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The secret afterlife of platelets

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Platelets express a wide variety of receptors and signaling molecules that enable responses to diverse physiological and pathological stimulants. For instance, in normal hemostasis, exposure of subendothelial collagen may elicit platelet activation at the site of injury via glycoprotein (GP)VI, integrin $\alpha_2\beta_1$, and, through plasma von Willebrand factor, the GPIb-IX-V complex. Moreover, GPIb-IX-V in tandem with protease-activated receptors mediate thrombin-induced platelet signaling and activation. GPIbα serves as a receptor for low concentrations of thrombin, transmitting a mechanosensory signal to mediate calcium-dependent 14-3-3 signaling while GPIb-IX–dependent Rac1/LIMK1 signaling is modulated by protease-activated receptors.[1,2] Upon activation, platelets aggregate and form clots that are interwoven with fibrin strands. Over the last several decades, much of the research effort has been focused on how platelets are rapidly activated by various agonists via their respective receptors and how activating, and sometimes inhibitory, signals amplify and propagate in the platelet. In most of these studies, the investigation ends at the cessation of blood flow, the formation of the clot, and/or the appearance of molecular signs that are well associated with platelet activation. A few minutes following platelet activation and aggregation, the blood clot contracts. In studies of clot contraction, the investigation often ends at the shrinkage of the platelet clot.[3] However, little is known about the platelets in the clot following the contraction of the platelet/fibrin clot. In other words, after the formation of a stable blood clot, where do platelets go?

A study by Kim *et al.*, published in this issue of *Haematologica*, demonstrates that after activation and contraction, thrombin-stimulated platelets break up into membrane particles, in a process termed platelet fragmentation.[4] Thrombin is a major nexus between coagulation and platelet activation, as it generates fibrin to form a crosslinked fibrin plug and concurrently activates aforementioned receptors on the platelet surface.[5] Platelet vesiculation and/or microparticle formation has been previously observed in response to thrombin and thrombin receptor activating peptide.[6-8] The role that these platelet fragments play in hemostasis or platelet clearance has yet to be elucidated. In this new study, interestingly, Kim *et al.* observed a bimodal distribution of platelet fragments, the size of which can be attributed to the origin of the fragment. Filopodia as well as the main platelet body are two sources of platelet fragmentation, as smaller fragments were generated by filopodia, and larger fragments were generated from the cell body. Thus, it appears that platelet breakdown in response to thrombin stimulation is a regulated process of drastic morphological changes, platelet fragmentation, loss of function, and metabolic exhaustion. Platelet fragmentation may be a relatively newly discovered platelet behavior, adding to the ever-growing list of what

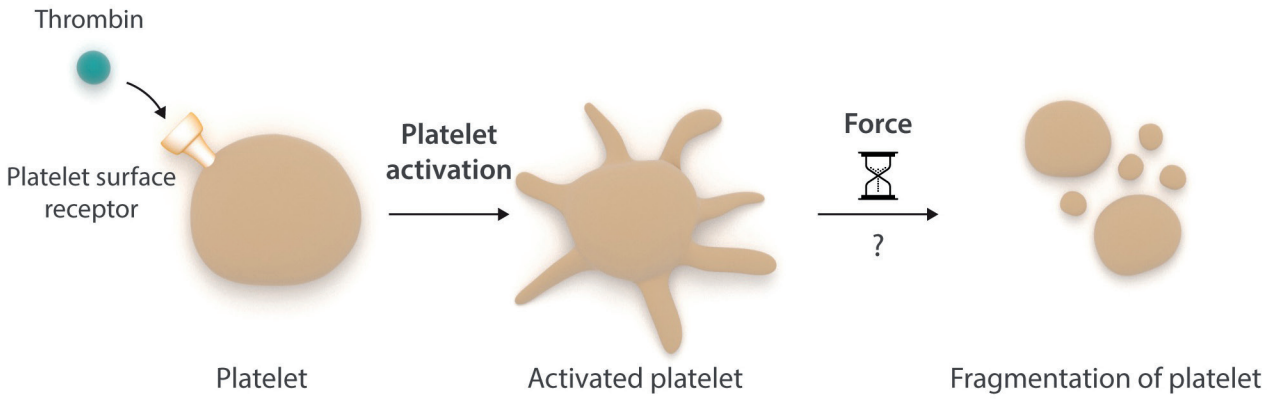

Figure 1. An illustration of thrombin-induced effects on platelets over time. Thrombin produced through coagulation binds to and activates platelet receptors, leading to platelet activation, degranulation, and filopodia formation. At a certain time after the hemostatic plug has contracted, platelets break down into membrane-bound fragments of defined sizes through a distinct mechanism.

happens to platelets after activation, and how they may play a role in hemostasis or clearance. This paradigm shift may help to elucidate novel mechanisms of platelet behavior and clarify the functional roles of such fragments of previously activated platelets.

Using transmission and scanning electron microscopy, Kim *et al.* observed that after 15 min of thrombin treatment, platelets broke up into separate membrane-bound particulates that contained granules, mitochondria, and vacuoles. Remarkably, this fragmentation was not seen after exposure to other platelet agonists such as collagen or ADP, although the platelets exhibited morphological changes associated with activation such as filopodia formation and spreading. This finding suggests that platelet activation creates agonist-specific behavior, and only when thrombin is being generated can platelets start to break up into fragments.

As actin is necessary for cytoskeletal rearrangement and filopodia extension,[9] the authors investigated localization of actin after exposure to thrombin. After fragmentation, actin was retained in the particulates, and gradually disappeared as the fragments became smaller. It is also worthy of note that intracellular levels of calcium correlated with fragmentation, such that calcium levels dropped as the platelets disintegrated. Mitochondrial function also decreased as the platelets fragmented. Mitochondria appeared to translocate to the periphery of the cell, or even escape to the extracellular space. Clot contraction force plateaued and the generation of reactive oxygen species coincided with the initiation of platelet fragmentation, suggesting that clot contraction is stopped by the loss of cellular energy in the form of ATP due to mitochondrial dysfunction. Actin-myosin is an ATP-driven motor, and these results support the previously seen loss of the actin-regulated cytoskeleton.

The authors noted that platelet fragmentation is different from typical necrosis, as fragmentation seemed to be a regulated process that did not entail cellular rupture as the membranes remained intact. To assess whether these platelet fragments were due to apoptotic signaling, caspase activity was assessed. Surprisingly, platelets exposed to up to 5 U/mL thrombin did not appear to activate caspases. If this is not apoptosis, the question is what is responsible for platelet fragmentation? The authors identified calpain, a cysteine protease that is believed to recognize tertiary structure as a cleavage site instead of sequence-specific activity,[10] as one enzyme responsible for these processes. Interestingly, maximal calpain activity coincided with the initiation of fragmentation and functional mitochondrial loss. ALLN, a calpain inhibitor, was able to delay thrombin-induced fragmentation and inhibit mitochondrial loss. However, ALLN was unable to inhibit calpain cleavage products completely. Also, it is clear that inhibition of calpains alone is not enough to prevent platelet fragmentation but the observations suggest that proteases are vital for the fragmentation process to occur.

While Kim *et al.* provided an elegant *in vitro* characterization and outlined the mechanism of platelet fragmentation, the biological significance of this process awaits further elucidation. For instance, do these platelet fragments play a role in the breakdown of platelet-rich clots, and can aberrant fragmentation play a role in thrombosis? Moreover, it is worth noting the time delay in fragmentation upon treatment with thrombin and after platelet contraction, which may be significant to its function. While the GPIb-IX-V receptor complex does not typically lead to a fast and strong intracellular signal, protease-activated receptors, like most G protein-coupled receptors, can rapidly induce full activation of platelets. Thus, what is the mechanism in the platelet that causes fragmentation to proceed *only* after platelet activation and contraction events have run their courses? The authors demonstrated that force and time are likely important factors in the process of fragmentation (Figure 1). It would be extremely interesting to understand how signaling and cytoskeletal proteins in the platelet respond to these forces and temporal factors. Furthermore, perhaps there is a balance between traditional apoptotic pathways and fragmentation during exposure to a combination of agonists. When would a platelet undergo apoptosis rather than fragmentation in response to multiple agonists? Finally, it remains to be addressed how platelet frag-

mentation relates to platelet clearance, or what percentage of activated platelets undergo fragmentation *in vivo*. It is widely accepted that activated platelets are quickly cleared from the body, but the actual molecular mechanism has been elusive. Recent work on platelet clearance has focused on investigating how platelets expose a 'clear-me' signal, perhaps through desialylation of platelet surface proteins.[11] The occurrence of platelet fragmentation following activation has raised the possibility that in some cases the reduction in platelet counts, which has been uniformly used as the indicator of platelet clearance, may be attributed to some extent to platelet fragmentation. It remains to be seen whether certain receptors responsible for platelet clearance can also recognize and clear platelet fragments.

If this fragmentation can be observed and tracked *in vivo*, perhaps the question of where fragmented platelets go after activation can be answered. A recent publication by Tomaiuolo *et al.*[12] included high resolution images of hemostatic plugs in response to a puncture in the jugular vein. What can be gleaned from these images is the notable presence of small platelet fragments in both the intravascular and extravascular boundaries of the injury site. Determining the roles that these fragments play in hemostatic plug formation and/or thrombus formation would be crucial to a complete understanding of *in vivo* platelet plug formation. Potentially, this mechanism could also be a pharmacological target to reduce thrombus formation or aid in thrombolysis in pathological conditions. This exciting finding may point to a novel mechanism of platelet behavior and has major implications for thrombus dissolution and platelet clearance in general.

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