR-B1) and $Ldlr^{-/-}$ (double knockout, *dKO*) deficiency on atherogenesis and the development of occlusive coronary artery atherothrombosis in response to four different dietary challenges(1). They observed that *dKO* mice had increased spontaneous coronary artery disease and decreased survival in response to diets containing high fat and high cholesterol (22% fat, 0.15% cholesterol), high cholesterol (2% cholesterol), and diets high in fat, cholesterol and choline (i.e. the Paigen diet)(1, 2). Diet-induced mortality in the *dKO* mice was likely attributed to abundant platelet accumulation surrounding atherosclerotic plaques as assessed by CD41 staining. Inflammatory markers and plaque-associated monocytes also correlated with increased plaque associated platelets. These studies suggest that diet not only contributes to the development of atherosclerosis, but also the degree of platelet accumulation within plaques.

Overproduction of platelets is a major risk factor for cardiovascular disease (CVD), yet the mechanisms that govern excessive platelet production in hyperlipidemic conditions are widely uncharted. Recent findings indicated the lack of ATP-binding cassette (ABC) transporters (e.g. ABCG4 and ABCB6) leads to increased platelet production and atherosclerosis in hypercholesterolemic mice(3, 4). Specifically, the lack of ABCB6 in the bone marrow leads to unrestrained platelet production, enhanced pro-inflammatory platelet activity, and accelerated atherosclerosis in Ldlr$^{-/-}$ mice(4, 5). The significance in this work lies in the possibility for a novel thrombolytic approach in part by tempering the production

of reactive platelets. Because thrombocytosis is a major risk factor for CVD, further studies dissecting mechanisms underlying maladaptive platelet production and hyperactivity are of immense interest.

Circulating activated platelets adhere to the endothelium and facilitate leukocyte recruitment and extravasation, promoting the development of atherosclerotic lesions(6, 7). Activated platelets also form complexes with circulating leukocytes, and these platelet-leukocyte aggregates (PLAs) are a central feature of inflammatory diseases. Platelet-monocyte aggregates (PMAs), in particular, have been shown to be an early predictor for cardiovascular events(8). Indeed, recent studies have indicated a fundamental role for platelets and their interaction with leukocytes in the development and progression of atherosclerosis. In hypercholesterolemic apolipoprotein E-null (ApoE$^{-/-}$) mice, activated platelets and PLAs accumulate in athero-prone regions of the murine carotid artery(9). This accumulation of activated platelets and PLAs are thought deliver pro-inflammatory factors that in turn amplify the recruitment of monocytes and accelerate atherosclerosis. Another study by Badryna et al. indicated that oxLDL stimulated PMA formation, which promoted phenotypic changes in monocytes, increased monocyte extravasation and enhanced foam cell formation *in vitro* and *in vivo*(10). To further probe the mechanism, these investigators explored whether blocking cyclooxygenase (COX) by aspirin (ASA) would have an effect on oxLDL stimulated PMAs. Indeed ASA suppressed PMA formation in response to oxLDL through an undefined mechanism(10). ASA has anti-inflammatory actions that cannot be solely attributed to its ability to inhibit prostanoid biosynthesis(11, 12). In this regard, ASA can jumpstart the production of specialized pro-resolving mediators (SPMs) that are known to temper inflammation, enhance tissue resolution/repair and in some cases even possess anti-platelet actions(13, 14). Importantly, SPMs have been shown to decrease PLA formation(15, 16) and may be a mechanism underlying ASA's ability to block oxLDL-stimulated PMA formation. Further mechanistic studies addressing how ASA or SPMs block PLAs are of interest.

Platelets are activated by thrombin via the protease activated receptors (PARs) and also figure prominently in the terminal occlusive arterial atherothrombotic event. Research by Kuipers et al. and van Montfoort et al. focused on the distal events of plaque rupture and thrombus development using an ultrasound atherosclerotic plaque rupture model(17). These investigations centered on the contribution of the contact activation pathway proteases Factors XI and XII on occlusive thrombus formation. Using the ApoE$^{-/-}$ mouse model, FXI and FXII deficiency were investigated in the context of atherosclerotic plaque rupture. Van Montfoort et al. demonstrated the importance of FXI in atherothrombotic disease by showing that the thrombus formation on an acutely ruptured atherosclerotic plaque is dependent upon FXI and that thrombus development and platelet accumulation can be decreased by reducing FXI levels by antisense oligonucleotides(18). Similarly, using the same plaque rupture model, Kuijpers et al. demonstrated that another contact activation protease, FXII, regulates the process of thrombus formation on ruptured plaques through the use of two FXII pharmacological inhibitors(19). This group also demonstrated that FXII binds to immobilized plaque homogenates. Taken together, these studies suggest that the contact activation pathway plays an important role in the late events of atherothrombosis and thus may be a viable therapeutic target for preventing occlusive thrombus formation in the

context of plaque rupture(20). Targeting these molecules may be especially attractive because FXI and FXII deficiencies are not associated with increased bleeding tendency.

Platelet granules and granule exocytosis

Platelets are known to contain at least 4 different types of granules: dense, alpha, lysosomal and the recently described T granules. However, additional subtypes may yet be identified and the heterogeneity within each platelet granule type is also not clearly defined. Dense granules contain non-protein prothrombotic, pro-inflammatory and vasoconstrictive molecules, while the alpha granular cargo consists of hundreds of proteins both synthesized and taken up from the plasma(21). Thus the upkeep of platelet alpha granules is likely a dynamic process. Alpha granules function to deliver necessary "just in time" coagulation factors to sites of bleeding or tissue injury. As a measure of control against inadvertent or accidental activation, platelets also carry antithrombotic molecules.

In an attempt to decipher which granular proteins are jettisoned by the platelets and which remain associated, Wijten et al. used a sophisticated reversed releasate proteomics approach involving tandem mass spectrometry to quantitate the proteins released from the platelet granular contents(22). These investigators stimulated washed platelets from three human subjects with thrombin and collagen to release their granular contents and carried out proteomic analysis of the releasate compared to non-stimulated platelets. They observed that 124 proteins were significantly released, which represented less than 3% of the 4,500 platelet proteins they were monitoring. The released proteins were highly enriched in secretion tags and contained all known releasate factors, such as von Willebrand factor, factor V, platelet factor 4 and plasminogen activator inhibitor-1, at high concentrations. Interestingly, in the lower concentration range of the releasate many novel factors were identified. Thus, Wijten et al. devised a new method for measuring platelet releasates that is likely to be very useful for future diagnostic approaches. As examples, this new method may be useful for identifying and assessing patients with platelet abnormalities and also as a research tool for analyzing platelets from patients with known genetic abnormalities in platelet specific genes.

The mechanisms underlying the platelet granule release measured in the Wijten study remain of interest. The granules must fuse with the platelet plasma membrane and are thought to release their contents through a dynamic fusion pore(23). Koseoglu et al. investigated this mechanism by using a variety of *in vitro* and *in vivo* methods including the use of single cell amperometry to measure dense granular serotonin release. These investigators previously discovered the existence of dynamin-related protein-1 (Drp1) in platelets through a compound screen of the NIH molecular libraries (http://mli.nih.gov/mli/) using chemical genetic analysis of a platelet granule secretion-probe(23). Drp1, a GTPase most commonly known for its role in mediating mitochondrial fission and fusion, has also been reported to play a role in degranulation of Mast cells.

Koseoglu et al. observed that Drp1 is not only found in platelets, but is also phosphorylated in an activation-dependent manner and localizes to both membranes and cytosol. Further experiments showed that blocking Drp1 via specific small molecule inhibitors impairs the

stability of the fusion pore and leads to inhibition of platelet granule exocytosis. Importantly, inhibition of Drp1 also disrupted platelet accumulation during thrombus formation *in vivo*. These studies demonstrate a new role for platelet Drp1 and shed light on a potential new therapeutic strategy.

Alternative splicing regulates platelet specific expression of TFPI isoforms

Based on the data of the Wijten paper, tissue factor pathway inhibitor (TFPI) is present in platelets but is not currently known to reside in any of the described granules. Despite its uncertain cellular location, platelet TFPI still plays an extremely important role in hemostasis(24). TFPI is a Kunitz type protease inhibitor, which is the sole inhibitor of the initial events of blood coagulation. The TFPI gene undergoes alternative splicing to produce several isoforms, including TFPI-α and TFPI-β. TFPI-α, or full length TFPI, contains an acidic N-terminal region followed by three Kunitz domains and a C-terminal region. Kunitz 1 and 2 reversibly bind and inhibit the TF/FVIIa/FXa complex. The Kunitz 3 domain binds to protein S(25, 26). Rounding out the functional domains is a C-terminal region that has been shown to bind to FVa/prothrombinase. The binding of TFPI to FVa is highlighted by the FV-short isoform of FV predominant in the East Texas bleeding disorder(27, 28). FV-short lacks a basic region in its B domain that normally competes with the homologous TFPI-α C-terminal region for binding to an acidic region also in the FV B domain(27, 28). TFPI-β lacks the third Kunitz and C-terminal domain and is attached to the cell surface via a GPI anchor. In humans and mice the primary platelet isoform is TFPI-α, whereas TFPI-β is the predominant endothelial isoform. Total absence of TFPI activity leads to thrombosis and early death in mice(29).

Two papers in the ATVB by the Mast group have investigated the splicing regulation and function of platelet TFPI. Ellery et al. characterized the long known but unstudied alternative splicing of human TFPI exon 2(30, 31). This splicing event creates mature TFPI mRNA isoforms including or excluding exon 2 in the 5' untranslated region of the mRNA. Exon 2 was found to negatively regulate the translation of TFPI-β, with no effect on TFPI-α. Variability in TFPI-β expression in human endothelial cells suggests that exon 2 splicing may play a role in tissue specific control of TFPI-β expression. This novel mechanism of 5' untranslated region splicing regulating translation of a specific protein isoform produced via a second independent splicing event sheds light on the regulatory potential of alternative splicing likely to be found in the control of gene expression of other genes as well.

Wood et al. probed the role of the protein S cofactor function for TFPI-α mediated inhibition of Factor Xa(32). It was previously found that Kunitz 3 binds to protein S and that protein S inhibits prothrombinase activity via direct interactions with factors Va and Xa in humans(33). However, in this study it was found that protein S is a cofactor for platelet TFPI-α and acts by tethering soluble TFPI-α to a membrane surface, where it effectively inhibits membrane bound FXa(32). The activity of TFPI-β and another GPI-anchored form of TFPI containing Kunitz 3 was not altered by protein S. Protein S also was found to stabilize the TFPI-α FXa inhibitory complex delaying thrombin generation by prothrombinase. This study advances the ever-evolving scope of TFPI function by describing how it can delay prothrombinase assembly. One important question is how the recently

described TFPI-α interaction with FV fits with this entire process, since it has been well documented that protein S is a cofactor for the activated protein C mediated inactivation of FVa(34).

Actin cytoskeletal defects influence the platelet reactivity

Actin is the most abundant protein present in platelets and reorganization of the actin cytoskeleton is essential for platelet activation. There are a number of proteases such as gelsolin, actin-related proteins such as the ARP2/3 complex as well as adapter molecules, which serve to translate platelet cell signals into cytoskeletal changes(35). Filamins (FLN) are one such class of adapter molecule. These actin-binding proteins act as extended homodimers to reversibly crosslink the actin cytoskeleton and connect it to the cellular membrane via interactions with membrane proteins(36). Platelets have been shown to express predominantly the FLNa gene and transgenic mice with a targeted deficiency of megakaryocyte/platelet *Flna* are characterized by macrothrombocytopenia and increased tail bleeding time resulting from impaired alpha granule secretion as well as problems translating activation signals from integrin αIIbβ3, collagen receptor glycoprotein VI (GPVI) and the C-type lectin-like receptor 2(37).

In humans, the FLNa gene resides on the X chromosome at Xq28 and mutations in this gene display striking phenotypic heterogeneity with several human genetic disorders ranging from periventricular heterotopia to terminal osseous dysplasia (http://www.omim.org/entry/300017). Bleeding and thrombocytopenia have been noted in patients with periventricular heterotopia due to mutations in FLNa. However, the specific role of FLNa in platelet function has not been established. Barrou et al. identified 4 unrelated female patients with FLNa mutations and extended their previous studies on the effect of these mutations on platelet function(38, 39). In fact, they observed that the three patients with truncating FLNa mutations displayed thrombocytopenia and abnormal responses in platelet aggregation and adhesion. These FLNa mutations resulted in no detectable truncated FLNa protein production. Interestingly, non-mutated FLNa produced from the other intact allele ranged from 37–82% and lower levels of FLNa directly correlated with the severity of the observed platelet phenotypic defects. The fourth patient in their study had a nonsynonymous p.Glu1803Lys mutation. The platelet functional defects in this patient were consistent with a dominant negative effect on FLNa. Thus, this study determined that FLNa plays a distinct role of in platelet function, including aggregation, spreading and granule release in humans.

Similar to the more recently described FLNa-αIIbβ3 interaction, Kindlins 1–3 are well known to interact directly with αIIbβ3 integrin and are thought to mediate its transition from a low to a high affinity binding state for fibrinogen. In order to probe the functional significance of specifically Kindlin-3 in this process, Xu et al. created a transgenic knock-in mouse model with nonsynonymous mutations in two consecutive amino acids (p.Arg597Ala and p.Trp598Ala) in the F3 subdomain of Kindlin-3(40). They observed defective interactions between their Kindlin-3 mouse mutants and αIIbβ3 that resulted in defective platelet spreading, aggregation and thrombus formation *in vitro* and *in vivo*. Thus, these experiments support the notion that Kindlin-3 contributes significantly to platelet activation via the direct interaction of its F3 subdomain with the cytoplasmic tail of αIIbβ3.

Therapeutic strategies – challenges, promises and the way forward

Since the discovery of platelets over 130 years ago(41), great advances have been made in the understanding that these cells are integral to a variety of physiologic and pathologic processes. These insights led to the development of several early anti-platelet therapies including the P2Y12 and αIIbβ3 antagonist class of drugs that potently block platelet activation(42). Although these drugs have not been widely administered as prophylactic agents for cardiovascular disease due to safety issues, they have been important in procedures such as percutaneous coronary interventions and have represented a first line in the attempt to control platelet-mediated disease processes(43). New strategies are emerging as we learn more about platelet signaling and biology that could lead to improved safety, efficacy and specificity for diseases involving platelets. For example, the glycoprotein receptor GPVI is only expressed in platelets and megakaryocytes and loss of GPVI function is not associated with severe bleeding complications *in vivo* and thus represents an attractive therapeutic target(44). Insights from elegant studies delineating the complex interactions between GPVI and the PARs on platelet activation and thrombosis are refining the basis of this approach(45). Recent papers also suggest that blocking P2Y1 may prove amenable as an alternative anti-platelet strategy(46) and that inhibition of phosphodiesterase type 4 (PDE4) can block platelet-neutrophil interactions at sites of vascular injury(47). Another possible anti-platelet therapeutic is the SPM resolvin E1 (RvE1) because of its ability to temper adenosine diphosphate (ADP) and thromboxane-stimulated platelet aggregation(14). Interestingly, RvE1 was recently shown to block atherogenesis in rabbits(48) and because of its ability to reduce platelet activation, may be useful in curtailing atherothrombotic events. The mechanisms and cellular targets underlying RvE1's protective actions in this model are unknown and are of interest. Further, pro-resolving mediators are not immunosuppressive and may be a particularly attractive therapeutic strategy for a disease like atherosclerosis where long-term treatment is needed.

In addition, several platelet-derived molecules could eventually result in targeted therapies for cardiovascular conditions such as intimal hyperplasia(49, 50), atherothrombosis(20) and calcific aortic valve sclerosis(51). The research topics highlighted in this article are likely to contribute to advances that will lead to targeted platelet-specific therapeutics for diseases in which platelets participate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Significance

Platelets are unique anucleate blood cells produced by megakaryocytes that, in addition to being the primary effectors of hemostasis, are rapid responders to injury and infection. Therefore, platelets have a diverse functional repertoire in their ability to regulate inflammation, host defense and tissue repair. This review highlights some of the recent ATVB publications regarding the role of platelets in inflammatory vascular disease, the mechanisms of platelet thrombotic action, and insights into new anti-platelet therapies.