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Thrombosis-on-a-chip: Prospective impact of microphysiological models of vascular thrombosis

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Abstract

The most common pathology of the blood-vessel organ system is thrombosis or undesirable clotting of the blood. Thrombosis is life threatening as more than 25% of such cases lead to sudden death from stroke and myocardial infarction. Even though the process of thrombosis has been extensively investigated with animal models, its exact pathobiology in different blood vessels is not yet fully understood and drug assessment remains unpredictable. This is primarily because the cause for thrombus formation is multifactorial and depends on the interplay of flow patterns within the blood vessel, the vessel wall or endothelium, extracellular matrix, parenchymal tissue, and the cellular and plasma components of the blood. Current *in vitro* and animal models do not mimic or dissect this organ-level complexity faithfully. However, microfluidic technology has recently been deployed to effectively recapitulate blood-endothelial–epithelial interactions in the onset of thrombosis in blood vessels. This technology is promising because it permits inclusion of primary human cells and blood obtained from patients, which is currently lacking in other *in vitro* models of thrombosis. In this review, we summarize the current state-of-the-art and practices in microfluidics and expected improvements in this field that will impact basic understanding of thrombosis, drug discovery and personalized medicine.

Keywords

Organ-on-a-chip; Microphysiological systems; Thrombosis; Hemostasis; Endothelium; Disease model

Background

Under physiological conditions, the blood remains in a fluid state by maintaining a balance between the procoagulant and the anticoagulant factors present in the blood. When this balance is impaired, a shift towards anticoagulant factors leads to unexpected bleeding and blood loss whereas a shift towards procoagulant factors leads to thrombus formation that occludes blood vessels eventually becoming a cause of stroke [1]. Such undesirable clotting of blood is the most common pathology found in the vasculature. Pathologic clotting of blood, or thrombosis, in the vasculature has been studied extensively since the 18th century. In the earliest studies, the initiation of thrombus formation (thrombogenesis) was related to one of the following factors: hypercoagulability of blood, blood stasis and endothelial damage. However, Rudolf Virchow in 1856 was the first to make a profound observation that all these three factors together are critical to thrombogenesis. These factors that collaboratively contribute to thrombosis are known as the Virchow's triad [2]. Blood can become hypercoagulable due to the activation of blood cells (such as platelets) and release of clotting factors (such as thrombin) into plasma [3]. Stasis of blood flow may occur due to occlusion of the blood vessel resulting from plaque formation in arteries (atherosclerosis) or due to impairment of valves in the deep veins (deep vein thrombosis) [4]. Vascular activation occurs when the endothelium is exposed to circulating stimulants, making it procoagulant and pro-inflammatory [5]. Endothelial cell inflammation increases cell permeability contributing to thrombus formation by the interaction of platelets and clotting factors with the exposed components of the vascular surface [6]. Since the discovery of Virchow's triad, its factors have been extensively investigated independently at molecular and cellular levels, but the mutual interplay of these factors (and others) has not been fully understood because it requires dissectable organ-level modeling of the physiology of thrombosis. Current methods that are used to investigate the multivariate factors that lead to thrombosis do not permit analysis of the organ-level prothrombotic responses with incremental level of complexity that underlies in several diseases. While several sophisticated animal models of thrombosis exist, and have contributed to our basic understanding of certain specific mechanisms, integrated models that can separate contributions of platelet–endothelial interactions versus tissue–tissue (e.g., epithelial–endothelial) interactions are still underdeveloped and significantly non-predictive. Most current *in vitro* assays of hemostasis and thrombosis on the other hand, do not incorporate vascular function. Several recent studies have enabled microfluidic flow chambers to include endothelial lumen within which whole blood can be perfused and platelet–endothelial interactions can be visualized with high resolution [7–10]. This development is promising as it opens opportunities to include the more complex organ-level interactions in the assessment of thrombosis as well as anticlotting drugs. There are further advantages of using such microphysiological systems (or organs-on-a-chip) due to their micro scale, for e.g. these devices require little amount of blood samples, tight control of dimensions, lower cost per device and easy fabrication and repeatability of large number of experiments [11]. Also, with organ-on-a-chip technology, assessment of thrombosis at a patient-specific level is possible by including specific vessel anatomy, vascular endothelial cells and blood, all derived from the same patient. Here, we succinctly discuss current animal and *in vitro* methods applied in modeling arterial, venous and

microvascular thrombosis, followed by an overview of utilization of microphysiological devices in assessing complex features of thrombosis and future directions that could improve status quo.

Current models of thrombosis: the gold standard

Current models used to investigate thrombosis include *in vivo* animal models ranging from zebra fish to nonhuman primate models, and *in vitro* models in the form of parallel plate flow chambers and cone-and-plate viscometers.

Animal models

The most common animal model of thrombosis is the mouse. Numerous murine thrombosis models have been developed to study the arterial, venous, and microvascular thrombosis [12]. In these models, arterial thrombosis is generally induced using injury of the carotid arteries (for example, ferric chloride or Rose Bengal-plus-light), venous thrombosis is induced slowly by stasis or stenosis (both by ligation) or rapidly by an acute injury (using free-radicals) of the inferior vena cava, and microvascular thrombosis is induced by free-radical injury or laser injury of the mesenteric veins. A few other models also exist, like direct electric injury, anastomosis, ultrasound disruption or intraluminal collagen, which are less preferred due to the difficulty in obtaining repeatable results between labs. These diverse mouse models have contributed immensely over the last few decades in decoding several key mechanisms that govern thrombosis [12–16], as well as capturing the thrombus dynamics in human-relevant conditions [17]. They have also been effective in studying the role of genetic variation and different clotting factors in thrombus formation. However, the physiological and genetic differences of these models with respect to humans limits them considerably [18,19]. This is mostly true because several drug trials that succeeded in such animal models have failed in human clinical trials [20], thus contributing to high healthcare costs and stroke remaining to be the biggest cause of patient mortality. While small animal models (zebra fish, mice etc.) are preferred in thrombosis research mostly due to their easy availability and established genetic data [13], the large animal models (dogs, pigs, nonhuman primates etc.) offer increased physiological relevance to humans. However, large animal studies cannot be easily conducted in academic laboratories and impose several ethical restrictions thus making small animals or *in vitro* models the first choice to model thrombotic diseases.

In vitro models

A commonly used *in vitro* thrombosis system, the parallel plate flow chamber is a hollow rectangular space with millimeter or centimeter scale dimensions, through which blood or its components can be transported to induce physiological wall shear stresses over purified proteins (such as, von Willebrand factor (VWF) or tissue factor), extracellular matrix (ECM like collagen, laminin or fibronectin) or cultured vascular endothelial cell monolayers [21]. The flow rates are often controlled using constant-flow pumps, although pressure and gravity are also used to transport blood. While these systems have been useful in studying the effects of shear and recirculating flow [22] on platelet function and coagulation [23], accurate blood vessel anatomy is not replicated in these chambers. For example, these

flow chambers have not included the complex vascular features like venous valves where abrupt shear stresses may contribute to the onset of deep vein thrombosis (DVT). The other commonly used *in vitro* system, the cone-and-plate viscometer, works by rotating a cone shaped insert above a stationary plate containing proteins or cultured vascular cells [24]. The rotational speed of the cones in these devices are controlled to generate shear stresses. These devices are bulky and need large amounts of blood, cultured cells and reagents for each experiment, making them low throughput. Also, the experiments in these devices are conducted over 2D monolayers of proteins or cells and therefore, they do not mimic the function of a 3D round vascular lumen and natural blood flow. These limitations have been countered by the recent development of microfluidic devices that can be designed to offer more reliable physiologically-relevant readouts of vascular thrombosis.

Microfluidic models of thrombosis: state-of-the-art

Microfluidic devices are typically hollow rectangular channels of micrometer scale dimensions that can be designed using standard microfabrication methods (such as, soft lithography [10]), through which whole blood can be perfused. A major advantage of the microfluidic devices is that they can be perfused at both high arterial wall shear rates ($1000\text{--}5000\text{ s}^{-1}$), as well as low venous wall shear rates ($100\text{--}200\text{ s}^{-1}$), thus incorporating the diversity in the composition and behavior of a thrombus that is dependent on the type of blood vessel [25]. In addition, different anatomical structures like atherosclerotic plaques, bifurcations etc. observed in blood vessels can be relatively easily included while designing the flow path of these devices. It is also possible to introduce complex flow patterns (pulsatile motion, rapid accelerations and decelerations etc.) as they exist *in vivo*. Finally, a more recent development in microfluidics has been the incorporation of vascular cells in the process of thrombosis in various diseases [8,26].

Arterial thrombosis

A major arterial thrombotic disease is atherosclerosis, where rupture of plaques leads to release of inflammatory cytokines and rapid thrombus formation [27]. These plaques are largely formed at vessel branching points and large bends where disturbed or stagnating flow is present [28]. Formation of these plaques creates stenosed regions in the artery that further augments the disturbed flow which is already present in this region. Microfluidic channels can be easily designed to incorporate channel geometries (such as stenosis) and disturbed flow conditions relevant to atherosclerosis. For example, a plaque-like geometry was modeled in a microfluidic conduit showing thrombus mostly forms in specific post-stenosed region [29]. This phenomenon was further utilized to model an arterial vascular bed and assess clotting time in extracorporeal devices and patients [30]. Further advancement in microfluidics permitted endothelialization of a similar geometry which showed that cell-secreted VWF further exacerbates platelet recruitment and adhesion [31]. More recently, a 3D printed microfluidic channel containing an atherosclerotic plaque using computed tomography angiography data of patients has been demonstrated [32]. Taken together, these studies have surveyed geometry-dependent arterial thrombosis due to atherosclerosis but several advancements can potentially be made to more faithfully mimic more complex biology of atherosclerosis. For example, pulsatile flow may be introduced in these devices

to mimic arterial blood flow more closely. Secondly, extracellular matrix and residing smooth muscle cell signaling also influences plaque formation and thrombosis, which can potentially be included with organ-on-a-chip technology.

Venous thrombosis

Human veins are macro-fluidic and are mostly of size 5 mm and above. Yet, downscaled microfluidic models of venous thrombosis (DVT) that match murine sizes could serve as a more efficient source to study shear-dependent mechanisms in DVT using very low volumes of human tissue and blood samples. Notably, the Virchow's triad is more dominant in the setting of venous thrombosis [33]. Also, venous thrombosis initiates near venous valves where abrupt and pulsatile flow conditions, and hypoxic endothelium contribute to the propagation of thrombus and its embolism [34]. Due to the scale difference and larger complexity, there have been relatively very few efforts to design microfluidic models of DVT. In one study, it was predicted in a microfluidic device that clot propagation from superficial veins to deep veins is regulated by fluid shear rate, which might explain the correlation between superficial thrombosis and the development of DVT [35]. Another study showed using murine blood that venous thrombi have lower platelet density compared to arterial thrombi because platelet aggregation and adhesion over ECM is dependent on the wall shear rates [36]. In future, microfluidic models of DVT containing endothelium and venous structures could contribute to a better understanding of the unique thrombus pathology in veins and specific drug–tissue interactions.

Microvascular thrombosis

Several diseases (like sickle cell disease, diabetes, cancer, and pulmonary injury) lead to fatal thrombosis in the microvascular arterioles and venules of various organs (lung, kidney, brain etc.). Modeling intravascular thrombosis can be very difficult because it is regulated by the several local mechanical and biological conditions of the blood flow, vessel wall, ECM and the epithelium. Nevertheless, microfluidic devices offer a huge opportunity to model this complexity due to their ability to use blood, tissue and vessel architecture derived from such patients to make *in vitro* models that are as close as possible to the *in vivo* disease microenvironment. Recent efforts to mimic vascular physiology *in vitro* leverage the microfluidic device technology that enables creation of arteriole-sized channels capable of endothelialization and blood perfusion [10,37]. In one example, a microfluidic device with arteriole-sized channels was lined with endothelial cells and when blood from sickle cell disease (SCD) patients or blood spiked with a bacterial toxin that causes hemolytic uremic syndrome (HUS) were perfused, it revealed increase in occlusion and clot formation [8]. The same system also showed clots when endothelial cells were activated with an inflammatory cytokine TNF- α . More recently, a microfluidic device containing stimulated and fixed endothelial cells could result in thrombosis upon whole blood perfusion and was proposed to be used as a diagnostic device in clinical settings [7].

Microphysiological models of thrombosis: new directions

The process of thrombosis in diseases can be more complex than the interactions between the endothelium, extracellular matrix and the blood cells (Fig. 1A and B). For example,

in cancer, the tumor cells release inflammatory factors that result in endothelial activation and platelet activation, eventually leading to the onset of blood clots [38]. Similarly, in acute lung injury or pneumonia, the alveolar epithelium may secrete factors that lead to platelet recruitment and thrombosis [39]. Organ-on-a-chip technology could offer a platform in order to mimic this thrombotic microenvironment and dissect tissue-tissue and drug-tissue interactions for systematic analysis of thrombosis (Fig. 1C). Recently, a model of lung thrombosis [26] showed 3 new features that support organ-level functional design: a) human primary alveolar and endothelial cells are co-cultured in adjacent microfluidic conduits, separated by thin layer of matrix, b) the endothelial cells cover all sides of one chamber to form a complete vascular tube, and c) when human whole blood is perfused at a designated shear stress through the stimulated vascular lumen of this device, the thrombus formed *in vitro* is identical to *in vivo* (Fig. 1D and E). With this device, it was discovered that the alveolar epithelium is critical to thrombosis that is initiated by lipopolysaccharide (LPS). Further, an endothelium-specific therapeutic effect of an antithrombotic compound was identified in the presence of alveolar epithelial endothelial-blood cell signaling, not easily possible with animal models. Finally, a critical mechanism of action of this compound was discovered and shown that it performs via the natural β -arrestin pathway of activated protein-C (APC) [40]. These recent developments in microphysiological systems are highly promising and it may be expected that the blood vessels in these organ-chips may be designed to take complex shapes (branches, valves, stenosis etc.) through which blood may be transported under physiologically-relevant fluid dynamical states. A major opportunity offered by these systems is that the biological processes can be visualized using microscopy, measured using biosensors, and quantified using analytical algorithms and genomic screening. However, there are still some limitations in the current microfluidic designs that limit the extent to which human physiology can be reconstituted. For example, a fundamental limitation of most microfluidic devices is that they are made of rectangular channels with a high surface-to-volume ratio. While the rectangular shape is easy to fabricate and image on microscopes, the flow near the edges of the rectangular walls does not recapitulate the flow inside a cylindrical vascular tube. Therefore, the analysis of thrombus can only be done distant from the wall [7]. Another limitation is that even though soft-lithography is most widely used in developing the aforementioned microfluidic devices, there is high initial setup cost, low throughput and limited 3D capability as layered 2D designs have to be used for 3D designs [41]. Also, due to small and rigid channel sizes, it is difficult to create turbulence (high Reynolds number) that may be present in large and elastic blood vessels in pathophysiological conditions and therefore, their contributions to thrombotic occlusion cannot be easily assessed [42]. Finally, microfluidic devices may not mimic all the biological processes of vascular thrombosis and therefore, *in vivo* models may still be needed for some studies. Whole blood perfusion without significant anticoagulation in organ-on-a-chip can only be done for a short-term duration and therefore, it is currently not possible to study long-term systemic thrombotic processes, that may also include vascular remodeling and embolism. With 3D-printing technology advancing fast in resolution and biocompatibility, it is an exciting option to fabricate microfluidic systems in future due to easy process automation, assembly-free 3D fabrication, and high throughput. Tissues and blood obtained from patients and induced pluripotent stem-cell (iPSC) technology could potentially personalize such systems and provide patient-

specific readouts that can never be obtained from animal models or current hemostasis and thrombosis assessment devices. In summary, the prospects of such microphysiological models are promising. As progress is made in this direction, they are expected to mimic the complex pathophysiology of thrombosis at an organ-level and permit dissection of mechanism of action of various cells and tissues to the extent that it will ultimately predict therapeutic responses and toxicities to drugs, thus directly impacting the health outcomes of patients, as well as cost of treatment.

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Figure 1. Organ-level complexity of thrombosis and microphysiological modelling.

A) A blood clot formed *in vivo* may contain contributions from endothelial cells, blood cells, extracellular matrix and parenchymal cells, along with released clotting factors and cytokines **B)** The three factors of Virchow's triad: inflamed or activated vascular endothelial cells (and activated epithelial cells), change in flow dynamics and blood rich in clotting factors, determine the dynamics of thrombosis. **C)** The organ-on-a-chip technology has been able to model the complex signalling in pulmonary thrombosis *in vitro*. **D)** Confocal micrograph of an alveoli-on-a-chip device showing co-culture of primary alveolar epithelial cells (purple) and endothelial lumen (green), with whole blood flow (not visible). **E)** Identical morphology of a thrombus formed *in vivo* (left) and inside the device (right). Bar: 10 μm .