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Is the endothelial cell responsible for the thrombus core and shell architecture?

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ABSTRACT

Ischemia leading to heart attacks and strokes is the major cause of deaths in the world. This report explores the possibility that intracellular material from ruptured endothelial cells is partially responsible for the heterogeneous core-and-shell blood clot architecture, typically observed using intravital microscopy. As evidence, we present a fluid dynamic argument that platelet agonists emanating from the injury cannot activate platelets in the thrombus core, given that they would have to travel *against* flow of blood escaping into the extravascular. Furthermore, we demonstrate visual evidence that the core material appears to be continuous and originating from the damaged endothelium. Finally, we present a mechanism, illustrating the steps of platelet recruitment into the thrombus and sealing of the injury. If correct, the model presented herein will be beneficial to the understanding and treatment of heart attacks, strokes and hemophilia.

Background and significance

Achieving hemostasis following penetrating injuries is essential for the survival of organisms that possess a closed high-pressure circulatory system. However, pathological manifestation of blood clot formation (a.k.a., thrombogenesis) and breakup (a.k.a., embolism) can potentially lead to life-threatening complications when occurring in the heart (i.e., a heart attack), brain (i.e., a stroke), or lungs (i.e., deep vein thrombosis/pulmonary embolism). Among these, thrombo-embolic infarction is the leading cause of mortality and morbidity in the United States, while stroke is the fifth [1]. Yet, despite tremendous efforts by the medical research community (e.g., ~$3 billion of annual expenditure on heart attack and brain stroke research alone [2]), the problem remains largely unsolved to this day.

Bottlenecking the progress is the fact that the clot formation is a fast microscopic process, which is not easily accessible to observation *in vivo*. Although, it has been studied since at least 1882 [3], only recently have developments in intravital microscopy [4,5] began to unravel the true mechanism behind it. Among the revelations is a recent discovery [6] that the clots consist of two distinct parts: a densely packed "core" of highly activated platelets, and a loose "shell" of less activated ones (see Fig. 1). This structural heterogeneity is important, because the biological differences between the regions could potentially be utilized to circumvent excessive bleeding associated with existing antithrombotic treatments [7,8].

Specifically, the thrombus core appears to be responsible for clot attachment to the blood vessel wall, and more importantly, for the cessation of blood loss; while the shell serves no apparent biological function. Yet, since the latter part of the clot is exposed to the blood flow, it serves as the source of the pathogenic embolism that leads to the life-threatening complications of thrombosis. Hence, a new class of drugs could be developed that would target just the dangerous outer shell of the blood clot, while leaving the useful core intact. However, the mechanism behind the formation of the core-and-shell is still an open question in the field of hematology. Therefore, gaining deeper insight into its origin would be beneficial for public health, as it could pave the way towards better pharmacological treatments.

Why isn't the core-and-shell architecture affected by different blood flow patterns and injury types?

As summarized in Fig. 1, the disparity in platelet activation between the two parts of the thrombi is attributed to molecular transport limitations of strong platelet agonists. Specifically, it has been proposed [9,10] that thrombin generated at the injury site propagates into the core and activates the platelets in that region. However, such large molecules do not reach the shell, because its diffusion is restricted by the tightness of the core's porosity. Instead, the activated platelets in

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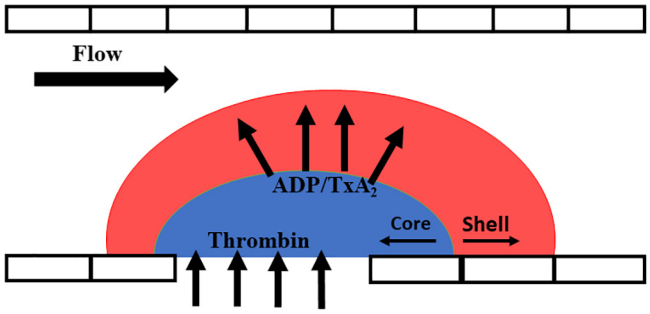

Fig. 1. Conventional model of the role of transport on agonist distribution and thrombus architecture: The core is a result of a gradient of soluble agonists emanating from the site of injury. Namely, thrombin generation at the injury site drives platelet activation in the core region, resulting in high platelet packing density, decreased solute transport, α-granule exocytosis, and fibrin deposition. This leads to the retention of larger agonists within the core, limiting the thrombin's propagation into the shell. The restriction of thrombin to the core contributes to the shell consisting of loosely adherent platelets, high solute transport, reduced platelet activation, and no fibrin. Instead, a gradient of smaller agonists, such as ADP and potentially TxA2, emanating from the activated core extends farther, and results in the recruitment of additional platelets forming the shell region. Furthermore, additional platelet signaling, such as contact-dependent pathways could reinforce the shell's architecture via local positive feedback.

this region release their granules, and form a secondary gradient of weaker agonists, such as adenosine diphosphate (ADP) and thromboxane A2 (TxA2). In turn, these smaller molecules emanate from the core outwards, and result in the recruitment of additional platelets that form the shell. However, their already diminished activation potential is further weakened by the washing out of the agonists by the surrounding blood flow, which is able to penetrate into the shell due to its high porosity [11]. Consequently, the degree of platelet activation is insufficient to elicit α-granule release in this region.

In summary, the conventional explanation of the core-and-shell formation relies on the platelet agonists fluxing *upward* from the injury, and into the body of the thrombus. However, intravital microscopy experiments [12] that use albumin coupled to caged fluorescein (cAlb) molecules as a transport biosensor show that different blood flow patterns do not affect the resulting thrombus architecture. Specifically, there are two extremes of the laser injury types typically achieved under *in vivo* conditions: Fig. 2-A and Supplemental Video 1 show an injury that is *non-penetrating* (i.e., no blood escapes to the extravascular); while Fig. 2-B and Supplemental Video 2 show a fully *penetrating* injury (i.e., blood does escape to the extravascular). Yet, the core-and-shell thrombus architecture forms in both cases, and at similar time scales, despite the drastic differences in blood flow regimes.

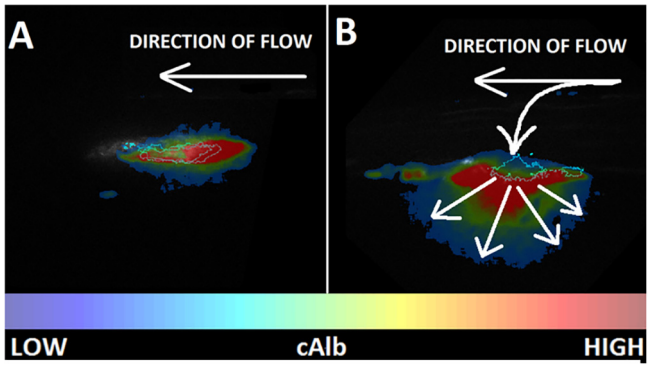

Fig. 2. Different injury types visualized via fluorescently labeled caged albumin (cAlb) in microcirculation of live mice injured via a laser: (A) Non-penetrating – cAlb is trapped in a punctured muscle cell, encircling the blood vessel; and (B) Fully Penetrating – cAlb escapes into extravascular. The images correspond to the initial time of the injury, before the thrombus has formed. The cyan outline shows the steady state outline of the clot's core, after the thrombus will have formed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, Fig. 3 shows that for the *penetrating* injury type, the platelet activation (visualized via P-selectin fluorescence) in the clot core increases steadily during the escape of blood to the extravascular. In other words, although the blood is flowing *downward*, the conventional model requires that the platelet agonists should flux *upwards*, in order to activate the core. And given that thrombin is a large molecule with a small diffusivity, it is rather unlikely that it would be able to travel *against* the convective flows of blood. Yet, by the time (see $t^* = 0.35$) half of the clot (red curve) and its core (blue curve) have formed already, the flow rate of the blood to the extravascular (green curve) has only halved from its initial value. Hence, there is a significant flow of blood in the direction opposite to the supposed diffusion, throughout most of the clot formation process. Therefore, this observation directly contradicts the conventional model.

Furthermore, another curious observation from Fig. 3 is that activation of the core ceases upon sealing of the injury at dimensionless time $t^* = 1$ (the scale is described in our prior publication [13]). Although it is true that the primary agonists would no longer be able to make their way from the injury into the thrombus, the diffusion of any molecules resident in its body should continue the activation process. Yet, the expansion of the core does not continue. Therefore, it is not apparent whether the conventional model of the core-and-shell formation's dependence on the agonist transport is entirely reliable.

Could the damaged endothelium be responsible for the resulting clot structure?

A critical element of the *in vivo* injury experiments mentioned above is that while the platelets themselves are labeled via anti-CD41, the degree of what is thought to be platelet activation is quantified via fluorescence of an anti-P-selectin (an adhesive molecule found in platelet α-granules) marker [6,14–16]. For example, the core is P-selectin positive, while the shell does not display a P-selectin signal. Thus, the validity of the core-and-shell model rests upon the *assumption* that the P-selectin expression is a signature of platelet activation only. However, the platelets are not the only source of P-selectin in the endovascular system: in fact, endothelial cells (EC) also contain P-selectin in their granules, called the Weibel-Palade bodies [17–19]. So, it is possible that the P-selectin signal observed in the *in vivo* injuries originates from the "guts" of the damaged ECs (rather than just from activated platelets), leaving room for an alternative interpretation of the core-and-shell microscopy images.

Furthermore, Sema4D$^{-/-}$ mice [20] have been used to show that the formation of the dense packing in the core can occur asynchronously to the P-selectin expression in the same region [12]. Specifically, Sema4D is a semaphorin family member on the surface of platelets that supports contact-dependent amplification of Syk activation, downstream of the platelet collagen receptor, GPVI. Injuries induced in the Sema4D$^{-/-}$ mice showed a significant delay in the P-selectin expression in the core, yet cAlb retention in the same region showed that it is still much denser than the shell (see Fig. 6 in Ref. [12]). One interpretation of this observation is that there could be *partial* activation of the platelets, which is not affected by the lag in the α-granule exocytosis required for the expression of P-selectin. Yet, another possibility is that the low porosity observed in the thrombus core is due to a presence of a non-platelet material (which is of EC origin). After all, a receptor for the Sema4D was recently identified in the ECs also [21]. Furthermore, the thrombi in the mouse microcirculation are dimensionally similar to the ECs: both are roughly 100 μm in length and 20 μm in width.

Following this line of reasoning, the next section develops the EC idea further, and presents our hypothesized mechanism of how thrombogenesis may involve its participation.

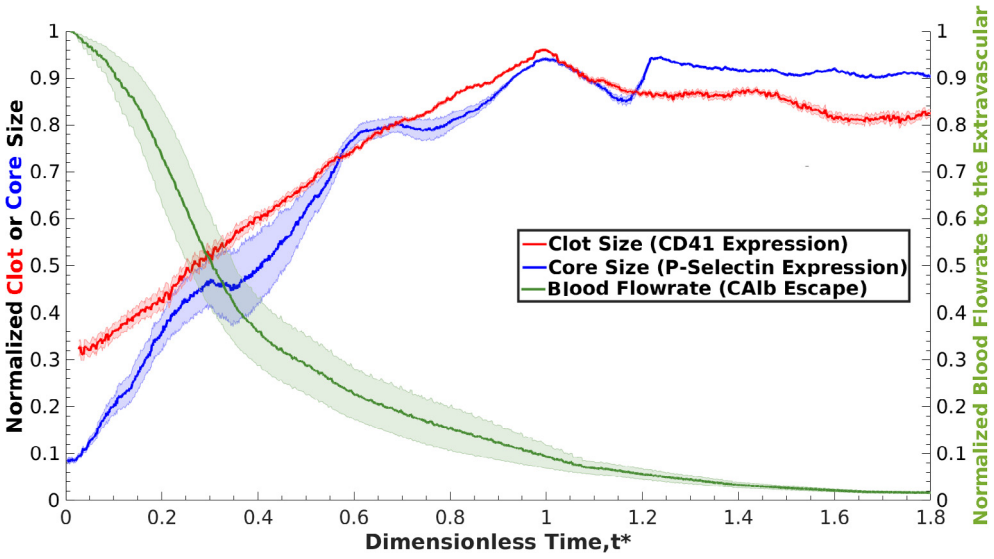

Fig. 3. Time course of blood flow into the extravascular space (green) versus P-selectin expression in the clot core (blue) and CD41 expression in the clot (red). P-Selectin is quantified by measuring the area of CD62P in the intravital microscopy videos. CD41 expression is quantified by measuring the area of CD41 in intravital microscopy videos. Blood flow rate estimate is based on the changes in the area of the fluorescently labeled cAlb plume in the extravascular space. Dimensionless time scale is achieved via normalization by the characteristic time at which the clot size is maximum (described in our prior publication [13]). Likewise, the clot and clot core sizes are normalized by their respective global maximums for each experiment. All curves are moving averages with windows of 0.5; and the error bars represent the moving variance for 10 different laser injury experiments on wild type mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Hypothesized role of endothelium participation in thrombogenesis

Fig. 4 illustrates our hypothesized mechanism of how the EC could be contributing to the core-and-shell structure, which forms as a result of thrombogenesis. As with the conventional models, the injury is initiated at dimensionless time, $t^* = 0$ by rupturing an EC. However, in our model the EC's interior is immediately exposed to the blood flow contents in the lumen (see Fig. 4-LEFT). Next, a gelatinous substance emanating from the damaged EC captures the platelets from the blood flow and recruits them into the thrombus. It is this substance that displays the majority of the early P-selectin signal and is perceived as the thrombus' core in the intravital microscopy videos.

As the thrombus grows further, it begins to occlude the passage of other blood contents. Ultimately, at some critical time ($t^* = 1$), the thrombus achieves its peak size (see Fig. 4-CENTER) and is consequently deformed by the fluid shear. At this point, the platelets from its front are transferred to the downstream side. When this happens, the upstream portion of the thrombus wraps around, creating the classical "comet" tail pattern (see Fig. 4-RIGHT). Finally, the gel retracts back into the damaged vessel wall, ultimately resulting in a consolidation of the platelets at the site of the injury and in sealing the wound (see the black arrows in Fig. 4-RIGHT).

This movement of intra-thrombus platelets towards the injury site has been observed previously *in vivo*: for example, see in Fig. 1A–B and supplemental Video 1 of Ref. [22]. Furthermore, in a different study, the collective retraction of the platelets has been shown to occur independently of their migration within the clot, because the latter is significantly slower and lacks the directionality towards the injury (see Fig. 1D-RIGHT, 1F, and 1H-RIGHT in Ref. [23]). Therefore, these *in vivo* observations are consistent with our model of thrombogenesis, in which the EC's guts exposed to the blood flow are withdrawn back into the damaged vascular wall, together with the trapped platelets.

Furthermore, Supplemental Video 3 shows that the P-selectin positive core (green) extends from the injury like a protrusion that gets stretched out and retracts back like a rubber band. This behavior strongly resembles a continuous substance, rather than discrete platelets visible in the shell. This is consistent with our hypothesis about a gel emanating from the injury site and wrapping about the thrombus, as a result of the structural deformation after $t^* = 1$. It would also explain how the shell is held together without considerable platelet activation, as the cells could be bound together by P-Selectin negative EC material.

Thus, our hypothesis is that there is an additional contribution of the EC material participating in the body of the clot. We suspect that it is this material that is responsible for the majority of the *early* P-selectin signal detected in the core by the previous studies.

How to test the hypothesis

In summary, we have hypothesized a thrombogenesis model that involves the participation of intra-EC material in the clot's core. This modification to the conventional model helps to explain why thrombi formation is not affected by blood flows into the extravascular, which should entrain the platelet agonists responsible for activating the core. It also helps explain how the thrombus shell is held together, despite no apparent platelet activation being detected in this region of the clot. If correct, the presence of the EC material in the core could open up the possibilities for new pharmaceutical strategies, capable of selectively dissolving the dangerous shell, while leaving intact the useful core. In

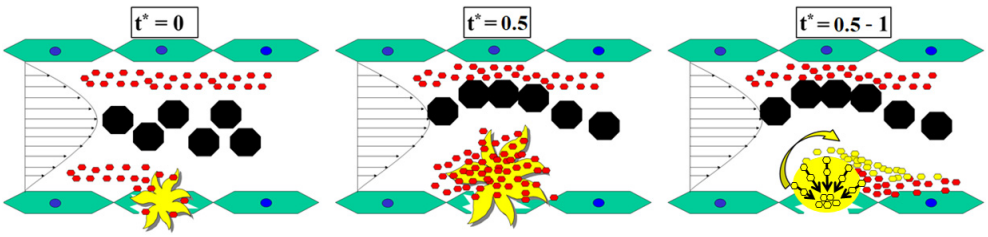

Fig. 4. TOP: Hypothesized thrombogenesis mechanism, as a function of dimensionless time, t^*. Green = intact ECs; Red = Platelets; Black = Red & White Blood Cells; Yellow = damaged EC's "guts". TOP LEFT – EC guts are exposed to the blood contents as a result of thrombogenesis initialization via injury. TOP CENTER – platelets are captured by the exposed EC guts in the lumen. TOP RIGHT – transfer of upstream mass to the downstream side, resulting in a comet shape. Values of t^* corresponding to each stage of thrombogenesis are hypothesized based on our prior work [13]. Black arrows indicate the direction of platelet retraction towards the injury. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

principle, this should be possible by taking advantages of the differences between the platelet [8,24] and the EC [23] drug targets.

However, given the speculative nature of this manuscript, further evidence is needed to test this hypothesis. Either proving or disproving this idea would serve a great benefit to the public health, given that pathogenic thrombosis is the leading cause of death worldwide. Therefore, exploring this idea further is warranted. And the most direct way of doing that is by looking for EC-specific markers [23] in the thrombus via fluorescence labeling. Furthermore, given that the P-selectin has multiple sources within the endovascular system, it would be better to use a marker of platelet activation that is unique to these cells. Another approach would be to deplete the test subject of its platelets and inject it with artificial substitutes. If the thrombus core is formed, this would demonstrate that the P-selectin in it is originating from something other than the platelets. Finally, even if the EC is shown not to participate in the thrombus formation, the contradiction of platelet agonists fluxing against the blood flow still needs to be resolved. This can be done by fluorescently labeling the agonists and tracking their transport within the thrombus.

Declaration of Competing Interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mehy.2019.109244.

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