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Microvascular platforms for the study of platelet-vessel wall interactions

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Summary

Platelets interact with the endothelium to regulate vascular integrity and barrier function, mediate inflammation and immune response, and prevent and arrest hemorrhage. In this review, we describe existing tools to study the flow-dependent interactions of platelets with the vessel wall. We also discuss our work on building engineered microvessels to study the roles of platelets on endothelial barrier function, endothelial sprouting, and thrombus formation on both quiescent and stimulated endothelium. In particular, we will show the advantage of using a cell-remodelable matrix in the studies of platelet-vessel wall interactions.

Platelets are anucleate blood cells that circulate at the periphery of the blood stream, being displaced there by the larger and denser erythrocytes. From this position, they constantly survey the endothelium for defects, either larger injuries that would result in hemorrhage, or smaller defects that perturb the vessel's barrier function. Under normal circumstances, the platelets do not adhere to intact endothelium, attaching to the vessel wall only upon exposure of subendothelial proteins at sites of vessel injury. These adhesive interactions, coupled with exposure to platelet agonists generated at the site or released from other platelets, cause the platelets to attach to each other and form an occlusive plug. Under some unusual pathological circumstances, the platelets can also form thrombi on intact endothelium, sometimes leading to occlusion of small blood vessels.[1]

Endothelium, another crucial component involved in hemostasis and homeostasis, serves as an interface between the blood and surrounding tissue.[2] If thought of as a single organ, the

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endothelium is one of the largest organs in the body, with a combined surface area of approximately 1000 m² in an adult human.[3–4] The endothelium is involved in many vital functions, including the regulation of tissue perfusion and blood pressure, fluid and solute exchange, hemostasis, inflammation, and angiogenesis. Healthy endothelium provides a non-adhesive and anti-thrombotic surface for the transport of blood through the vessel, largely by expressing molecules that inhibit platelet adhesion (prostacyclin, nitric oxide, and ecto-ADPase) and blood coagulation (*e.g.*, thrombomodulin). During inflammation or injury, however, the endothelial cells become activated, and release the contents of their granules, Weibel-Palade bodies (WPBs),[5] the major component of which is von Willebrand factor (VWF),[6] and express adhesion molecules such as P- and E- selectins on their surfaces. A portion of the newly released VWF remains attached transiently to the endothelial surface, and recruits platelets from the blood stream until the VWF is proteolytically removed from the endothelial surface by the plasma metalloprotease ADAMTS13.[7–8] If removal of VWF from the endothelial surface is delayed, the formation of thrombi and subsequent shedding of relatively large emboli may occlude downstream vasculature. In larger vessels, this mechanism can contribute to pathologies such as myocardial infarction, stroke, and deep vein thrombosis; whereas microvascular thrombi contribute to organ dysfunction and failure, [9] either in isolated organs, or systemically in conditions such as thrombotic thrombocytopenic purpura (TTP).[10]

The adhesion and aggregation of platelets largely depend on the blood flow and shear conditions at sites of vascular activation or injury.[11] The flow velocity in blood vessels is greater at the center than near the vessel wall, the velocity difference creating a shearing effect between adjacent fluid layers. This shear stress, defined as force per unit area, is greatest near the vessel wall and decreases to zero at the center of the vessel. For blood, the shearing effect is frequently represented by the shear rate, the ratio of shear stress to blood viscosity. This parameter is easier to determine for blood because blood is non-Newtonian, its viscosity changing as a function of shear rate. Typical wall shear rates are in the range of 300–800 s⁻¹ in large arteries and of 500–1600 s⁻¹ in arterioles, and can exceed 10,000 s⁻¹ in stenotic vessels.[12–13] Platelets are thus exposed to a wide range of shear stresses as they circulate near the vessel wall, from 10–60 dyne cm⁻² in the arteries and arterioles to 0.8–8 dyne cm⁻² in the venous circulation.[14]

At wall shear rates greater than 500 s⁻¹, the initial tethering and adhesion of platelets to the vessel wall is primarily mediated by the interaction of the glycoprotein (GP) Ib- IX-V complex to VWF.[11] At wall shear rates above 5000 s⁻¹ or in the presence of sharp shear gradients, endothelial cells may be activated to secrete more VWF and further trigger GPIb-IX-V-dependent thrombus formation.[15] Platelet adhesion mediated by the GPIb-IX-V–VWF interaction is unstable, with platelets rolling on VWF-coated surfaces without stably adhering.[16] Stable adhesion requires platelet integrins to be activated to conformations capable of binding VWF or other immobilized ligands such as fibrinogen.[17–18]

Shear-dependent platelet–vessel wall interactions have been studied using various *in vivo* and *in vitro* models. Animal models are fully physiological, and genetically modified mice represent very useful tools to dissect the molecular mechanisms underlying the complex multistep process leading to the formation of a stable platelet plug,[19–20] particularly

employing intravital video microscopy.[21] In spite of these advantages, the complexity of whole organisms is such that dissecting the contributions of individual molecular or cellular components to the process of thrombus formation is exceedingly difficult.[22] Ex vivo studies are in some cases a good alternative for the study of platelet–vessel wall interactions. Large blood vessels can be isolated from an animal and the vessel can then be perfused with blood components and other substances.[23] This model has the advantage that native blood vessels with the proper cellular and matrix composition are being studied, and the interactions of blood components and the vessel intima can be examined under physiological flow. The model is suitable for examining thrombus formation in large blood vessels due to its accessibility, easy of study, and the fact that large arteries are involved in common and relatively easily definable diseases. Unfortunately, a similar system for studying small blood vessels is currently not feasible. Small blood vessels are hard to isolate in a way that maintains their architecture, and often contain bifurcations and junctions, resulting in complex profiles for blood flow and shear stress. Systems that model small blood vessels are in great need as there is increasing appreciation for the role of the microvascular thrombosis in a large number of human diseases.

Engineered platforms that mimic the vasculature have been developed as an alternative way to study flow-dependent thrombus formation and platelet–vessel wall interactions *in vitro*, particularly targeting processes occurring in small blood vessels. One such approach has employed parallel-plate flow chambers.[24–25] These flow chambers usually have depths of 0.1 – 0.5 mm, widths of 2 – 10 mm, and lengths of 20 – 50 mm, and therefore require large blood volumes (in the order of 10 mL) for each experiment. The endothelial cells used in the chambers are usually cultured as a monolayer on glass or plastic, sometimes with an added underlying matrix. These types of flow chambers allow for the assessment of the interactions of platelets with stimulated or unstimulated endothelium under flow, and, to a certain extent, can be used to probe the role of hydrodynamic forces on these interactions. However, these chambers generally use fixed conditions of shear stress and substrate conditions within individual experiments, and have relatively low throughput to allow study of different experimental parameters across the full range of physiological and pathological shear stresses. Furthermore, the flow chambers usually have simple geometries, *i.e.* straight channels with unidirectional flow, making it impossible to mimic the complex flow patterns found at vessel bifurcations and in regions of high curvature that occur *in vivo*. Parallel-plate chambers have largely been eclipsed over the past ten to fifteen years by advances in microfluidic technologies, which offer new possibilities to study shear-dependent phenomena related to platelet-vessel wall interactions.

Conventional microfluidics for the study of platelet-vessel wall interactions

Microfluidic devices have several advantages over conventional flow chambers, including the very small volumes they require, their capacity for high-throughput testing resulting from the possibility of using multiple channels within a single device, and the ability to use several geometries in one small chip. Numerous studies have been carried out with these types of devices to study platelet adhesion and aggregation and blood coagulation under flow, although generally the role of the endothelium has not been taken into account,[26–27] until recently.[15, 28–30] Most relevant microfluidic devices have been made of glass or

polydimethylsiloxane (PDMS), a silicone elastomer, which is transparent and biocompatible. The microfeatures were usually created by silicon or SU-8-based lithography, with the size of features ranging from several microns to a few hundred microns. Features that have been incorporated into microfluidic devices include parallel microchannels of uniform size,[28–29] microchannels with varying dimensions, including incorporation of “stenotic” regions,[15] or channels with bifurcations and junctions.[30] Once these microfluidic chambers are made, they can be seeded with endothelial cells, which can be grown under flow until they reach confluence. Isolated platelets, platelets in plasma, or whole blood can then be perfused through these chambers to evaluate platelet adhesion and aggregation on the endothelialized surfaces under different flow conditions[15, 31] and stimuli.[28] These studies can be valuable to screen for drugs to promote or inhibit platelet adhesion to an endothelium.[29] They can also be used to study the biophysical mechanisms of microvascular occlusion in diseases such as sickle cell disease and hemolytic-uremic syndrome.[30]

Though much progress has been made using microfluidic chambers, the chambers have limitations in their ability to mimic real blood vessels. One disadvantage is that conventional microfluidic chambers have square or rectangular cross sections resulting from the limitations of the fabrication procedures. These features result in inhomogeneous and unphysiological shear conditions on the vessel wall, as the shear stresses are very different at corners than at the center of the walls. This inhomogeneity creates large artifacts that hinder interpretation of studies of shear-dependent platelet-vessel wall interactions. Development of microfluidic chambers with cylindrical cross sections[32] would provide more physiological tools for vascular research, particularly for the study of flow-dependent platelet-vessel wall interactions. Another limitation of this system is that the walls of conventional microfluidic chambers are rigid and non-distensible, preventing vessel wall remodeling in response to pressure, flow, or interactions with blood cells. Such remodeling can either be a passive change of vessel size or active growth secondary to cell proliferation. Furthermore, many current microfluidic chambers use PDMS as the base material, which, although convenient and easy to use, is impermeable to liquid, unlike the diffusive exchange between the circulation and interstitial space that occurs *in vivo*. This drawback precludes studies of the role of platelets in endothelial barrier function or angiogenesis, which are among the many essential functions of platelets.[33]

Remodelable microvascular platform for platelet-vessel wall interactions

The combination of remodelable biomaterials and conventional microfluidics provide new methods to generate endothelialized structures that support the diffusion of water and gases. Matrix biomaterials used for this purpose include native matrices such as type I collagen [34], fibrin [35], elastin and decellularized matrices[36–37]. Such a system overcomes many or most of the limitations of conventional microfluidic systems and mimics more closely conditions found *in vivo*. Fabrication methods similar to those described above for glass and PDMS can also be used in these remodelable platforms to create microfeatures within the channels. Tien and his group [38–39] have developed methods to create single channels or tubes within the matrix by placing a cylindrical needle in the matrix before it is allowed to gel, then removing it afterwards. The diameters of the tubes may be varied by varying the

diameter of the removable needles. The lumen of the tube can be endothelialized and the endothelial cells cultured under flow. Although these investigators did not use these tubes to study the interactions of platelets with the vessel wall, they have shown that the barrier function of the endothelium can be altered by flow at either very low and very high shear stresses [40], or by chemical reagents such as plasma expanders[41] that elevate cytoplasmic cyclic AMP [39]. This type of microvessels should be amenable to the study of interactions between blood cells and the endothelium.

Recently, Zheng *et al.* [42–43] combined traditional photolithography and soft lithography with injection molding techniques to engineer microchannel networks with defined architecture within three-dimensional collagen matrices (Figure 1A). These channels were endothelialized by seeding endothelial cells into the microfluidic circuits through the inlet and outlet. The channels were formed within a matrix of native type I collagen (6–10 mg/mL), which is of the appropriate stiffness to support the structural integrity of microvessels and allow for remodeling by endothelial cells through matrix degradation or deposition. This new process has advantages and more flexibility over earlier methodologies in that it is possible to produce microvessels with complex geometries such as bifurcations and vessel junctions, it is possible to control cellular, chemical, and physical stimuli, and the walls of the microvessels can be remodeled by the endothelial cells. Another advantage of this system is that multiple cell types can be incorporated. In addition to the endothelial cells that line the vascular lumen, perivascular cells and tissue stromal cells can also be incorporated into the collagen matrix as a way of beginning to approximate tissue microenvironments. Once the channels are formed and the introduced endothelial cells are given a short time to adhere to the matrix, the vessel lumen can then be perfused with medium under continuous flow. Without additional treatments, the vessels mature to gain full barrier function within about a week of endothelial seeding. They remain patent and functional for over two weeks and perhaps longer. Because of the ability to employ complex geometries and controllable flow, these synthetic microvessels are ideal tools to study in detail the interactions between platelets and the vessel wall in a way that mimics various scenarios of microvascular thrombosis.

Interaction of platelets with activated endothelium

Microvascular occlusion occurs in many diseases including the thrombotic microangiopathies (such as thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome) and related disorders, including sickle cell disease and malaria. These disorders often feature extensive endothelial activation, with the endothelial surface becoming adhesive and capable of binding platelets and other cells, and the adherent platelets allowing further attachment of other blood cells. We have shown that microvessel networks containing HUVECs can be stimulated by phorbol ester and other inflammation-mimicking molecules to secrete large amounts of VWF (Fig. 2A) from abundant WPBs[43]. In microvessels of complex geometry, VWF can self-associate into enormous strands that align in the direction of flow. These VWF strands potently bind platelets through their A1 domains, which bind the GPIb-IX-V complex on the platelets (Fig. 2B). The platelets adhere firmly to the strands, and such adhesion can be eliminated by blocking the GPIba subunit of the GPIb-IX-V complex. Unlike adhesion of platelets to surfaces coated with the plasma

form of VWF, where the platelets roll but do not firmly adhere, the platelets immediately adhere firmly to the VWF strands (Fig. 3). In regions of complex geometry such as vessel junctions, platelet–VWF strands may diverge or converge depending on the flow patterns (Fig. 3A). At stagnation points in the region of bifurcations, platelets are deposited and can firmly adhere, producing thrombi at the bifurcation. Adherent platelets can become activated and release their granule contents, which include hemostatic and adhesive proteins, pro- and anti-angiogenic factors, and chemokines. The platelet surface also changes its adhesive character, exposing P-selectin, which provides a counter-receptor to attract leukocyte adhesion (Fig. 4) through leukocyte P-selectin glycoprotein ligand–1 (PSGL-1). After an hour of whole blood perfusion through the stimulated vessels, the vessel showed multiple layers of adherent blood cells on the vessel wall, first platelets, in a single layer or multiple layers, and then leukocytes adherent to the platelet surface (Fig. 4A–B). Prominent platelet–VWF strings could also be found at vessel bifurcations (Fig. 4C–D). These features likely model platelet–VWF mediated microvascular thrombosis that occurs in TTP and HUS.

Platelets interact with quiescent endothelium

In addition to platelet adhesion on activated vessels, platelets sometimes also adhere to non-stimulated vessels. We previously showed that when whole blood was perfused through non-stimulated vessels, the majority of platelets flowed past the endothelial surface without adhering. A small portion of platelets adhered reversibly and rolled along the endothelial surface. Platelets adhered firmly only at the junctions between endothelial cells (Fig. 5A), or at the site of defects where the subendothelium might be exposed (Fig. 5B). Ultrastructural analysis showed that on non-stimulated endothelium, platelets attached singly or formed small aggregates (less than 10 μm) (Figs. 5C–D).

We also studied the influence of platelets on endothelial barrier function by perfusing platelet-rich plasma through leaky non-stimulated vessels that had been engineered to have scattered defects, and allowing the platelets to remain in the vessels overnight. Interestingly, vessels with extensive leakage before platelet incubation showed dramatically enhanced barrier function and underwent remodeling with expanded diameters and thick abluminal linings. As part of this process, platelets in some instances appeared to become incorporated into the vessel wall by fusing with endothelial membranes. Fusing platelets demonstrated few signs of activation (unpublished results).

Platelet-induced endothelial sprouting

When our microvessels were cultured with growth medium containing proangiogenic factors (vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and phorbol ester) for 7 days, the endothelial cells and microvessels sprouted from existing vessels and invaded the collagen matrix [43]. These angiogenic sprouts, however, were leaky, lacking the barrier function of the non-sprouted vessels. In contrast, when we incubated microvessels with good barrier function overnight with platelets, the platelets induced potent vessel sprouting. Platelets could be found at the tips of most of the new sprouts. More interestingly, the platelet-induced vessel sprouts did not leak, unlike the ones formed with the soluble angiogenic stimuli. These findings were consistent with the animal studies of Kisucka et al. [44], who showed that platelets support angiogenesis without

hemorrhage. Platelets appear to be mildly activated, possibly indicating that they have secreted pro-angiogenic molecules to induce vessel sprouting, while remaining quiescent in terms of their thrombotic activities (unpublished results). Many questions remain to be investigated in this process.

Future of microvascular platforms and perspectives

Our studies represent a step toward development of a system that can mimic physiological flow and vessel wall conditions for the study of platelet-vessel wall interactions. Nevertheless, technologies need to be further advanced to develop the capability to generate hierarchical vascular structures that can fully recapitulate the characteristics of flow and transport from a large artery to small arterioles, and further into capillaries, then back into venules and veins. To achieve this, a full 3D vessel bed must be built, with vessels of the various diameters and proper bifurcating order, and 3D branching. Recent work by Miller et al.[45] represents a step towards achieving this goal. These investigators applied 3D printing techniques to generate a cubic filament network made from carbohydrate glass, and used this network as a cytocompatible sacrificial template in engineered tissues containing living cells. After surrounding the template with matrix, the glass network was dissolved and the resultant tubes were endothelialized, generating cylindrical 3D grid vessels capable of being perfused with blood under high pressure.

In summary, *in vitro* microvascular platforms have been shown to be an important model with which to study the interactions of individual components of blood with the vessel wall. Because many therapies developed using animal models fail to be effective in human patients, these platforms provide an advantage in that entirely human components can be studied, thus benefitting the development of therapies directed at disrupting the interactions between blood components and the vessel wall, increasing or inhibiting angiogenesis, and improving endothelial barrier function and preventing vascular leak.

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Figure 1. Microvessel networks[43]

A. Schematic diagram of microfluidic collagen scaffolds after fabrication. **B.** Z-stack projection of horizontal confocal sections of endothelialized microfluidic vessels - overall network. **C.** Perfusion of 70 kDa FITC-Dextran through the endothelium showing robust barrier function and low permeability across the endothelium. **D.** projection view and cross-sectional view of vessel corner. **E.** 3D reconstructional view showing the branching sections. Red: CD31, blue: nuclei. Scale bar: 100 μm .

Figure 2. von Willebrand factor (VWF) strands on endothelial cells

A. VWF secreted from stimulated endothelial cells assembled into fibers in the microvessels, and the VWF fibers were aligned to the flow direction. Arrow: Flow direction. Red, CD31; green, VWF; blue: nuclei. **B.** Platelet adhesion on VWF fibers. Arrow: flow direction. Green: VWF, red: CD41a.

Figure 3. Platelet interaction with stimulated microvessels

A. Platelets formed string structures and showed three-dimensional flow phenomena with converging and diverging strings near flow junctions. Scale bar: 50 μm . **B.** Platelet aggregation induced thrombi formation at vessel bifurcation, where flow stagnation points were, after 5 to 10 minutes of whole blood perfusion through the stimulated microvessels. Scale bar: 100 μm . Arrows: flow direction. Green: platelets.

Figure 4. Blood interactions with stimulated endothelium

A. z-projection images of the overall network showing the adhesion of platelets and leukocytes on stimulated microvessels after 1 hour of whole blood perfusion. Red: CD31, green: CD41a, white: CD45; and blue: nuclei. **B.** zoomed image of the microvessels near the inlet and first two bifurcations showing the adhesion of blood cell layers (platelets and leukocytes) on the stimulated endothelium. **C–E.** Scanning electron microscopic images of (C) platelets strings at bifurcations (flow toward bifurcation from the bottom), (D) zoomed platelet strings (red box in C), and (E) zoomed images of leukocyte adhesion on platelets and endothelial membrane.

Figure 5. Blood interactions with non-stimulated endothelium

A–B. Platelet adhesion on endothelial junctions of non-stimulated microvessel network after 15 mins of whole blood perfusion: (A) zoomed view and (B) z projection of confocal sections. Red: CD31, green: CD41a; and blue: nuclei. **C–D.** Scanning electron microscopic images of platelets adhesion on non-stimulated endothelium to fix the endothelial junctions in (C) and zoomed platelets on endothelial cell junctions (D).