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Threshold Response of Initiation of Blood Coagulation by Tissue Factor in Patterned Microfluidic Capillaries Is Controlled by Shear Rate

Feng Shen, Christian J. Kastrup, Ying Liu, Rustem F. Ismagilov

Objective—Blood flow is considered one of the important parameters that contribute to venous thrombosis. We quantitatively test the relationship between initiation of coagulation and shear rate and suggest a biophysical mechanism to understand this relationship.

Methods and Results—Flowing human blood and plasma were exposed to cylindrical surfaces patterned with patches of tissue factor (TF) by using microfluidics. Initiation of coagulation of normal pooled plasma depended on shear rate, not volumetric flow rate or flow velocity, and coagulation initiated only at shear rates below a critical value. Initiation of coagulation of platelet-rich plasma and whole blood showed similar behavior. At constant shear rate, coagulation of plasma also showed a threshold response to the size of a patch of TF, consistent with our previous work in the absence of flow.

Conclusion—Initiation of coagulation of flowing blood displays a threshold response to shear rate and to the size of a surface patch of TF. Combined with the results of others, these results set the range of shear rates that limit initiation of coagulation by small surface areas of TF and by shear activation of platelets. This range fits the relatively narrow range of physiological shear rates described by Murray's law. (*Arterioscler Thromb Vasc Biol.* 2008;28:2035-2041)

Key Words: blood coagulation ■ blood flow ■ coagulation ■ vascular biology

This article analyzes initiation of coagulation of blood flowing over a patch presenting tissue factor (TF) in the regime where platelets are not activated by shear alone. TF is the primary stimulus for initiation of coagulation *in vivo*.¹⁻³ We demonstrate that initiation of coagulation displays a threshold response to shear rate and to the size of the patch of TF, and we suggest a biophysical mechanism that explains these threshold phenomena. While low blood flow and endothelial damage are known risk factors for coagulation,⁴ the precise effects of blood flow on the initiation of coagulation are not fully characterized, possibly because of the difficulty in experimentally decoupling the effects of volumetric flow rate, flow velocity, and shear rate.

The effects of flow and shear rate on the activation of coagulation factors have been studied previously,⁵⁻¹² but whether high or low shear rates promote initiation of coagulation remains unclear. Intuitively, high blood flow and shear rates may promote coagulation by increasing the rate of delivery of coagulation proenzymes to sites of vascular damage. Indeed, high shear rates have been shown to increase the generation of factor Xa from surfaces coated with the TF and factor VIIa complex (TF-VIIa).¹¹ Moreover, high shear rates increase the potential for shear activation of platelets.^{13,14} On the other hand, numeric simulations have pre-

dicted that low blood flow could promote initiation by reducing the removal of activated coagulation factors from surfaces of TF-VIIa,¹⁵ and, clinically, stasis and low blood flow are considered risk factors for deep vein thrombosis (DVT).¹⁶ Low shear rates have also been found to increase fibrin deposition and appearance of fibrin monomers, indicating that low shear rates may promote initiation of coagulation.^{17,18} Similarly, chemical models^{19,20} have also predicted that low shear rates promote the initiation of coagulation attributable to reduced transport of activated coagulation factors away from the surface stimulus. We have previously observed a similar phenomenon for propagation of a clot from one channel to another in the presence of flow.^{21,22}

In addition to the fundamental question of whether high or low shear rates promote initiation of coagulation, the question of whether initiation of coagulation can be predicted by either flow rate or flow velocity remains. It is known that shear rate is a better parameter than either flow rate or flow velocity to consider when analyzing coagulation in the presence of flow, because transport phenomena near surfaces are governed by shear rate and not by flow rate or flow velocity.²³ Shear rate describes the change of flow velocity with increasing distance from the surface and is usually applied to explain transport phenomena near the surface.^{24,25} While *in vivo* experiments

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can be used to observe clotting at sites of vascular damage,^{26,27} a quantitative description of the relationship between initiation of coagulation and shear rate at patches of vascular damage would be difficult to obtain in vivo because of the difficulty of precisely controlling the dimensions of patches of vascular damage, volumetric flow rate, flow velocity, and shear rate. Here, we used microfluidics to decouple the effects of volumetric flow rate, flow velocity, and shear rate to independently determine the effects of each parameter on initiation of coagulation on patches of TF in vitro.

Methods

Methods given below are brief summaries of experimental procedures. Sources of materials and detailed methods for all procedures are given in supplementary information (<http://atvb.ahajournals.org>).

Patterning Inert Silica Capillaries With Patches of TF

Detailed procedures for forming layers of phospholipids containing TF on glass surfaces and for patterning phospholipid patches have been previously described by others.^{6,28} A monolayer of neutral phospholipid, 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC), was formed in silanized silica capillaries. Portions of this lipid and silane surface were selectively removed by deep-UV photolithography. Then, phospholipid vesicles containing TF and anionic phosphatidylserine were flowed through the capillary to form a bilayer only in the irradiated region.

Preparing Blood Samples (Whole Blood, PRP, NPP)

Whole blood, platelet rich plasma (PRP), and normal pooled plasma (NPP) were prepared as described in supplemental information. All blood and plasma samples were incubated with CTI (100 $\mu\text{g}/\text{mL}$) to inhibit the factor XII pathway of initiation of coagulation.²⁹

Measuring Clot Time of Human Blood and Plasma on Patches of TF

Blood or plasma was flowed into the patterned capillary at 37°C. To monitor coagulation, fluorescence microscopy was used to detect the generation of thrombin via cleavage of a fluorogenic substrate for thrombin, Boc-Asp(OBzl)-Pro-Arg-MCA,³⁰ and to detect the accumulation of fibrin labeled with the fluorescent tag Alexa 488. Also, brightfield microscopy was used to detect the formation of the fibrin mesh and aggregation of platelets.

Results

Initiation of Coagulation Displayed Threshold Responses to Shear Rate and the Size of a Patch of TF

We designed a capillary-based microfluidic system to monitor the coagulation of blood flowing at different shear rates over patches of TF in capillaries with a circular cross section (Figure 1). This technique of patterning TF has been well characterized for planar surfaces without flow.^{20,31} In capillaries of this shape, the flow velocity and shear rate are easily controlled and uniform across the patch. Blood or plasma was flowed into a microcapillary patterned with a patch of TF (red) in an inert background (gray), and initiation of coagulation on the patch resulted in cleavage of a thrombin-sensitive fluorogenic substrate, manifested by a substantial increase in blue fluorescence (Figure 1A). In this article, we considered initiation of coagulation to be the time point when a burst of thrombin was generated and the initial formation of

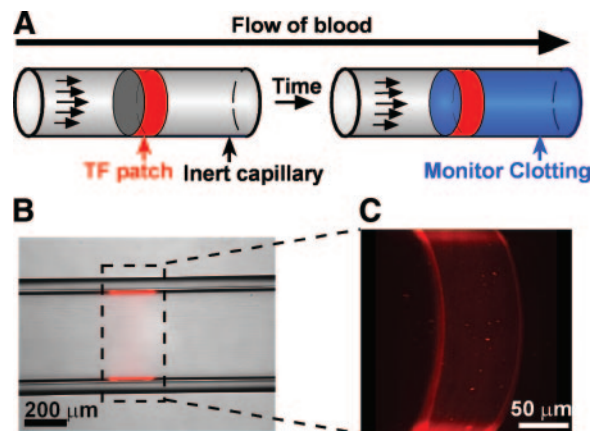


Figure 1. Microfluidic device used to monitor initiation of coagulation on patches of TF. A, Schematic of the device. B, A photograph shows a microcapillary patterned with a patch of TF (red) in a lipid bilayer. C, A 3D reconstructed confocal image shows a portion of the patch.

cross-linked fibrin appeared. Previous control experiments demonstrated that the presence of TF is required for the procoagulant activity of patterned lipid patches.^{20,31}

Initiation of coagulation of NPP on patches of TF of a given size demonstrated a threshold response to shear rate. The time required for coagulation of NPP to initiate (“clot time”) on a 200 μm patch of TF was monitored and recorded for various shear rates. Initiation of coagulation did not occur when NPP was flowed over a 200 μm patch of TF at a high shear rate of 40 s^{-1} (Figure 2A), but initiation did occur at a low shear rate of 5 s^{-1} (Figure 2B). The fluorescence images on the left in panels A and B show the intensity of blue fluorescence at 30 and 300 seconds of blood flow. The intensities of the blue fluorescence, measured by using a linescan along the green dashed line drawn on the fluorescence images on the left, are shown in the graphs on the right in panels A and B. The threshold shear rate in these experiments was approximately 25 s^{-1} (Figure 2C).

Previous results demonstrated that a patch of TF must be above a critical size to initiate coagulation and that this phenomenon occurs over a wide range of conditions in the absence of flow.³¹ To test whether this phenomenon is also observed in the presence of flow, the clot time of NPP flowing at a constant shear rate of 40 s^{-1} was measured on patches of TF of various sizes. These results show that clotting in the presence of flow also displays a threshold response to the size of a patch of TF (Figure 2D). The threshold patch size in this experiment was approximately 400 μm . We observed a large variance in the clot times on patches near this threshold size, which is expected for threshold behavior.

Proposed Mechanism for the Threshold Response of Initiation of Coagulation to Shear Rate

The threshold response of initiation of coagulation to both shear rate and the size of a patch of TF can be understood by considering a physical mechanism based on the competition between reaction and transport of activated coagulation factors. This mechanism may be understood by using

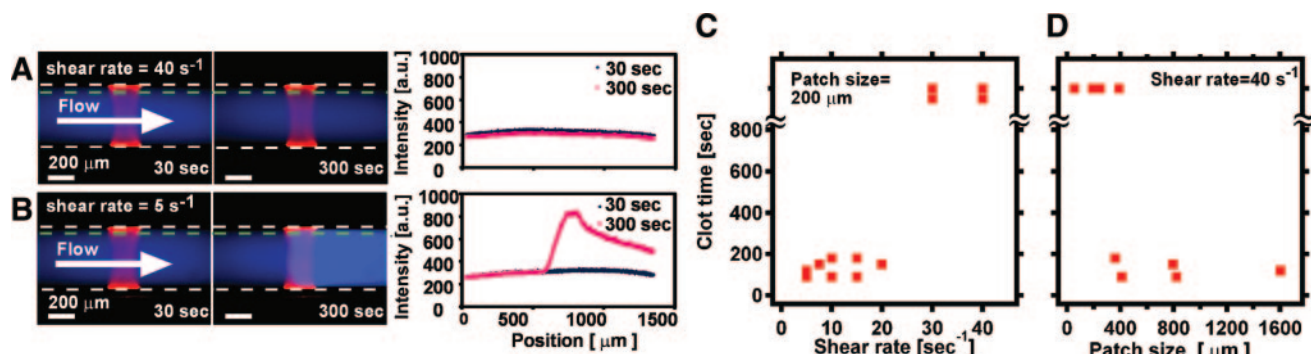


Figure 2. Initiation of coagulation of NPP displayed a threshold response to shear rate and the size of a patch of TF. A and B, Coagulation, as seen by the intensity of the blue fluorescence, did not initiate at a shear rate of 40 s⁻¹, but did initiate at a shear rate of 5 s⁻¹. C and D, NPP displayed a threshold response to shear rate at constant patch size (C) and to patch size at constant shear rate (D).

simple models such as those provided by modular mechanisms.^{20–22,31,32} In these models, initiation of coagulation is determined by the competition between reactions that produce activated factors at the patch and transport phenomena that remove activated factors from the patch. Initiation occurs only when the concentration of activated factors exceeds a critical concentration (Figure 3). In the context of this article, this mechanism assumes that experiments are conducted at a high value of the Peclet number, *Pe*, which describes the relative importance of transport by convective flow versus transport by diffusion. *Pe* is defined as $Pe = U \times R / D$, where *U* is the mean velocity (m/s), *R* is the radius of the capillary (m), and *D* is the diffusion coefficient for activated factors (m²/s). In this article, *Pe* was always greater than 1000. In vivo, *Pe* is

usually greater than 1000 in vessels larger than capillaries, as calculated from reported values of shear rate and vessel diameter.³³ However, *Pe* can be much lower in capillaries.³⁴ In addition, because the diameter of red blood cells is comparable to the diameter of capillaries, flow in capillaries is not laminar; rather it is dominated by recirculation typical of segmented flows.³⁵ In these cases, the mechanism described in Figure 3 has to be modified to take these effects into account. At high shear rates, flow dilutes the activated factors by “stretching” and diluting the plume of activated factors produced at the patch (Figure 3A). Conversely, at low shear rates, activated factors accumulate at the patch, exceed the critical concentration, and initiate coagulation (Figure 3B). Previous results have also demonstrated that higher TF surface density will cause the threshold patch size to decrease.³¹ Thus, we anticipate that higher TF surface density will also lead to a higher threshold shear rate. Here, we propose that this mechanism is applicable during the initiation phase of coagulation when activated factors are readily transported to and from the surface in solution. However, this mechanism may be less applicable during the propagation stage of coagulation when reactions are also occurring in the solid-phase, such as in the fibrin mesh, where transport is impeded.

With large *Pe* and high surface reactivity, transport of the activated coagulation factors from the surface is described by the classical Leveque problem.^{24,25} The mass transfer of activated coagulation factors is described by the following equation, $c \times v^{-1} \times (R/D)^{-1} \sim [z/(R \times Pe)]^{1/3}$, where *Pe* = $U \times R / D$ and $U \sim \gamma \times R$. In equations, *c* (mol/m³) is the concentration of activated coagulation factors near the surface at the downstream end of the patch, *v* is the reaction rate on the surface (mol/m² · s), *z* is the patch length (m), and γ is the shear rate on the surface (s⁻¹). We derived this equation by using scaling arguments, and it is equivalent to the equations given previously for concentration of product formed from surface-bound enzymes at various shear rates.^{33,36,37} This equation disagrees with the Equation 3 in reference 8, but it appears that a minor typographic error was introduced into that equation (units of “flow” should be flow velocity, not volumetric flow rate). Therefore, when *D* and *v* are held constant as follows, $c \sim v(z/\gamma)^{1/3}/D^{2/3}$, the ratio of the patch length, *z*, and shear rate, γ , should describe whether the

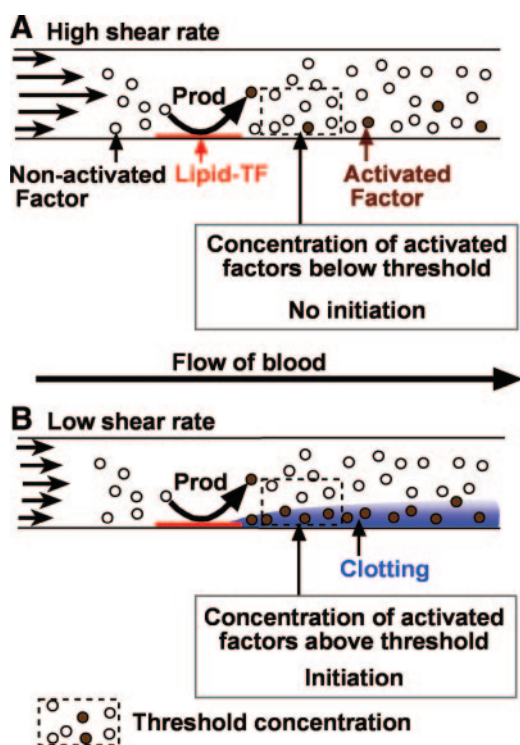


Figure 3. Schematic illustration of the proposed mechanism. A, At high shear rates, the concentration of activated factors cannot exceed the critical (threshold) concentration. B, At low shear rates, activated factors can accumulate, exceed the critical (threshold) concentration, and initiate coagulation (blue).

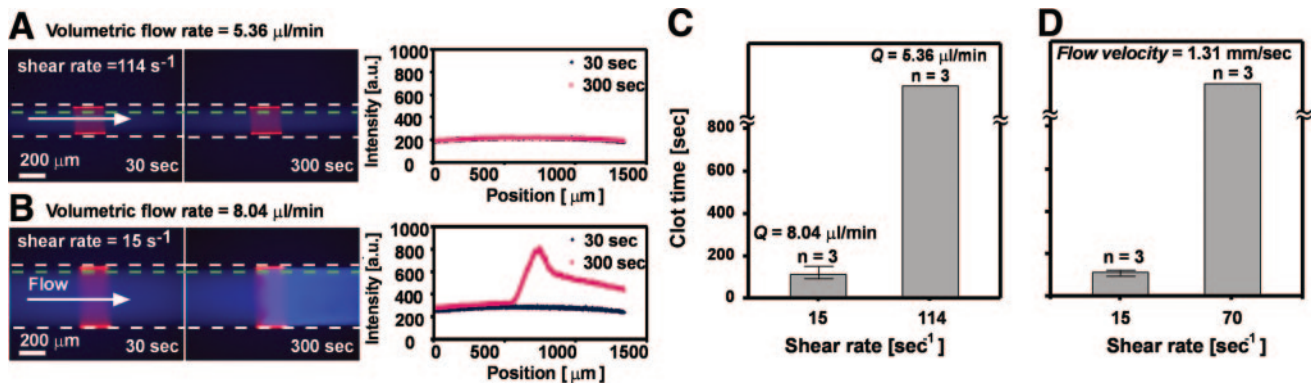


Figure 4. Initiation of coagulation of NPP depends on shear rate, not volumetric flow rate, Q , or flow velocity. A–C, Shear rate regulates initiation of coagulation, not volumetric flow rate. D, With constant flow velocity, initiation occurred only at a low shear rate. Error bars represent the range.

concentration of activated factors reaches the threshold. Although the data presented here are consistent with this prediction, additional experiments would be needed to test this prediction quantitatively. Nevertheless, it provides a guideline for thinking about these phenomena.

This simplified mechanism also assumes that the enzymatic production of activated factors at the surface of the patch is constant and does not depend strongly on the rate of delivery of substrate, such as when TF-VIIa on the patch is saturated with factor X. However, if this complex is not saturated, high shear rates may increase the rate of production of factor Xa by supplying factor X more rapidly. Also, the concentration of tissue factor pathway inhibitor (TFPI) bound to TF-VIIa is assumed to remain constant. However, an increase in the rate of inhibition of TF-VIIa with an increase in shear rate would also contribute to reduced coagulation at high shear rates. While detailed analysis of these effects is outside of the scope of this article, we expect that realistic numeric simulations^{38–40} may be used to quantify these effects.

An alternative mechanistic explanation of the decrease in coagulation observed at high shear rates may be damage to the patch of TF by the high shear force generated under these conditions. However, two control experiments ruled out this alternative mechanism by showing that the surface containing TF remained active after exposure to high shear force (see supplemental information).

Initiation of Coagulation Depended on Shear Rate, Not Volumetric Flow Rate or Flow Velocity

Capillaries with different inner diameters (I.D.) were used to independently control and analyze the effects of different

parameters—volumetric flow rate, flow velocity, and shear rate—on initiation of coagulation. At a given volumetric flow rate, shear rate was increased by reducing the diameter of the capillary. In a capillary with an inner diameter of 200 μm , initiation of coagulation did not occur when NPP was flowed over a 200 μm patch of TF at a low flow rate of 5.36 $\mu\text{L}/\text{min}$, which generated a high shear rate of 114 s^{-1} (Figure 4A and 4C; Table). These results indicate that even at a low flow rate, a high shear rate prevented initiation of coagulation by removing activated coagulation factors from the patch. In a capillary with an inner diameter of 450 μm , initiation of coagulation of NPP occurred even at a higher flow rate of 8.04 $\mu\text{L}/\text{min}$, which generated a low shear rate of 15 s^{-1} (Figure 4B and 4C; Table). These results indicate that even at a high flow rate, activated factors were not removed from the patch by shear rate fast enough to prevent the accumulation of the critical concentration of activated factors and initiation of coagulation. The plots to the right in Figure 4A and 4B show the intensity of blue fluorescence (thrombin) of a linescan along the green dashed line drawn on the fluorescence images on the left.

In addition to flow rate, we also tested the effect of flow velocity on initiation of coagulation. When the flow velocity was constant and the shear rate was varied by changing the diameter of the microcapillaries, initiation of coagulation depended on shear rate. With a constant flow velocity of 1.31 mm/s, coagulation did not initiate in a capillary with an inner diameter of 150 μm (high shear rate of 70 s^{-1}), but coagulation did initiate in a capillary with an inner diameter of 700 μm (low shear rate of 15 s^{-1}) (Figure 4D; supplemental Figure III; Table).

Table. Parameter Values in Experiments on Initiation of Coagulation in NPP, PRP, and Whole Blood

Case	Flow Rate ($\mu\text{L}/\text{min}$)	Flow Rate (pL/sec)	Capillary I.D. (μm)	Flow Velocity (mm/sec)	Shear Rate (sec^{-1})
Fig 4A and 4C	5.36	89	200	2.85	114
Fig 4B and 4C	8.04	134	450	0.843	15
Fig 4D	1.39	23	150	1.31	70
Fig 4D	30.3	505	700	1.31	15
Fig 5B	10.72	179	450	1.12	20
Fig 5A	42.88	715	450	4.50	80

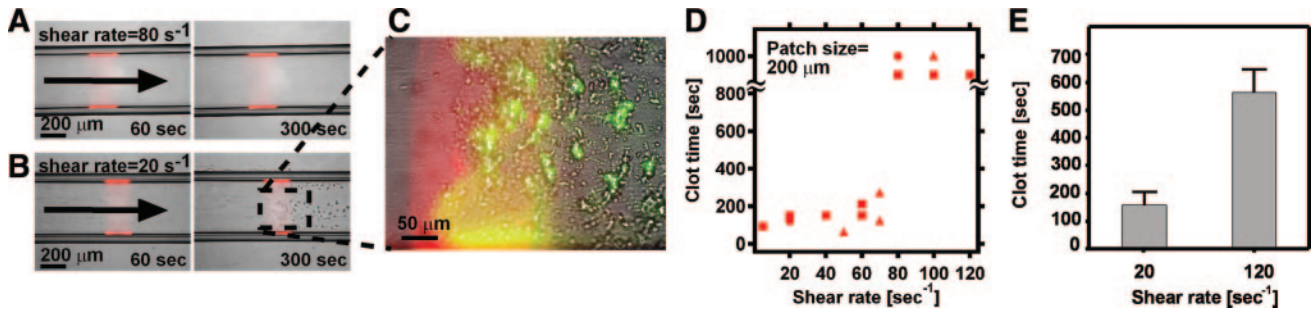


Figure 5. Initiation of PRP and whole blood is regulated by shear rate. A and B, Coagulation of PRP did not initiate at high shear rates, but did at low shear rates. C, A photograph shows the correlation between platelet aggregation (brightfield), a patch of TF (red), and fibrin mesh (green). D, PRP displayed a threshold to shear rate at constant patch size, shape of data points corresponds to 3 donors. E, Whole blood initiated faster at low shear rate than at a high shear rate. Error bars represent standard error ($n=4$, $P<0.01$).

Initiation of Coagulation Displayed a Threshold Response to Shear Rate in the Presence of Platelets

The above experiments were performed in the absence of platelets. However, platelets are known to play a major role in the kinetics of coagulation.⁴¹ Therefore, we tested whether a threshold response to shear rate existed in the presence of platelets. Experimentally, we measured the clot time of whole human blood and platelet rich plasma (PRP) on a 200 μm patch of TF at various shear rates.

Initiation of coagulation of PRP did not occur when PRP was flowed over a 200 μm patch of TF at a high shear rate of 80 s^{-1} (Figure 5A; Table), but initiation of coagulation occurred at a low shear rate of 20 s^{-1} (Figure 5B; Table). At this low shear rate, platelets aggregated, stuck to the walls of the capillary, and a fibrin mesh formed (Figure 5B). Black solid dots ($\approx 20 \mu\text{m}$) are aggregated platelets (Figure 5B). In a separate control experiment, Alexa Fluor 488 labeled fibrinogen (Figure 5C, green) was used to visualize fibrin formation. Fluorescence from localized fibrin correlated with aggregated platelets and with blue fluorescence from thrombin (not shown). Initiation of coagulation of PRP also displayed a threshold response to shear rate on 200 μm patches of TF (Figure 5D). Although platelets are known to be activated at high shear ($> 3000 \text{ s}^{-1}$),¹³ the shear rate in our experiments was not high enough to activate platelets. In a second control experiment, PRP was flowed over the TF patch at a high shear rate of 80 s^{-1} and no fibrin formation or platelet aggregation of PRP was observed at the patch or 3 mm downstream from the patch (see supplemental Information). The clot time of whole blood was significantly longer at a high shear rate of 120 s^{-1} than at a low shear rate of 20 s^{-1} (Figure 5E). However, this threshold response was difficult to quantify, because whole blood had short spontaneous clot times²⁹ of 8 to 10 minutes in our system. These experiments have been performed with both sodium citrate and CTI, as has been previous done in a number of studies.^{5,42–44} Others have pointed out that different kinetics of coagulation may be obtained when only CTI was used as anticoagulant.⁴⁵ To confirm that our results with whole blood are not dependent on the method of anticoagulation, we performed additional control experiments using whole blood without addition of sodium citrate, which still demonstrated a similar response to shear rate (see supplemental information).

Discussion

These experiments provide insight into the regulation of initiation of blood coagulation by shear rate, volumetric flow rate, and flow velocity in vitro. They also establish a threshold response of initiation of coagulation to shear rate and to the size of a patch of TF, but not to volumetric flow rate or to flow velocity. These results suggest a biophysical mechanism that explains these threshold responses based on reaction and transport of activated coagulation factors. These results emphasize the importance of shear rate, not flow rate or flow velocity, in the prevention of unwanted coagulation as well as the applicability of microfluidics to understanding the spatial dynamics of hemostasis. In addition to regulating initiation of coagulation, previous studies indicate that shear rate is also important in regulating propagation of blood coagulation.^{21,22} A growing clot will stop propagating when it encounters a shear rate above a threshold value, but a clot will continued propagating in the presence of a shear rate below the threshold.

Blood flow is considered one of the important parameters that contributes to venous thrombosis according to the classic Virchow's triad.^{4,46} Although various anticoagulants contribute to the prevention of venous thromboembolism,² increased blood flow rate and velocity are usually interpreted as possible mechanisms by which compression stockings prevent DVT.^{16,47} However, we hypothesize that shear rate may be a better predictor of initiation of coagulation than volumetric flow rate or flow velocity, because a considerable amount of coagulation is initiated on or near vessel walls, and the flow velocity approaches zero on the vessel walls. In this respect, large veins may be more risky because, for a given flow rate or flow velocity, their shear rates are disproportionately low. In addition, the lower surface-to-volume ratio in large veins and the reduced effectiveness of surface-bound coagulation inhibitors⁴⁸ must also be considered. Our analysis (shear rates were calculated here from the vein diameter and flow rate values measured by Jamieson et al⁴⁷) of published data⁴⁷ shows that the shear rate in the common femoral vein is increased by $\approx 50\%$ by gradient compression stockings, a result that is consistent with this hypothesis. Although shear rate can be estimated by dividing the velocity of blood flow by the diameter of a blood vessel, assuming a circular, noncompressible tube,³³ the actual shear rate in blood vessels in vivo may be more difficult to calculate because of

variations in vessel geometry, vessel compressibility, the non-Newtonian nature of blood, and variations in flow such as turbulent and pulsatile flow and recirculating flow in capillaries. New methods for accurately measuring the shear rate in vessels in vivo could be useful for predicting the likelihood of initiation of coagulation and for testing this hypothesis.

These results also point out an intriguing coincidence. According to Murray's law, although flow rates vary widely throughout the vasculature ($\approx 10^{-5}$ – 10^2 mL/s), shear rates remain in a narrower range ($\approx 10^2$ to 10^3 s $^{-1}$) to minimize work and achieve efficient oxygen transport.⁴⁹ This physiological range of shear rates is within the range that limits coagulation: it is below the $\approx 10^3$ to 10^4 s $^{-1}$ limit that causes direct activation of platelets by shear¹³ and above the $\approx 10^1$ to 10^2 s $^{-1}$ limit set by this work for coagulation on a patch of TF of a few hundred microns. Physiological shear rates are also in the range that suppresses propagation of clotting from one vessel to another.^{21,22} Did the coagulation cascade evolve to respond by clotting to deviations above or below the physiologically normal range of shear rates? Or was the evolution of vasculature affected, in part, by the requirement to maintain shear in a range that suppresses both initiation and propagation of undesirable coagulation? Further work will be required to answer these fascinating questions.

The in vitro experimental setup described here provides control of the shear rate and the size and location of the patch of TF, low variability between experiments, and the ability to monitor the formation of clots at the patch of TF in real time. The disadvantages of this system include the absence of endothelial cells and the vascular microenvironment, which influences local TF activity.⁵⁰ Shear stress is another parameter that needs to be considered in this context. In the presence of endothelial cells, shear stress regulates initiation of coagulation by alternating the TF gene expression.⁵¹ Shear stress may also contribute to reducing enzymatic activity, by mechanisms such as conformational changes in the TF complex, at sites of vascular damage and on patches in our experiments. Another limitation in the experimental setup was absence of flow rate dynamics, such as pulsatile flow. Pulsatile flow should affect whether the threshold concentration of activated factors is reached. These considerations must be addressed by in vivo experiments before the physiological significance of the threshold phenomena is clinically applicable to control thrombosis and hemostasis.

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Disclosures

None.

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Supplemental Information for

Threshold Response of Initiation of Blood Coagulation by Tissue Factor in Patterned Microfluidic Capillaries is Controlled by Shear Rate

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Methods

Materials

All solvents and inorganic chemicals used in buffers were purchased from commercial sources and used as received unless otherwise stated. Fibrinogen labeled with Alexa Fluor 488 and Texas Red®, 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) were purchased from Molecular Probes/Invitrogen (Eugene, OR). 1,2-Dilauroyl-sn-glycero-3-phosphocholine (DLPC) and L- α -phosphatidylserine from porcine brain (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). T-butylloxycarbonyl- β -benzyl-L-aspartyl-L-prolyl-L-arginine-4-methyl-coumaryl-7-amide (Boc-Asp(OBzl)-Pro-Arg-MCA) was purchased from Peptides International (Louisville, KY). Human recombinant tissue factor (TF) was purchased from Calbiochem/EMB Biosciences (La Jolla, CA). Corn trypsin inhibitor (CTI) was purchased from Haematologic Technologies (Essex Junction, VT). Silica capillaries (inner diameter, I.D., of 150 μ m, 200 μ m, 450 μ m, and 700 μ m) were purchased from Polymicro Technologies (Phoenix, AZ). Teflon tubing (I.D. of 305 μ m) was purchased from Weico Wire & Cable, Inc. (Edgewood, NY). Albumin from bovine serum (BSA), anhydrous hexadecane, and butyltrichlorosilane (BTS) were purchased from Sigma Aldrich (St. Louis, MO). Sodium Chloride and anhydrous methyl sulfoxide (DMSO, 99.7% purity) were purchased from Fisher Scientific (Pittsburgh, PA). Human normal, platelet poor, pooled plasma (NPP) was purchased from George King Bio-Medical, Inc. (Overland Park, KS). Human whole blood was obtained from individual healthy donors

in accordance with the guidelines set by the Institutional Review Board (protocol # 12502A) at the University of Chicago.

Generating patches of tissue factor (TF) surrounded by an inert phospholipid monolayer

Lipid vesicles were prepared as previously described.¹ Detailed procedures for forming layers of phospholipids containing TF on glass surfaces and for patterning phospholipids patches have been previously described by others.^{2,3} Our procedure is briefly described here. A monolayer of inert neutral lipids of DLPC was initially formed in silanized silica capillaries. Then, this monolayer and silane were selectively removed by deep-UV photopatterning, and a bilayer of clot-inducing lipids was formed in the irradiated region. Silica capillaries were first made hydrophilic by flowing a solution of “piranha” (3:1 sulfuric acid:hydrogen peroxide, WARNING: piranha reacts violently with organic substances, including all human tissues) for 20 min, followed by rinsing with deionized water (18.2 MΩ.cm) and drying with nitrogen gas. A solution of BTS (4 μL) in anhydrous hexadecane (1 mL) was flowed through the capillary at a flow rate of 0.01 mL min⁻¹ for 45 min. Additional hexadecane was flowed through the capillary to remove excess BTS. The silanized capillary was then rinsed with ethanol (2 mL) and dried with nitrogen gas. A monolayer of DLPC was formed on the silanized surface by filling the capillary with a solution containing DLPC vesicles (1.25 mg mL⁻¹ vesicles in phosphate buffered saline (PBS, pH = 7.4)) and incubating at room temperature for 40 min. Excess vesicles were removed by rinsing with a 150 mM NaCl solution (saline). These monolayers were photopatterned within 24 hr.

A photomask patterned with rectangular windows was placed over the capillary substrate and irradiated with deep UV light.^{1,3} The patterned capillary was backfilled with vesicles containing 79.5 mol % of DLPC, 20 mol % of PS, 0.5 mol % of Texas Red DHPE, and TF. The concentration of TF in the vesicle solution was 0.4 nM¹ in all the experiments except two: 1) the control experiments comparing the volumetric flow rate, flow velocity, and shear rate (as in Figure 4), in which the concentration of TF was 1.6 nM and 2) the control experiments on whole blood without sodium citrate, in which the concentration of

TF was 0.8 nM. The capillary was incubated at room temperature for 2 min and then extensively rinsed with saline at a shear rate of 2000 s^{-1} to remove excess lipid vesicles.

Preparing blood samples (whole blood, PRP, NPP)

Whole blood, PRP, and NPP were prepared as previously described.⁴ Whole blood obtained from individual healthy donors was collected in Vacutainer® tubes (Franklin Lakes, NJ) containing 3.2 % sodium citrate (9:1 by volume). PRP with approximately 3×10^5 platelets μL^{-1} was obtained by centrifuging whole blood at 300 g for 10 min.⁵ Human normal, platelet poor, pooled plasma (NPP) was purchased from George King Bio-Medical, Inc. (Overland Park, KS). According to the manufacturer, platelet counts in NPP samples were less than 10,000 per μL before freezing. Samples were stored at -80 °C and thawed immediately before the experiments. All blood and plasma were incubated with CTI ($100 \mu\text{g mL}^{-1}$) to inhibit the Factor XII pathway of initiation of coagulation.⁶ CTI was added to NPP during the thawing of the frozen plasma. CTI was added to whole blood approximately 30 minutes after collection, although adding CTI during the collection may help extend the background clot time even further in this experimental setup.⁷

For the control experiment on whole blood without sodium citrate, blood was drawn into 10mL BD syringes. The first 10mL blood was discarded, and CTI was added during collection to reach a final concentration of $100 \mu\text{g mL}^{-1}$.

Measuring clot time of human blood and plasma on patches of TF

Citrated NPP and PRP (600 μL) were recalcified by adding 200 μL of a solution of CaCl_2 (40 mM), NaCl (90 mM), and a thrombin sensitive fluorescent substrate, Boc-Asp(OBzl)-Pro-Arg-MCA (0.4 mM).¹ Citrated whole blood (500 μL) was recalcified by first adding Boc-Asp(OBzl)-Pro-Arg-MCA (6.75 μL , 10 mM in DMSO) and then a solution of CaCl_2 (31.2 μL , 200 mM).⁸ The recalcified blood or plasma sample was placed into a 1 mL plastic syringe blocked with BSA and connected to the capillary with Teflon tubing. The patterned capillary was kept under NaCl solution (150 mM) in a Petri dish in a 37 °C incubator. The blood or plasma was flowed into the capillary at a rate of $1.39 \mu\text{L min}^{-1}$ to

64.32 $\mu\text{L min}^{-1}$ by using a syringe pump (Harvard PHD 2000, Harvard Apparatus, Holliston, MA). For fully developed laminar flow through a circular capillary, the shear rate ($\dot{\gamma}$) can be calculated from the volumetric flow rate (Q) and the radius of the capillaries (R) as $\dot{\gamma} = 4 \times Q / (\pi \times R^3)$.⁹ Thrombin generation was monitored by fluorescence microscopy to detect the cleavage of Boc-Asp(OBzl)-Pro-Arg-MCA.¹⁰ The formation of fibrin and the aggregation of platelets were monitored by brightfield microscopy.

Monitoring fibrin formation with Alexa 488 labeled fibrinogen

Fibrinogen labeled with Alexa 488 was added to 1mL PRP to a concentration of 100 $\mu\text{g/mL}$, and the sample was incubated at room temperature for 30 min.¹¹ This PRP sample was flowed into a microcapillary (I.D. = 450 μm) at a shear rate of 20 s^{-1} . After aggregation of platelets occurred, excess fibrinogen was rinsed away with saline, leaving the crosslinked fibrin mesh. Fluorescence microscopy was used to detect the presence of fibrinogen/fibrin labeled with Alexa 488.

Image acquisition and preparation

Fluorescence and brightfield images of fibrin labeled with Alexa 488 were acquired at 37 $^{\circ}\text{C}$ by using a digital camera (C4742, Hamamatsu Photonics, Japan) mounted to a Leica DMI6000 B epi-fluorescence microscope (Leica Microsystems GmbH, Germany) with a 20 \times 0.7 NA objective. The confocal image of the bilayer patch was acquired by a digital camera (C9100, Hamamatsu Photonics, Japan) mounted on a Visitech VT Infinity 3 multi-point confocal system (VisiTech International Ltd., United Kingdom) attached to Leica DMI 6000 B microscope with a 20 \times 0.7 NA objective at 24 $^{\circ}\text{C}$. All the other fluorescence and brightfield images were acquired by using a digital camera (C4742, above) mounted to a Leica DMI 6000 B epi-fluorescence microscope with a 10 \times 0.4 objective at 37 $^{\circ}\text{C}$.

Image analysis was performed as previously described.¹ The original grayscale fluorescence images were collected and false-colored by using MetaMorph[®] software (Molecular Devices Corporation, Downingtown, PA). For each figure, the levels of all

wavelengths were adjusted to the same values. Images were overlaid by using Adobe Photoshop software. The confocal images were collected and false-colored by using SimplePCI® software (Hamamatsu Corporation, Sewickley, PA), and the brightness and contrast were adjusted to be better visualized in grayscale by using Adobe Photoshop software.

Supplemental experiments

At an above-threshold shear rate, coagulation does not initiate downstream of TF patches.

In experiments where coagulation did not initiate on the patch of tissue factor (TF), we wished to confirm that coagulation did not initiate downstream of the patch. We also monitored coagulation downstream of the patches in all experiments. As an example, we performed the following control experiment. Platelet rich plasma (PRP) was flowed into a microcapillary (I.D. = 450 μm) over a 200 μm patch of tissue factor (TF) at a shear rate of 80 s^{-1} and flow velocity of 4.5 mm/s at 37 °C. Details and procedures are described in the experimental section. Fibrin formation and platelet aggregation were monitored both at the patch and 3 mm downstream from the patch by using brightfield microscopy. No fibrin formation or platelet aggregation was observed on the patch which is in agreement with the results as in Figure 5A of the main text. At shear rate of 40 s^{-1} and flow velocity of 2.25 mm/s, clotting initiated no further than 100 μm downstream from the patch. If the sole role of increased shear rate and flow rate were to move the site of initiation of coagulation further downstream, then this initiation would have occurred within 200 μm of the patch or closer. These results agree with the previous study on effects of shear in propagation of clotting,¹² where clotting downstream from a channel was not observed.

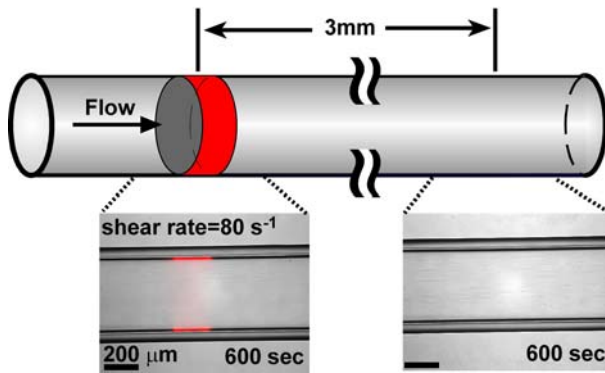


Figure S1. Coagulation of PRP did not initiate 3 mm downstream of the patch of TF at a high shear rate of 80 s^{-1} . PRP was flowed into a microcapillary patterned with a patch (red) of TF in an inert background (gray). Brightfield images show that coagulation of PRP did not initiate at the patch or downstream of the patch within 600 sec (10 min).

Control experiments for shear force

High shear force could wash TF away from a patch, preventing the initiation of coagulation. However, two control experiments ruled out this alternative mechanism. In one experiment the capillary was extensively rinsed with a saline solution at a shear rate of 2000 s^{-1} , which generated a shear force of approximately 40 times higher than NPP at a shear rate of 40 s^{-1} .¹³ When NPP was flowed into this capillary at a shear rate of 5 s^{-1} , coagulation initiated on a $200 \mu\text{m}$ patch of TF in approximately 210 sec. In a second control experiment, NPP was flowed over a $200 \mu\text{m}$ patch of TF at an above-threshold shear rate of 40 s^{-1} for 300 sec, and then the shear rate was reduced to a below-threshold shear rate of 5 s^{-1} . Coagulation initiated on the patch in 100 sec, indicating that the TF stimulus was still present and active.

Control experiment of whole blood without sodium citrate

Whole blood with CTI ($100 \mu\text{g} / \text{mL}$) was flowed into the device within 6 min after blood was collected from a donor. No sodium citrate was added to the blood sample. When whole blood was flowed over a $200 \mu\text{m}$ patch of TF, the clot time of whole blood was

noticeably longer at a high shear rate of 120 s^{-1} than at a low shear rate of 20 s^{-1} (Figure S2).

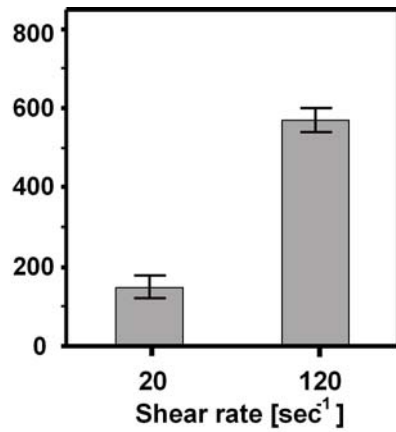


Figure S2. Whole blood without sodium citrate initiated clotting faster at a low shear rate than at a high shear rate. Error bars represent maximum and minimum ($n = 2$).

Supplemental Figure

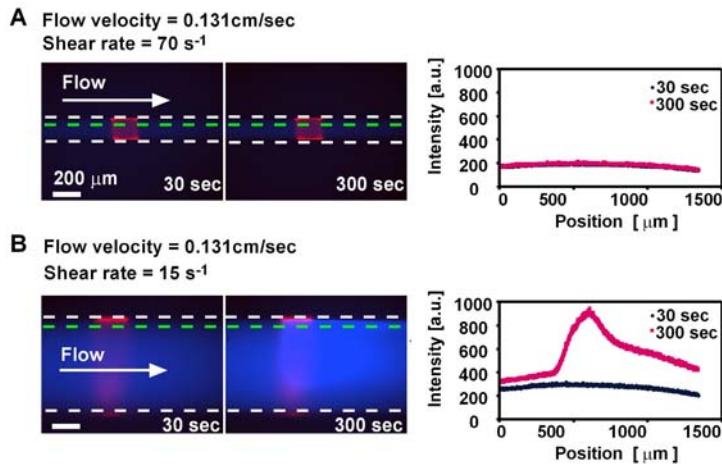


Figure S3. Initiation of coagulation of blood plasma depends on shear rate, not flow velocity. (A-B) Blue and red fluorescence is explained in the caption of Figure 1. Time-lapse, fluorescence images demonstrate that with same flow velocity, coagulation of NPP did not initiate in a capillary with an inner diameter of 150 μm at a high shear rate of 70 s⁻¹ (A), but coagulation initiated in a capillary with an inner diameter of 700 μm at a low shear rate of 15 s⁻¹ (B). The plots to the right in panels A and B show blue fluorescence intensity (thrombin) of a linescan along the green dashed line drawn in the fluorescence images on the left. The image of the capillary with an inner diameter of 700 μm gave rise to increased background fluorescence in panel B due to increased out-of-focus background fluorescence of the uncleaved fluorescent substrate in this thick capillary.

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