Published in final edited form as:

Thromb Res. 2012 May ; 129(Suppl 2): S54–S56. doi:10.1016/j.thromres.2012.02.034.

Imaging coagulation reactions *in vivo*

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Abstract

Significant gaps remain in the understanding of how blood cells and the vasculature differentially support coagulation enzyme complex function leading to regulated thrombus formation *in vivo*. While studies employing knock-out or transgenic mice have proved useful many of these scientific gaps partly result from the lack of molecular approaches and analytic tools with appropriate sensitivity for incisive conclusions. Over the past decade, studies employing state of the art videomicroscopy to image hemostasis *in vivo* following laser injury to the mouse cremaster arteriole have begun to bridge these gaps and provide remarkable insight into the early events of the hemostatic process. Many of these new insights have started to question some of the long-standing concepts that were driven by *in vitro* approaches. This review provides an overview of this technology, describes insights that have been made using it, and discusses limitations and future directions.

Introduction

Vascular damage triggers the hemostatic response to limit the loss of blood by the formation of a blood clot. The normal hemostatic response is localized to the site of damage and is commensurate with the magnitude of injury. Extensive biochemical work using purified and reconstituted systems has established a detailed mechanistic understanding of the various steps of blood coagulation [1]. Despite this strong biochemical foundation, *in vitro* studies generally fail to appreciate how the ensemble of vascular and blood cells combined with flow participate with the coagulation system to regulate thrombus formation. Thus, major gaps remain in integrating knowledge of blood coagulation reactions to understanding the overall behavior of the hemostatic system *in vivo*.

Intravital imaging of laser-injury induced thrombus formation

Studies with knock-out and transgenic mouse models have greatly expanded vistas for the investigation of coagulation *in vivo*. Apart from monitoring coagulation parameters, bleeding and thrombosis models are also useful; however, they typically lack the analytic power required for incisive conclusions related to molecular detail. Over the past decade this drawback has been overcome, in part, by pioneering work employing intravital imaging

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Financial & competing interest disclosure: RMC receives research support and royalties from Pfizer for technology related to FXa. The other authors have no financial interest to disclose related to the contents of this article.

methods to examine the contribution of various blood and vessel components in thrombus formation [2–4]. This technique offers direct visual insight into the early events of thrombus development in real time, enabling the characterization of this dynamic process. Because of the amenability of the mouse mesentery and cremaster muscle to intravital fluorescence microscopy, these tissues are used in a laser-induced injury model to visualize thrombus formation in real-time. Furthermore, detailed three-dimensional visualization to facilitate spatial localization of protein and cellular components can be performed by high-speed confocal microscopy. To visualize platelets and fibrin, non-inhibitory fluorescent antibody derivatives are infused prior to injury [3]. Following laser injury to arterioles or venules, brightfield and fluorescent images are rapidly acquired in real-time which can be subjected to quantitative analysis (Figure 1). An advantage of this experimental system is that vessel injury generally does not produce full occlusion of the lumen; rather endothelial cells appear to be activated instead of damaged with no apparent exposure of the subendothelium [2,5,6]. The precise mechanism by which the endothelium becomes activated still remains unclear. However, laser injury of these cells leads to Ca^{2+} -mobilization and granule secretion as well as fibrin formation suggesting phosphatidylserine is exposed which would allow for clotting factor binding [5].

New concepts on thrombus formation *in vivo*

Real-time visualization of thrombus formation in the living mouse following injury provides both spatial and temporal information about thrombus development. This information coupled with unique mouse models and specific probes to detect proteins and other cellular components in the vicinity of the thrombus have the potential to uncover new paradigms. Over the last decade, work using this technology has reshaped thinking on early hemostatic events in the microcirculation. For example, an open and unanswered question relates to which mechanisms contribute to platelet activation at the site of vascular injury. Upon injury platelets adhere to the extracellular matrix and/or other cellular components, become activated, and aggregate to initiate the hemostatic response [7]. These observations are dominant in *in vivo* thrombosis models where the endothelium is damaged and von Willebrand factor (vWF) and collagen, for example are exposed [8]. However, laser injury to the cremaster muscle in mice lacking the collagen or vWF receptor (Gblba) revealed that platelet activation is independent of these receptors [9,10]. Rather, tissue factor (TF) mediated thrombin generation, plays a central role in platelet activation in this model [9,10]. The TF and collagen/vWF pathways of platelet activation are clearly both relevant; however these important studies highlight the dependence of the hemostatic response on the extent of injury, pathological conditions, and makeup of the vessel wall.

Other important findings derived from work using intravital microscopy pertain to factors which contribute to the initial events of fibrin formation. Work from the laboratories of Bruce and Barbara Furie have shown that microparticle-derived TF, depending on the extent of injury, plays a key role in fibrin progression. Endothelial cells in culture can be activated by laser injury and may contribute a critical source of activated TF [5]. Another important contributor to the early events of thrombus formation appears to be protein disulfide isomerase (PDI). PDI is rapidly released from platelets or endothelium following activation and accumulates at the site of injury [11–13]. Inhibition of PDI completely blocks fibrin formation and platelet accumulation [12]. While the mechanism by which PDI influences thrombus formation may involve TF activation, mechanistic details remain controversial.

A key observation made using the intravital imaging technology was that platelet accumulation and fibrin generation occur simultaneously in this model [3]. This raises the important question as to which cellular surface contributes to thrombin formation at the site of vascular damage. The prevailing dogma is that activated platelets fulfill this role by

supporting intrinsic Xase and prothrombinase complex assembly. Data challenging this dogma come from mice deficient in Protease Activated Receptor 4 and therefore deficient in thrombin-dependent platelet activation [14]. These mice do not form a platelet thrombus following laser injury yet exhibit essentially normal fibrin accumulation. Furthermore, blocking platelet accumulation at the injury site with an $\alpha_{IIb}\beta_{III}$ inhibitor has little, if any, influence on fibrin formation [12]. These data show that membrane surfaces other than those provided by the activated platelet at the injury site must contribute significantly to fibrin formation. The most likely source of the cell surface involved in fibrin generation is the endothelium. Evidence to support this comes from recent work showing that in the laser injury model endothelial cells *in vivo* become rapidly activated [5]. Furthermore using endothelial cells in tissue culture the same study showed that these activated endothelial cells support fibrin formation in a TF-dependent manner. While more work needs to be done, these studies are provocative and challenge existing paradigms in which activated platelets are considered dominant contributors to thrombin and ultimately fibrin formation.

Current limitations of imaging coagulation in vivo

New intravital videomicroscopy techniques have permitted the study of the biochemistry and cell biology of the hemostatic process in a living animal. As the technology improves with higher speed cameras, faster confocal capabilities, more sensitive detectors, etc., a higher resolution picture of clot formation will emerge. As with any methodology there are limitations to this system. For example, while the laser-injury model produces a reproducible injury without significant blood loss and endothelial cell denudation, the relevance of heat or photon-induced damage to authentic clot-inducing vascular damage in the microcirculation is arguable. Other injuries within this model are currently being explored such as a poking or crushing model or even chemically induced injuries [6,15]. A concern however with these alternatives is reproducibility of the injury. Another limitation is the availability of observational tools. With few exceptions [16,17] visualization of cells, cellular components, and coagulation proteins have relied on infused fluorescent antibodies. In general, there is a lack of available antibodies which cross-react with murine proteins. To visualize these components without negatively impacting the hemostatic process the antibodies also need to be non-inhibitory. Furthermore, if the intent of the work is to quantitatively analyze a particular protein at the site of injury, the use of labeled antibodies may obscure the analysis as it is an indirect detection technique. Direct quantitative comparisons in the accumulation of a particular measured protein are limited because the fluorescent signal is dependent not only by their concentrations but is also impacted by the extent of antibody labeling, the affinity of the individual antibodies for their respective antigens and the amount of antibody infused. Furthermore, despite the use of antibodies that have no overt effect on function established by limited characterization, it is possible that their presence in the circulation produce subtle effects with a major consequence to interpretation of thrombus formation in the microcirculation. As a result, many of the conclusions using this approach remain in the qualitative realm.

Future directions—site-specifically labeled coagulation proteins

The expansion of intravital imaging techniques to study *in vivo* hemostasis requires novel analytic tools with appropriate sensitivity. A direct way to achieve this is to bioengineer recombinant coagulation factors, preferably murine, which can be site-specifically labeled with a single fluorescent probe without loss of biologic function. Over the past decade our group has established several recombinant approaches for the large-scale expression of recombinant coagulation protein variants from multiple species in mammalian cells. Taking advantage of these systems, we generated site-specifically labeled derivatives of factor Va, factor Xa, and prothrombin. To accomplish this, we relied on available free cysteines (Cys)

which are amenable to labeling or recombinant variants with an introduced Cys. For example, for factor Va we prepared a variant (FVa-810_{SYA}) that is constitutively factor Va-like with three free Cys mutated while leaving a single free cysteine at position 539 [18,19]. This site can be readily labeled with Alexa-maleimide fluorescent probes without affecting cofactor function. In preliminary work, site-specific fluorescent derivatives of factors Va and Xa can be visualized with appropriate sensitivity and represent powerful tools to establish their spatial distribution at the site of laser injury in the mouse cremaster model (Ivanciu, et al., unpublished observations and [18,19]). The novel application of these well-characterized and unique site-specific fluorescent probes is expected to provide both temporal and spatial information regarding the assembly and function of the coagulation enzymes during thrombus development.

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Figure 1.

Platelet and fibrin accumulation following laser-induced arteriole injury in wild-type (wt)-mice mice. *Top panels*, digital composite fluorescence and brightfield images of representative thrombi in wt-mice (Balb/c; 6 weeks old) prior to (0 sec) and 60, 120, and 180 sec after laser-induced injury of the blood vessel wall. Platelets (red) were detected by an Alexa₅₅₅-labeled rat anti- CD41 F(ab)₂ and fibrin (green) with Alexa₄₈₈-labeled anti-fibrin antibody; areas of overlap are depicted by yellow.

Bottom panels, confocal imaging of platelets and fibrin 240 sec after initial laser injury. Images depict different orientations of the same thrombus. Coloring scheme is same as top.