Imaging fibrin formation and platelet and endothelial cell activation in vivo

Lola Bellido-Martín1,*; Vivien Chen1,*; Reema Jasuja1,*; Bruce Furie2; Barbara C. Furie2

1Division of Hemostasis and Thrombosis, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA; 2Harvard Medical School, Boston, Massachusetts, USA

Summary
Over the past six decades research employing in vitro assays has identified enzymes, cofactors, cell receptors and associated ligands important to the haemostatic process and its regulation. These studies have greatly advanced our understanding of the molecular and cellular bases of haemostasis and thrombosis. However, in vitro assays cannot simultaneously reproduce the interactions of all of the components of the haemostatic process that occur in vivo nor do they reflect the importance of haemodynamic factors resulting from blood flow. To overcome these limitations investigators have increasingly turned to animal models of haemostasis and thrombosis. In this article we describe some advances in the visualisation of platelet and endothelial cell activation and blood coagulation in vivo and review what we have learned from our intravital microscopy experiments using primarily the laser-induced injury model for thrombosis.

Keywords
Animal models, thrombosis, intravital imaging

Introduction
Haemostasis has evolved in vertebrates to maintain a sound, closed, high pressure circulatory system. The haemostatic process must rapidly stanch blood loss upon vascular injury while avoiding obstruction of flowing blood within the vessel. Components of the haemostatic system include the endothelial cells lining the blood vessels, exposed constituents of the subendothelium including fibrillar collagen in the extracellular matrix and smooth muscle cells, and constituents of circulating blood-platelets and the proteins of the blood coagulation and its regulation. Following vascular injury components from these compartments interact in an integrated response that includes endothelial cell activation and granule secretion; platelet adhesion, activation, aggregation, and granule secretion; and activation of the enzymes of the blood coagulation cascade. The result is the spatially localised, temporally limited formation of a fibrin rich haemostatic plug. Thrombosis may occur when pathologic processes interfere with the regulatory mechanisms for spatiotemporal control of haemostasis.

Over the past six decades research employing in vitro-assays has identified enzymes, co-factors, cell receptors and associated ligands important to the haemostatic process and its regulation. These studies have greatly advanced our understanding of the molecular and cellular bases of haemostasis and thrombosis. However, in vitro-assays cannot simultaneously reproduce the interactions of all of the components of the haemostatic process that occur in vivo, nor do they reflect the importance of haemodynamic factors resulting from blood flow. To overcome these limitations investigators have increasingly turned to animal models of haemostasis and thrombosis. Many of these models, developed initially in larger mammals, have been adapted to use in mice (1, 2). Two factors have contributed to the appeal of the mouse as a model for the study of thrombosis. The first is the ability to generate genetically modified animals with targeted deletions or modifications of haemostasis and thrombosis. The second is the suitability of the mouse for study by recently developed intravital imaging methods to examine the importance of various components and their role in thrombus formation. Advanced real time intravital microscopy as a detection method provides the opportunity for more complete and detailed study of thrombus formation in vivo than has been available with the earlier end points of intravital thrombosis experiments. The commonly used Doppler or temperature sensing probes or direct visualisation with analog videomicroscopy detect changes in blood flow and primarily indicate time to thrombotic occlusion of the experimental vessel. There are a number of excel-
lent reviews of mouse thrombosis models (1–3). We limit this review to advances in the visualisation of platelet, and endothelial cell and blood coagulation activation in vivo and review what we have learned from our intravitral microscopy experiments using primarily the laser-induced injury model for thrombosis.

Intravitral imaging of thrombus formation

Thrombosis models

Diverse insults to the vasculature have been used to induce thrombus formation (1–3). We have focused on two methods that proved amenable to analysis of thrombus formation by intravitral imaging: the ferric chloride injury model and the laser injury model. Induction of thrombus formation by topical exposure of blood vessels to ferric chloride has been widely used in a variety of animals and on a variety of blood vessels (4, 5). Most recently this model has been adapted to use on the microvasculature in the mouse mesentery and cremaster, two thin membranous tissues which permit sufficient light transmission for intravitral microscopy (2, 6, 7).

Ferric chloride model

Exposure of the adventitial surface of a blood vessel to ferric chloride was believed to produce an oxidative injury that resulted in denudation of the endothelial cell lining of the vessel resulting in thrombus formation (5, 8). This mechanism of ferric chloride injury is supported by the demonstration that ferric ions penetrate the internal elastic membrane by an endocytic/exocytic pathway (9). Denudation of the endothelium after ferric chloride injury has been reported in several studies by showing loss of von Willebrand factor-containing cells in mesenteric arterioles or exposure of collagen in cremaster arterioles (6, 7). However, Tseng et al. reported that a ferric chloride insult to the carotid artery resulted in a range of responses from minimal cellular damage to total denudation (9). Using a modified application of the ferric chloride solution, Kawamura et al. observed that the injured endothelial cells in cremaster arterioles became rounded, exposing the subendothelial surface (10). In these experiments platelets initially bound at junctions of the rounded endothelial cells and endothelial cells were present in the occlusive thrombi that form in the cremaster arterioles. Some of the differences observed in response to ferric chloride injury may be due to differences in the blood vessels used. However, variation in the injury sustained after ferric chloride exposure is likely even with seemingly similar protocols, a property that suggests that when using this model of thrombosis it is useful to verify the nature of the injury achieved.

Laser injury model

The first laser-induced injury model of thrombosis used a ruby laser to initiate thrombosis in a neo-vascularised transparent chamber implanted on the rabbit ear (11). Subsequently, a mouse model of laser-induced thrombosis was developed that employed the native vasculature of the mouse ear and injury with an argon-krypton laser (12). The model has been further extended to the mouse mesentery and cremaster, two tissues amenable to intravitral fluorescence microscopy (13, 14). As with the ferric chloride thrombosis model, there is variability in the laser-induced thrombosis models currently in use with intravitral microscopy. In our laser-induced thrombosis model, performed in a warm, temperature and 

pH controlled preparation of the cremaster or mesentery, we generally use a single intensity arteriolar injury that spares the endothelium and generates a non-occlusive thrombus (13). In this model platelet activation leading to platelet thrombus formation and fibrin generation is mediated by thrombin (15). Gachet et al. have developed a laser-induced thrombosis model employing two different injury intensities (16). Their “superficial injury”, which results in exposure of collagens III and IV, generates a transient thrombus and does not lead to fibrin deposition (16). In this model platelet activation is collagen-dependent. Their “deep injury” model results in exposure of collagens I, III and IV, generates a nearly occlusive thrombus and leads to fibrin deposition (16). Both collagen and thrombin participate in platelet activation in the “deep injury” model. As with the ferric chloride thrombosis model, it is important to define the nature of the injury generated when employing these models.

Intravitral imaging

Real time visualisation of thrombus formation provides spatio-temporal information about events within the growing thrombus in the living animal. In small animal models, platelet thrombus formation has been imaged by digital contrast interference microscopy (17), with fluorescent dyes such as calcein (6, 18–21), with fluorophore-conjugated antibodies against platelet surface proteins such as CD41 or CD42 (13, 22), coagulation zymogens and proteases containing fluorescent active site labels (23), green fluorescent protein-containing platelets (24, 25) and more recently quantum dots (26). In large vessels, high absorption and scatter make the penetration of visible wavelengths difficult, so near infrared dyes have been used (19).

Quantitative data on platelet accumulation, activation and fibrin formation in near real time is achieved using widefield microscopy in an image-intensified system. Localisation of a protein to the vessel wall or platelet can be assessed using confocal microscopy. Advances in technology now allow us to do near real-time confocal analyses as well. Images are collected with a digital camera employing a charge coupled device (CCD) (18). The light intensity is digitised to allow extraction of quantitative data from the images. Thickness, transparency and auto-fluorescence of the tissue must be considered when designing the experimental model.

Current intravitral imaging techniques have improved our understanding of the process of thrombus formation in vivo. This review will concentrate primarily on the techniques of measuring platelet, endothelial cell and blood clotting protein activation in
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Vascular imaging of inflammation

Two pathways to platelet activation

Studies in genetically altered mice have suggested the concept of two independent pathways to platelet activation (7, 15, 27). In our laser injury model, where the insult does not result in significant collagen exposure, we have determined that platelet accumulation is tissue factor- and thrombin-dependent but is independent of the collagen receptor glycoprotein VI (GPVI) and von Willebrand factor (7, 28–31). In contrast, using thrombosis models where the endothelium is denuded and collagen is exposed, both GPVI and GPIbα are important for thrombus formation (32, 33). Taken together, these results suggest two independent pathways to platelet activation, either acting in parallel or separately. Platelet activation may occur either through subendothelial collagen exposure, or by thrombin generated by the tissue factor pathway of blood coagulation. The sequelae of platelet activation are the same in both pathways. It is not clear what recruits platelets to the laser-injured vessel wall when collagen is not exposed. We have shown that endothelial cells are activated by the laser injury (vide infra). Perhaps this activation results in expression of an adhesive molecule or platelet ligand on the surface of the activated endothelial cell. In human pathophysiology, the relative contribution of each of these mechanisms to platelet activation may be determined by the disease process or injury.

Platelet accumulation and fibrin formation are independent early events

It is widely thought that the activated platelet membrane is the critical surface for thrombin generation via the assembly of the tenase and prothrombinase complexes on the pathway to thrombin generation. The ability to visualise the two separate components of the clotting system simultaneously by intravital microscopy provides the opportunity to investigate this hypothesis. Dual channel fluorescence experiments measuring simultaneously platelet accumulation and fibrin deposition in wild-type mice after non-occlusive laser-induced injury showed that fibrin formation commences simultaneously with platelet accumulation in response to this injury (31). Intravital imaging in PAR4-/- mice demonstrated that fibrin formation was normal even when platelet accumulation was minimal due to lack of the PAR4 thrombin receptor (31).

Pharmacological inhibition of platelet accumulation by eptifibatide, an inhibitor of the interaction of glycoprotein $\alpha_{\text{IIb}}\beta_3$ with fibrinogen, or depletion of platelets using a mixture of anti-GP$\text{IIb}$ antibodies (34) confirm that thrombin generation, as measured in vivo by fibrin accumulation, is independent of platelet accumulation and activation ([35], Jasuja, Furie, Furie, unpublished data). These data suggest that activated endothelial cells or microparticles recruited to the site of injury may play an important role in thrombin generation and provide tools for parsing the role of the endothelium in the thrombotic response to laser-induced injury.

The source of the tissue factor that initiates thrombin formation in our non-occlusive, endothelium sparing laser injury model of thrombosis remains an unresolved question. On one hand tissue factor and fibrin accumulation are reduced in P-selectin-/- or P-selectin glycoprotein ligand-1-/- mice due to loss of binding of tissue factor-bearing microparticles to the growing platelet thrombus. This suggests that leukocyte-derived microparticles that bear both P-selectin glycoprotein-1 and tissue factor deliver blood-borne tissue factor to the site of injury (36). However laser-induced thrombosis experiments in mice receiving reciprocal bone marrow transplants between wild-type mice and mice expressing only low levels of human tissue factor suggested that tissue factor from two different compartments is important in thrombus formation in this model (29). Vessel wall-associated tissue factor appears to be important for initiating thrombin generation while haematopoietic-derived tissue factor leads to propagation of thrombin generation. The source of the non-haematopoietic-derived tissue factor in this model remains an open question. While fibroblasts, smooth muscle cells or other subendothelial cells are known to make tissue factor, the laser-induced injury model described here does not appear to result in exposure of these cells in the subendothelium. Whether endothelial cells express tissue factor in the short time frame of our laser-induced thrombosis experiments remains uncertain. Data described below indicate that endothelial cells are rapidly activated by laser injury, before accumulation of platelets at the injury site, and could contribute to early thrombin generation. Further studies, perhaps using mice with tissue-specific gene deletions of tissue factor, may provide answers to this important question.

Cell activation after laser-induced vessel wall injury

Activation of platelets

Elevation in intracellular calcium levels is associated with various events of cell activation, including cytoskeletal reorganisation, granular content release and integrin activation. Using calcium-sensitive dyes we have developed methods for observing calcium elevation in cells in vivo in response to activating stimuli that initiate thrombus formation (30, 37). Several lines of evidence indicate that not all adherent platelets in a platelet thrombus become activated. Calcium mobilisation after laser-induced injury was visualised in reporter platelets loaded with the calcium-sensitive dye Fura-2 and infused into a recipient mouse. We observed that pla-
Platelets binding at the site of laser injury have not initially undergone calcium mobilisation; only about 50% of the platelets recruited to the growing thrombus become activated (Fig. 1A) (30). Platelets in which calcium mobilisation occurs remain associated with the thrombus longer than those in which calcium mobilisation does not occur. Platelets that do not become activated do not appear to become stably incorporated into the thrombus and the length of time an activated platelet remains bound to the thrombus appears to be directly related to the duration of the calcium spike (Dubois, Panicot-Dubois, Furie and Furie; unpublished results).

Confocal intravital imaging has also demonstrated that not all platelets in a laser-induced thrombus are activated (Fig. 1B). Only the platelets in an activated core closest to the injury site express the activation marker P-selectin. Adherent platelets that do not express P-selectin, like the platelets that do not mobilise calcium, may ultimately disengage from the thrombus. The loss of unactivated platelets is likely responsible for the decrease in platelet thrombus size observed over time after laser injury.

Recent advances in the technology of spinning disc confocal microscopy enable confocal 4D time lapse imaging of platelet thrombus formation and fibrin deposition in relation to the vessel wall after laser injury (Fig. 1C). These images show fibrin deposition localising with a subset of platelets at the vessel wall-thrombus interface in the region upstream of the developing thrombus. In addition, fibrin deposition occurs circumferentially at the endothelium as well as upstream and downstream from the site of laser injury. These observations taken together with the data on calcium mobilisation, P-selectin expression and fibrin generation in the absence of a platelet thrombus further suggest that platelet thrombus formation and fibrin generation occur simultaneously.

**Activation of endothelial cells**

Endothelial cells at the site of laser injury seemed to us the most likely source of the cell surface that supports fibrin generation at sites of laser injury in the absence of platelet thrombus formation. We thus examined the effect of the laser pulse on endothelial cells in vitro and in vivo (37). Human umbilical vein endothelial cells in culture were loaded with Fluo-4, a calcium-sensitive fluorochrome, and a single cell targeted with a laser pulse. A rapid rise in intracellular calcium was observed in the targeted cell followed by a rise in calcium in surrounding cells, indicating activation of these cells. The intracellular calcium returns to resting levels and the cells can be reactivated with a laser pulse. Laser-induced injury of cultured human umbilical vein endothelial cells in the presence of corn trypsin inhibitor-treated, platelet- and microparticle-depleted plasma leads to fibrin deposition on the endothelial cell layer that is inhibited by anti-tissue factor antibodies (Fig. 2A) (37). These results indicate that the activation of endothelial cells in culture leads to expression of tissue factor activity.
Intravital imaging of inflammation

Fluo-4 AM was infused into the mouse circulation, resulting in dye uptake in the endothelium and circulating haematopoietic cells. Mice were treated with epifibatide to inhibit platelet accumulation (37). Rapid calcium mobilisation within the endothelium was detected after laser injury using this technique. Calcium elevation was apparent circumferentially from the site of laser injury (Fig. 2B), indicating that, as was observed in vitro, the activation signal is propagated to neighboring cells. Quantitative analysis of the intensity of calcium-induced fluorescence in widefield microscopy shows that calcium mobilisation at the endothelium is maximal at the site of injury (Fig. 2C and D). Lysosomal-associated membrane protein 1 (LAMP 1), which is found in many cells including endothelial cells, is translocated to the cell membrane upon cell activation and granule secretion. Rapid secretion of LAMP 1 is observed after laser-induced injury of the vessel wall (37). The appearance of this protein on the endothelium after laser injury indicates that the insult leads to later stages of cell activation.

Dual fluorescence experiments imaging both platelet accumulation and calcium mobilisation showed that at sites of laser-induced thrombosis endothelial cell activation precedes platelet accumulation (37). Our data further suggest that laser-activated endothelial cells can support assembly of the tenase and prothrombinase complexes responsible for the generation of fibrin observed in the absence of platelet thrombus formation. These cells may be a source of activated tissue factor as well. Hayashi et al. have used annexin V as a marker for phosphatidylserine exposure in the laser-induced thrombosis model (24). Their results suggest that the appearance of this phospholipid in the platelet thrombus, both close to the laser-induced injury site in the vessel wall and penetrating into the core of the thrombus, is correlated with fibrin generation (24). The kinetics of formation of the platelet thrombus, the pat-

Figure 2: Endothelial cell activation after laser-induced vessel wall injury. A) Cultured endothelial cells activated by a laser pulse are pro-coagulant. Washed cultured endothelial cells were overlayed with corn trypsin inhibitor treated, platelet and microparticle depleted human plasma. Cells were left resting or were activated by a laser pulse. Supernatant plasma was removed and the cells fixed and stained for fibrin with a fibrin-specific antibody (red). Cell nuclei are stained with DAPI (blue) and actin stained with FITC-phalloidin (green). Left hand panel, unactivated endothelial cells; right hand panel, laser-activated cells. B) Calcium flux in endothelial cells activated by laser injury in vivo. Mice were infused with the calcium sensitive dye Rhod-2 and with epifibatide to prevent platelet accumulation after laser-induced vessel wall injury. Time lapse confocal images show calcium elevation in endothelial cells at the site of injury (X) and in neighboring cells. C) Analysis of propagation of endothelial cell activation along the vessel wall following laser injury. Left panel: The intensity of the calcium elevation in the endothelial cells surrounding the site of laser injury (X) is indicated by a pseudocolor heat intensity map. Right Panel: The intensity of calcium fluorescence along the side of the vessel wall that sustained the laser injury and along the opposite vessel wall show a bell-shaped distribution.

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tern of calcium elevation in platelets within the thrombus, and the distribution of fibrin observed by these investigators are very similar to those we observe in our laser injury model. However, our studies indicating that fibrin generation at sites of laser injury is normal in the absence of platelet thrombus formation suggest that exposure of phosphatidylserine on the surface of activated platelets is not required for fibrin generation in this model (31, 35).

Role of protein disulfide isomerase in thrombus formation in vivo

The oxidation state of labile disulfide bonds in critical haemostatic proteins has been implicated in regulating the process of thrombus formation (38). Alterations in the oxidation state of these bonds are catalysed by an enzyme(s) of the thiol isomerase family including protein disulfide isomerase. Both platelets and endothelial cells secrete protein disulfide isomerase and display the enzyme on their surface (28, 35, 38–41). We used our laser-induced mouse thrombosis model to determine if protein disulfide isomerase had a role in thrombus formation in vivo. Protein disulfide isomerase appears rapidly after laser-induced vessel wall injury, prior to the appearance of the platelet thrombus (28, 35). Inhibition of protein disulfide isomerase activity with bacitracin A, a non-specific thiol isomerase inhibitor, or an inhibitory monoclonal anti-protein disulfide isomerase antibody inhibited platelet thrombus formation and fibrin generation (28). Infusion of an inhibitory monoclonal anti-protein disulfide isomerase antibody into the circulation of a Par4−/− mouse prior to vessel wall injury inhibited fibrin generation indicating that protein disulfide isomerase is required in vivo for both fibrin generation and platelet thrombus formation (28). If platelet thrombus formation is inhibited by the infusion of epitibatide into the circulation, protein disulfide isomerase is detected following vessel wall injury and fibrin deposition is normal. Treatment of mice with the function blocking anti-protein disulfide isomerase antibody completely inhibits fibrin generation in epitibatide–treated mice. These results indicate that, although both platelets and endothelial cells secrete protein disulfide isomerase following laser-induced injury, protein disulfide isomerase from endothelial cells is sufficient for normal fibrin generation in vivo (35). A role for protein disulfide isomerase in thrombus formation has also been demonstrated in the mouse using the ferric chloride and the carotid artery ligation models of thrombosis (Bellido-Martin, Furie, Furie, unpublished results, [42]).

Tissue factor, the initiator of coagulation, contains a surface exposed potentially labile disulfide bond. It has been suggested that modulation of the state of the cysteines that form this disulfide bond may regulate the pro-coagulant activity of tissue factor (encryption-decryption) (43–47). However, this concept remains controversial with some investigators attributing the role of PDI in blood coagulation to regulation of phosphatidylserine exposure (48–52). Although protein disulfide isomerase is important for tissue factor expression and thrombus formation, the mechanism by which it participates in this process remains unknown.

Conclusions

Visualisation of in vivo-platelet and endothelial cell activation and fibrin generation has increased our understanding of haemostasis and thrombosis. The continuing development and improvement of imaging techniques using widefield and confocal microscopy adapted to living animals provide powerful tools to study in depth physiologic processes associated with human cardiovascular diseases. No thrombosis model truly mimics the normal human physiologic process of haemostasis or pathologic processes of thrombotic disease. However, like the in vitro-assays of blood coagulation and platelet function that provided vital information for developing our current understanding of these processes, intravital thrombosis models inform our understanding of what can happen in vivo.

The results of studies in the laser-induced and other thrombosis models have provided new insights into the mechanisms of thrombus formation. The potential roles for the activated endothelium in initiation of platelet accumulation and as an important early surface for formation of the blood coagulation complexes that lead to thrombin generation, the concept of two independent pathways to platelet activation and the identification of an important role for protein disulfide isomerase in thrombus formation may provide novel targets for anti-thrombotic agents. In the decade or so that we have been doing intravital experiments the technology has improved dramatically, providing us with markedly increased data acquisition times and improved resolution in both widefield and confocal modes. Recently, two-photon intravital microscopy has been applied to a laser-induced injury model of thrombosis (53). Thus, it is likely that new modes of investigating thrombus formation in vivo will continue to provide novel insights into the process.

References


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