Determination of surface tissue factor thresholds that trigger coagulation at venous and arterial shear rates:

Amplification of 100 fM circulating tissue factor requires flow

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Running title: Circulating and surface tissue factor under flow
Abstract

Protein microarrays presenting spots of collagen and lipidated tissue factor (TF) allowed a determination of the critical surface concentration of TF required to trigger coagulation under flow. Whole blood supplemented with corn trypsin inhibitor (to inhibit Factor XIIa) was perfused over microarrays for 5 min. Immunofluorescence staining of platelet glycoprotein GPIbα and fibrin(ogen) revealed a critical TF concentration (EC50) of 3.6, 8.4, and 10.2 molecules-TF/µm² at wall shear rates of 100, 500, and 1000 s⁻¹, respectively. For collagen arrays where only the center lane of spots (in the direction of flow) contained TF, a downstream distance of 14 mm was required for the thrombus to widen enough to reach across a 300 micron gap to the adjacent TF-free lanes of collagen spots, in agreement with numerical simulation. To investigate the effect of low levels of circulating TF, whole blood (± 100 fM of added TF) was tested under static and flow conditions. After 5 min, the addition of 100 fM TF to whole blood had negligible effect under static conditions, but caused a 2.5-fold increase in fibrin formation under flow. This report defines the threshold concentrations of surface TF required to trigger coagulation under flow.
Introduction

Plaque rupture reveals tissue factor to flowing blood, resulting in coronary thrombosis and occlusion with consequent acute myocardial infarction. Despite the prevalence of this event, the critical concentration of surface tissue factor required to cause clotting at various hemodynamic conditions remains poorly defined. Also, the existence, source(s), and functional activity of circulating levels of tissue factor are not fully resolved in health or disease. The function of circulating TF in concert with wall derived TF may depend on prevailing flow conditions.

TF in a lipid surface serves as a cofactor for Factor VIIa (present at ~1 % of the 10 nM Factor VII concentration) resulting in ~10^5-fold enhancement of Factor Xa formation. Platelet deposition may reduce access of Factor X to the TF/VIIa complex formed on the damaged wall. Elevated TF antigen and activity is detectable in human atherosclerotic lesions and is expressed by various cell types. Bonderman et al. determined using ex vivo plaque disruption/scraping that the average TF site density underneath plaques is 33 pg TF/cm^2, corresponding to ~ 6 molecules-TF/µm^2. Drake et al. found that in human cardiac and skeletal muscle the TF levels were 7 and 119 ng TF/mg protein, respectively. Tissue factor pathway inhibitor (TFPI) is also elevated in atherosclerotic vessels in comparison to 10 to 20 ng TFPI/cm^2 in healthy vessels.

Blood-borne tissue factor antigen was first reported in a system using 5-min ex vivo perfusion of human blood over collagen-coated slides, a system in which fibrin deposition was blocked by inhibited Factor VIIa (FVIIa). Collagen-activated platelets are highly procoagulant and may present Factor VIIa cofactor activity susceptible to antagonism by antibodies or FVIIa. A recent study of 91 individuals using the Luminex assay indicated that most normal individuals had < 2 pM TF in plasma, a value lower than the average 4 pM TF obtained from a literature survey of plasma TF levels in healthy individuals measured by ELISA. Addition of increasing amounts of sub-picomolar levels of lipidated TF to corn trypsin inhibitor (CTI)-treated whole blood indicate that active TF in normal individuals is sub-picomolar, estimated to between < 20 fM and < 200 fM. Recently, rapid splicing of TF pre-mRNA and expression
of TF antigen has been reported in sonicated membranes obtained from activated platelets. Under flow conditions, the transfer of tissue factor may be of importance via leukocyte delivery to platelets via CD15 or capture of microparticles presenting TF and PSGL-1 or derived from platelets.

Mathematical simulations of the hemostatic response have also taken into account the importance of tissue factor site density. Kuharsky and Fogelson developed a full transport-reaction coagulation model that takes into account surface dependent reactions, transport of factors and cells due to flow, and populations of resting and activated platelets. In the Kuharsky-Fogelson model, an increase of the TF surface concentration from 2 to 8 fmol/cm² (12 to 50 molecules-TF/µm²) was predicted to cause a 4 to 5-order of magnitude increase in local thrombin production under flow from 100 to 1500 s⁻¹.

In prior work, we used a matrix protein microarray assay to spatially control surface composition by presenting collagen microspots to flowing platelet-rich plasma or whole blood. In the present study, we tested the effect of lipidated tissue factor in printed collagen microspots under defined laminar flow conditions. We also added low level exogenous TF to whole blood prior to perfusion in this assay. We determined the threshold for TF to trigger coagulation under flow. While sub-picomolar levels of lipidated TF had little effect under no-flow conditions at 5 min, this low level dramatically enhanced thrombosis in the presence of flow.

Materials and Methods

Materials

Mouse monoclonal anti-human CD42b (GPIbα) (Research Diagnostics Inc.; Flanders, NJ), FITC-conjugated rabbit anti-human fibrin/fibrinogen, FITC-conjugated Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit (Invitrogen Corporation; Carlsbad, CA), corn trypsin inhibitor (Haematologic Technologies, Inc.; Essex Junction, VT), human serum albumin (Golden West Biologicals; Temecula, CA), native collagen fibrils (type I) from equine tendons suspended in isotonic glucose solution of pH 2.7 (ChronoLog Corporation; Havertown, PA), bovine serum albumin and sodium citrate (Sigma-Aldrich Corp.; St. Louis, MO), HEPES (N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid) (Fisher Scientific;
Pittsburgh PA), lipidated recombinant human tissue factor (baculovirus expressed, amino acids 1-263; 43 kDa), and phosphate buffered saline (PBS) without calcium chloride or manganese chloride (Invitrogen Corporation; Carlsbad, CA) were stored according to the manufacturers’ instructions. IMUBIND Tissue Factor ELISA Test Kit (American Diagnostica; Stamford, CT) was used according to manufacturer’s instructions to determine the concentration of available tissue factor accessible to antibody in the intact lipidated TF preparation. The amount of available TF antigen of the stock solution was determined to be 18.2 nM.

Human blood was collected from healthy donors via venipuncture and anticoagulated with sodium citrate (9 parts blood to 1 part sodium citrate). Prior to perfusion, the plasma was treated with CTI (50 µg/ml) to block Factor XIIa and associated intrinsic pathway initiation and then was recalcified with CaCl₂ to a final calcium concentration of 20 mM. Phlebotomy was conducted according to the Declaration of Helsinki and under University of Pennsylvania Institutional Review Board approval.

**Matrix protein microarray perfusion assay**

Collagen matrix microarrays were prepared as previously described. Briefly, an OmniGrid Accent (Genomic Solutions, Ann Arbor, MI) robotic contact microarrayer was used for arraying with a 1 x 1 pin protocol with an ArrayIt™ Stealth Micro Spotting Pin SMP-4 (Telechem International Inc.; Sunnyvale, CA). Prior to printing, plain glass slides (25 x 75 x 1.0 mm, SuperFrost Plus, Fisher Scientific; Pittsburgh, PA) were incubated with 1 M NaOH (15 min), rinsed extensively using distilled water, ethanol rinsed, and vacuum-dried. Collagen (1 mg/ml) and tissue factor (0 TF, or 1 pM to 1 nM TF) in 5% (v/v) glycerol were printed via robotic contact printing (50% relative humidity). For the tissue factor/collagen microarrays, 14 columns (aligned in the direction of flow) by 30 rows were printed (spot center-to-center distance = 500 µm). Printed TF concentrations in the 14 columns were 0, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, 500, 750, or 1000 pM (1000 pM = 25 molecules-TF/µm² for the 1-nL printed features of 175-micron diameter). After printing, the slides were stored at 4°C until mounted on flow chambers.
A parallel-plate perfusion chamber was used as previously described.\textsuperscript{22} The wall shear stress, $\tau_w$ (dyne/cm$^2$) was calculated by $\tau_w = 6Q\mu/B^2W$ where $Q$ is the volumetric flow rate (cm$^3$/s), $\mu$ (Poise) is the viscosity of the fluid, $B$ is the separation between the plates (0.02 cm) and $W$ (1.11 cm) is the flow chamber width. The viscosity of whole blood was taken as 0.04 Poise at 37°C.\textsuperscript{23} Perfusion of 1% (w/v) human serum albumin (HSA) in HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) at a wall shear rate of 25 s$^{-1}$ for 5 min was used to rinse the glycerol from the microspots and to prevent nonspecific binding. Recalcified whole blood was then immediately perfused for 5 min at 37°C over the slide at various shear rates by withdrawal using a syringe pump (Harvard Apparatus; Holliston, MA).

**Immunostaining and microarray scanning**

After perfusion, the dismounted slides were immediately rinsed in 3% (w/v) BSA, fixed with 2% paraformaldehyde in PBS for 20 min, and rinsed with NH$_4$Cl to quench any unreacted aldehyde. Anti-CD42b was fluorescently labeled using Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit according to the manufacturers’ instructions. The labeled anti-CD42b and FITC conjugated anti-fibrin/fibrinogen was diluted 1:50 with 3% BSA solution. The slide was incubated with the antibodies for 1 hr and rinsed with 3% BSA. The slides were imaged using a cooled CCD microarray scanner (Alpha Array 8000; Alpha Innotech Corp.; San Leandro, CA) with Cy5 and FITC filter sets. The raw images were quantified for fluorescent intensity using ArrayVision 6.3 (Imaging Research; St. Catharines, Ontario). The fluorescent intensity was background subtracted for each spot by using background estimates from thirty regions around the array. For surface tissue factor EC50 analysis at each shear rate, a four parameter logistic equation, $y = A + [(B-A)/(1+10^{C-xD})]$ was fit with the Levenburg-Marquardt algorithm ($XLfit$) with: $A$, minimum y value; $B$, maximum y value; $C$, log (EC50); and $D$, slope factor. In calibration experiments, a maximum in fibrin production was reached at 12.5 to 25 molecules-TF/µm$^2$ (corresponding to $\sim 1.3 \times 10^9$ FI signal for fibrin), with a slight decline in fibrin formation when higher levels of TF > 25 molecules-TF/µm$^2$ were used. The decrease in fibrin deposition at ultrahigh levels of TF above 25/µm$^2$ may be related to fibrin shielding of collagen and reduced platelet GPVI signaling in the microthrombus spots.
Numerical simulation of TF microspot experiment

The steady-state three-dimensional mass conservation equation was solved using commercial finite element method software (COMSOL Multiphysics, Burlington, MA). The geometry consisted of a volume 1 mm in width, 14 mm in length, and 50 mm in height. Microspots with a diameter of 200 µm were spaced 0.5 mm apart along the y-axis at the center of the plane (physical properties and boundary conditions summarized in Table 1). A previously reported platelet thrombin flux\textsuperscript{24} (U/platelet-sec) was multiplied by 500 platelets\textsuperscript{20} and divided by the microspot area to calculate the thrombin flux for each microspot. The thrombin conservation equation was coupled to the velocity profile for flow between two parallel-plates: \( v_y(z) = \gamma_w (z^2/B - z) \), where \( v_y(z) \) is the velocity (cm/sec) in the direction of flow as a function of height from the bottom plate, \( \gamma_w \) is the wall shear rate (1/sec), and \( B \) is the gap height (cm) between the two plates.

Pseudo first-order kinetics were assumed for ATIII inhibition of thrombin because the concentration of ATIII (5 µM) is much greater than the highest concentration achieved within the simulated thrombi (~100 nM). The rate constant for thrombin inhibition was calculated by multiplying the second-order rate constant from Hockin et al.\textsuperscript{25} by the ATIII plasma concentration. The grid consisted of 58,625 prism elements. Mesh independence was evaluated by comparing the concentration profiles for grids consisting of 23,996, 58,625, and 93,800. There was no greater than 1.5% difference in the magnitude of the concentration between the three grid sizes at any position within the simulation geometry. The steady state mass conservation equation was solved in 12 minutes using a GMRES solver (COMSOL, 2007) on a PC workstation (2.2 GHz processor, 12 GB RAM).

Results

Surface TF titration

To determine the level of surface TF needed to cause combined platelet and fibrin deposition under physiological hemodynamic flows, microarrays presenting collagen and increasing levels of tissue
factor from 1 pM to 1000 pM TF (corresponding to 0.025 to 25 molecules-TF/µm²) were mounted in parallel plate flow chambers. Recalcified CTI-treated whole blood was perfused over 3 separate arrays at 3 different wall shear rates (\(\gamma_w = 100, 500, \) or \(1000 \text{ s}^{-1}\)) (Fig. 1A). On pure collagen lacking TF (left most column of each array in Fig. 1A), platelet deposition was greatest at the venous shear rate of 100 s\(^{-1}\) and was reduced at arterial shear rates of 500 and 1000 s\(^{-1}\), as expected for surfaces lacking von Willebrand factor.

At each wall shear rate, a marked increase in fibrin(ogen) staining was detected at the highest concentrations of TF tested. At 100 s\(^{-1}\), as surface TF levels increased across the microarray, platelet deposition also increased at subcritical levels of TF (< 2 molecules-TF/µm²) indicating that thrombin production can enhance platelet deposition prior to fibrin formation. Below 2 molecules-TF/µm², there was essentially no effect of surface TF on either platelet deposition or fibrin formation at 5 min at arterial wall shear rates (500 or 1000 s\(^{-1}\)). While collagen alone or collagen with low TF (< 2 molecules-TF/µm²) was insufficient to drive fibrin formation after 5 min of blood exposure, substantial quantities of platelets were deposited at each shear rate. At a venous wall shear rate of 100 s\(^{-1}\), moderate to high levels of TF ranging from 1.9 to 25 molecules-TF/µm² caused increasing deposition of platelets and fibrin, indicating that thrombin production facilitated platelet incorporation into the growing thrombi (up to 300 to 500 platelets/spot). The impact of thrombin production at 1.9 to 25 molecules-TF/µm² on platelet deposition was less pronounced at the highest arterial shear rate of 1000 s\(^{-1}\) (Fig. 1B). Consistent with prior studies with platelet rich plasma perfusion,\(^{20}\) essentially no platelet deposition was detected on the glass microarray surface lacking printed collagen features.

In the narrow range of 1.9 to 25 molecules-TF/µm², fibrin formation switched from negligible to maximal production at all shear rates tested (Fig. 1A-E). The calculated effective concentration to produce half maximal fibrin deposition (EC50) was 3.63 molecules-TF/µm² at 100 s\(^{-1}\), 8.40 molecules-TF/µm² at 500 s\(^{-1}\), and 10.2 molecules-TF/µm² at 1000 s\(^{-1}\) (Fig. 1C-E). At 100 s\(^{-1}\), fibrin formation at 5 min appeared to reach a maximal extent as surface TF increased to levels above 12.5 molecules-TF/µm². Based on fluorescence immunostaining, there was a >10-fold increase in fibrin density at the highest
concentration of TF tested. Below 1.9 molecules-TF/µm², there was little dependence of fibrin(ogen) staining on surface TF concentration, consistent with detection of platelet-bound fibrinogen. To test the role of wash-out of the lipidated TF from the collagen microspot, we compared thrombosis on the TF-laden collagen microarrays with and without 5 min pre-rinse at 25 s⁻¹. We found that pre-rinse had no effect on platelet deposition and caused a modest 29 % right-shift of the EC50 for fibrin formation (Supplemental data Fig. S1).

**Transport of species with and transverse to flow**

Transport of soluble reactive species in the flow stream can remove species from the local site of formation as well as propagate thrombosis beyond the site of wall disruption. We developed a specialized printed array to investigate transport effects of soluble species in the direction of flow (by convection) and transverse to the direction of flow (by diffusion/dispersion). This array had one column of collagen and 1000 pM TF (25 molecules-TF/µm²) surrounded by collagen spots. Thus, there was one ‘active’ lane of spots surrounded by ‘inactive’ lanes. Whole blood was perfused at $\gamma_w = 100 s^{-1}$ for 5 min at 37°C. The fibrin-rich thrombus extended well past the array length indicating that fibrin polymerization could continue well down stream of the location of printed features. The fibrin tail was notably depleted of platelets (Fig. 2A). However, the fibrin remained highly localized over and downstream of the TF-laden lane of spots with a width comparable to the original collagen/TF features (Fig. 2A and 2B). A substantial distance of 14 mm downstream was needed to achieve a maximum fibrin width of 350 µm from the centerline (250 microns from the outer radius of the feature). Even at a venous shear rate, it is difficult for a clot to propagate via diffusion or platelet deposition in a direction transverse to the flow, from a site of high surface TF to a nearby neighboring site of collagen lacking TF. Numerical simulation of constant thrombin release from a center lane of microspots (See Table 1) followed by inhibition with antithrombin III predicted that active thrombin can be detected downstream of the printed collagen/TF features, however diffusive/dispersive penetration of thrombin in a direction transverse to the flow is
severely restricted by the flow (Fig. 2C). Diffusive penetration of thrombin into the flow field normal to the surface was also equally restricted above the surface (not shown).

**Effect of 100 fM of added TF: Role of flow**

In normal or diseased individuals, low levels of circulating active TF may have a consequence on the propagation of the coagulation response. Since less is known about the interaction of circulating TF with wall-derived TF under flow conditions, we tested the functional significance of adding 100 fM lipidated TF to CTI-treated whole blood under static and flow conditions. Butenas et al.\(^\text{13}\) found that under rocking conditions the addition of ~100 fM lipidated TF to CTI-treated whole blood reduced the visual clot time (appearance of clumps) by about 50% to ~20 min., although thrombin-antithrombin or fibrin production were not measured. Using thrombin-antithrombin (TAT) ELISA or the fluorogenic thrombin substrate boc-VPR-MCA in CTI-treated whole blood in static well plate assay, we found that adding 100 fM TF produced no detectable thrombin within 5 min, but reduced the clotting time from greater than 60 min to between 20 and 40 min (Supplemental Data Fig. S2). Thus, addition of 100 fM lipidated TF to CTI-treated whole blood was a minimal perturbation of blood under static conditions with respect to thrombin production at 5 min post-TF addition. Next, CTI-treated whole blood with and without added 100 fM TF was introduced into two independent microarray flow chambers. For the static assay, whole blood was perfused into the chamber at 50 s\(^{-1}\). Once the chamber was filled with blood, perfusion was stopped and the chamber was incubated for 5 min at 37°C. Under static conditions, increasing surface TF above 2 molecules-TF/µm\(^2\) caused a marked enhancement of platelet and fibrin deposition at 5 min (dotted line, Fig. 3A and 3B). Interestingly, adding 100 fM of lipidated TF to whole blood caused a slight reduction in platelet and fibrin deposition on the printed microarray under the no flow condition. In the absence of flow, only platelets initially near the surface can interact with the microarray and a low buildup of platelets on the collagen was expected. The slight decrease in platelet deposition by added TF under no flow conditions shown in Fig. 3B was unexpected. Under no flow conditions, cell settling is the
dominant mechanism of platelet delivery to the surface. Subtle changes in red blood cell or platelet motions under gravity-driven settling could be altered by the presence of the added TF. Also, we note that the platelet structures formed under static conditions were not nearly as dense or stable as those formed under flow. Thus, platelet incorporation by settling to the surface may lead to more friable structures (analogous to structural differences due to diffusion-limited vs. ballistic aggregation) with potentially reduced interaction with collagen. For CTI-whole blood perfusion (no added TF), the presence of flow substantially enhanced platelet delivery and accumulation on the microarray, at all prevailing surface TF levels (solid line, Fig. 3B). In the presence of flow, adding 100 fM TF slightly enhanced platelet deposition by ~30 to 40% at all surface TF levels (Fig. 3B). Thus, addition of 100 fM TF did not enhance platelet deposition on collagen or collagen/TF microspots in the absence of flow, but did enhance platelet deposition on collagen or collagen/TF microspots in the presence of flow at 5 min. The effect of 100 fM lipidated TF was apparent under flow at < 2 molecules-TF/µm² as indicated by the number of activated platelets captured to the surface and by the fibrinogen staining of those captured platelets (Fig. 3A and 3B). Platelet deposition was largely independent of surface TF up to about 8 molecules-TF/µm². However, under flow conditions at > 10 molecules-TF/µm², the intensity of fibrin deposition was markedly enhanced by 2.5-fold by the addition of 100 fM circulating TF. Such elevation in fibrin deposition was not observed by adding 100 fM soluble TF under static incubation, even in the presence of 25 molecules-TF/µm² on the surface.

Discussion

We report that surface tissue factor functions over a very narrow concentration range between 2 and 20 molecules-TF/µm² to trigger rapid platelet and fibrin deposition on collagen between venous levels (γw = 100 s⁻¹) and arterial levels of flow (γw = 500 or 1000 s⁻¹). Below 2 molecules-TF/µm², tissue factor has relatively minor effect on fibrin formation or platelet deposition over 5 minutes. Above 20 molecules-TF/µm², thrombin and fibrin production appeared maximal. The level of 6 molecules-TF/µm² in human atherosclerotic carotid artery plaques is well within the range defined in the microarray
experiments to produce an intense and rapid coagulation response. A level of 0.05 U/ml thrombin (\(~0.5\) nM) is known to be sufficient to cause threshold activation and allow reliable detection of more than 10 % activated platelets within a platelet sample \(^{26}\).

The concept of a threshold TF concentration under flow conditions has been anticipated theoretically\(^3\). Also, patch size thresholds have been investigated under no-flow conditions where a very strong patch size threshold exists for plasma-based systems, but not in whole blood at 24°C (See Fig. 2F of Kastrup et al \(^{27}\)). We have extended these theoretical and experimental studies to define the critical surface TF needed to trigger whole blood clotting on collagen within 5 min under physiological flow conditions. In recent numerical simulations, Kuharsky and Fogelson\(^3\) predicted a very similar narrow concentration regime for surface tissue factor to trigger clotting under flow. In the Fogelson model and in our experimental results, the EC50 to cause 50 % maximal fibrin formation was only moderately sensitive to flow over the physiological regime. We observed that the surface TF EC50 right shifts only 2.8-fold (from 3.6 to 10.2 molecules-TF/\(\mu \text{m}^2\)) as wall shear rate increased 10-fold from venous to arterial levels (Fig. 1C-E). Convection and ATIII limit fibrin formation to zones above and downstream of focal locations of surface tissue factor (Fig. 2).

Low circulating levels of 100 fM tissue factor when added to flowing CTI-treated whole blood did not substantially left shift the dose response curve for fibrin formation triggered by surface tissue factor. Rather, the results shown in Fig. 3B indicated that 100 fM of circulating tissue factor served to amplify the response initiated by the surface TF, allowing for higher levels of fibrin to be formed under flow. The enhancing effect of adding 100 fM of circulating TF required flow and was not seen under stationary conditions. Convective delivery of circulating TF may cause its accumulation at the thrombotic site as well as facilitate Xa production at later stages of the clotting process.

A recent study has shown that citrate/recalcification protocols may alter the function of plasma coagulation and platelet aggregation responses \(^{28}\). In this recent study, the use of citrate had essentially no effect on platelet activation response to collagen. Collagen was the surface protein used in the current study and the use of citrate/recalcification is not expected to have any effect on the platelet responses to
collagen in the flow experiments shown in Fig. 1-3. Additionally, Mann et al.\textsuperscript{28} showed that the use of citrate had no effect on the clotting time of CTI-treated whole blood (no added Tf) as measured by the authors using thromboelastography ($R^*$) [CTI-citrate whole blood, $R^* = 66 \pm 19$ min vs. CTI (no citrate) whole blood, $R^* = 66.1 \pm 18.7$ min]. Thus, we conclude that recalcified, citrated CTI-treated whole blood treated with a minimal perturbation of 100 fM tissue factor displays kinetics of fibrin production and formation that are very weakly affected by the use of citrate.

Estimates of functional or antigenic blood-borne TF concentrations range from femtomolar to low picomolar levels.\textsuperscript{6,12,13,29} The purpose of the experiments shown in Fig. 3 was not intended to resolve the functional role of blood-borne tissue factor in normal individuals. Rather, the intent of the experimental design shown in Fig. 3 was to understand the conditions by which a low level of circulating TF (100 fM) may demonstrate functionality during a coagulation event. We conclude that fibrin formation in flow systems presenting surface TF may be useful for detecting the effects of low level circulating TF. The role of blood-borne tissue factor remains an area of intense investigation and various phenomena including platelet synthesis or release, de-encryption, secreted splice-variants, or microparticle capture may be usefully examined under hemodynamically relevant conditions.

Flow chambers coated with various proteins (fibrinogen, collagen, vWF, selectins, etc.) have been used for decades to study blood function.\textsuperscript{24,30-32} However, most of these studies typically use anticoagulated blood or isolated blood cells to study events related to adhesion, independent of the \textit{in situ} production of thrombin and fibrin. Since the advent of CTI to antagonize contact activation in vitro,\textsuperscript{33,21} we are not aware of any in vitro flow studies to have included the effects of surface TF on platelet and fibrin deposition over collagen. The collagen/lipidated TF microarray assay allows a single variable to be titrated in a single flow experiment. An additional aspect of this assay is that it provides an in vitro mimic of focal ‘patches’ of subendothelial proteins exposed to blood, analogous to thrombosis triggered via laser injury in mouse models,\textsuperscript{18} albeit an \textit{in vitro} assay lacking endothelial functionalities such as nitric oxide, prostacyclin, or thrombomodulin. While lipidated TF can be washed out of the collagen microspot to a limited extent (~30 \%) over the 5-minute time frame of this in vitro experiment, it is important to
note that TF washout from damaged vessels also occurs in vivo, but is poorly quantified with respect to rates and sensitivity to flow.

The printed microarray mounted in parallel-plate flow chambers allows the spatial control of platelet adhesion with collagen and thrombin/fibrin production triggered by lipidated TF under physiological flow. This approach may also be particularly useful for evaluating the pharmacological efficacy of anti-adhesion agents, anti-platelet agents, anti-coagulants, and thrombolytic or clot imaging agents under a range of flow and procoagulant surface stimuli.

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Authorship

U.O. conducted all flow experiments. K.N. conducted numerical simulation. W.D. and M.C. conducted well plate experiments. S.L.D. oversaw all aspects of study and wrote the manuscript with contributions from all authors.

Conflict of Interest Disclosure

The authors declare no competing financial interests.
References


Physical and biochemical parameters

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<td>Viscosity of whole blood</td>
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<td>Diffusivity of thrombin</td>
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<td>First-order ATIII inhibition</td>
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Boundary conditions

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Table 1. Physical properties and boundary conditions used in numerical simulation
Figure Legends

Figure 1. Titration of surface tissue factor required to trigger fibrin formation. Immunostaining of platelet GPIbα (top) and fibrin(ogen) (bottom) after 5-min whole blood perfusion over collagen/TF arrays at wall shear rates of 100, 500, and 1000 s⁻¹ (A). Surface tissue factor concentration in each feature increased from left to right from 0 to 25 molecules-TF/µm² (Scale bar = 1 mm). Increased magnification of the first row of each array for each shear rate for surface TF concentration ranging from 1.9 to 25 molecules-TF/µm² (Scale bar = 300 µm; yellow, colocalized GPIbα and fibrin(ogen)) (B). Column averages of fluorescent intensity (FI) of the fibrin(ogen) staining are plotted against the TF surface concentration for 100 s⁻¹ (C), 500 s⁻¹ (D), and 1000 s⁻¹ (E) for 3 independent experiments (3 x 30 spots per plotted data point). Data was fit to a 4 parameter logistic equation to determine the TF EC50 values for each shear rate.

Figure 2. Convection-diffusion processes in coagulation. Immunostaining of platelet GPIbα and fibrin(ogen) after 5-min whole blood perfusion over ‘center lane’ array at 100 s⁻¹ (A) Bottom arrow indicates column where collagen and 25 molecules-TF/µm² spots were printed (Scale bar = 1 mm). Magnified images are of the regions indicated by the white squares (Scale bar = 300 µm). Identical results observed in 3 separate experiments. Growth of fibrin width from center of “center lane” plotted against distance downstream from first spot (B). Steady state numerical simulation of thrombin at 10 µm above the surface for the center lane experiment shown in A using constant flux of thrombin from each microarray feature into a flow field containing ATIII (See Table 1 for parameters) (C). Magnified images are the same areas as Panel A magnified images.

Figure 3. Amplification of surface TF by circulating 100 fM TF requires flow. The effect of exogenously added lipidated TF (100 fM) was studied in the presence and absence of venous wall shear rate of 100 s⁻¹. Immunostaining for platelet GPIbα and fibrin(ogen) after 5-min whole blood perfusion (with or without 100 fM TF) over TF titration arrays (A). Column averages of fluorescent intensity of the GPIbα staining (top) and fibrin(ogen) staining (bottom) are plotted against the TF surface concentration.
for static (dashed line) or venous flow (solid line) for perfusion of CTI whole blood without (○) or with 100 fM of added lipidated TF (●) for 3 independent experiments (3 x 30 spots per plotted data point) (B).
Figure 1

A

Whole Blood + 50 μg/ml CTI, 5 min, 37 °C
100 s⁻¹  500 s⁻¹  1000 s⁻¹

B

100 s⁻¹
500 s⁻¹
1000 s⁻¹

TF molecules/μm²
1.9  2.5  6.3  12.5  18.8  25

C

D

E

Log₁₀ [TF]  Log₁₀ [TF]  Log₁₀ [TF]
Figure 2

A

WB (50 μg/ml CTI)

γw = 100 s⁻¹, 5 min (37°C)

AF 647 anti-GPIba

FITC anti-fbg

25 TF molec/μm²

B

Fibrin width

Spot Radius

Distance from first spot (mm)

Flow

C

[Thrombin] nM

γw = 100 s⁻¹

Ωᵣ = 4.16 × 10⁻⁷ cm²/sec

Jᵣᵣ = 1.11 × 10⁻¹³ nmol/μm²·sec
Figure 3

A

\[
\gamma_w = 100 \text{ s}^{-1}, \ 5 \text{ min (37°C)}
\]

WB (50 µg/ml CTI)  WB (50 µg/ml CTI) + 100 fM TF

Flow

AF 647
anti-GPIIb\alpha

FITC
anti-fbg

B

Graph showing fluorescent intensity against surface TF density. The y-axis represents fluorescent intensity (×10^5), and the x-axis represents surface TF density (TF molecules/µm²). Three conditions are compared:
- WB (CTI)
- WB (CTI) + 100 fM TF
- Static

The graph illustrates the increase in fluorescent intensity with increasing surface TF density for each condition.
Determination of surface tissue factor thresholds that trigger coagulation at venous and arterial shear rates: amplification of 100 fM circulating tissue factor requires flow

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