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Blood clots are rapidly assembled hemodynamic sensors: Flow arrest triggers intraluminal thrombus contraction

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Abstract

Objective—Blood clots form under flow during intravascular thrombosis or vessel leakage. Prevailing hemodynamics influence thrombus structure and may regulate contraction processes. A microfluidic device capable of flowing human blood over a side channel plugged with collagen ($\pm$ tissue factor) was used to measure thrombus permeability (κ) and contraction at controlled transthrombus pressure drops.

Methods and Results—The collagen ($\kappa_{\text{collagen}} = 1.98 \times 10^{-11} \text{ cm}^2$) supported formation of a 20- μm thick platelet layer, which unexpectedly underwent massive platelet retraction upon flow arrest. This contraction resulted in a 5.34-fold increase in permeability due to collagen restructuring. Without stopping flow, platelet deposits (no fibrin) had a permeability of $\kappa_{\text{platelet}} = 5.45 \times 10^{-14} \text{ cm}^2$ and platelet-fibrin thrombi had $\kappa_{\text{thrombus}} = 2.71 \times 10^{-14} \text{ cm}^2$ for $\Delta P = 20.7$ to 23.4 mm-Hg, the first ever measurements for clots formed under arterial flow (1130 s^{-1} wall shear rate). Platelet sensing of flow cessation triggered a 4.6 to 6.5-fold ($n=3$, $P<0.05$) increase in contraction rate, which was also observed in a rigid, impermeable parallel-plate microfluidic device. This triggered contraction was blocked by the myosin IIA inhibitor blebbistatin and by inhibitors of thromboxane (TXA_2) and ADP signaling. In addition, flow arrest triggered platelet intracellular calcium mobilization, which was blocked by TXA_2/ADP inhibitors. As clots become occlusive or vessels rupture, flow around developed clots diminishes facilitating full platelet retraction and hemostasis.

Conclusion—Flow dilution of ADP and thromboxane regulates platelet contractility with prevailing hemodynamics, a newly defined flow sensing mechanism to regulate clot function.

Keywords

thromboxane; ADP; thrombosis; hemodynamics; coagulation

INTRODUCTION

During thrombosis or hemostasis under flow conditions, platelets rapidly deposit at a site of vascular injury. The vessel wall and subendothelium quickly become connected mechanically to the developing thrombus.¹ During vessel wound closure, this interwoven

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DISCLOSURES

None.
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assembly prevents further blood loss by platelet-mediated clot contraction and stiffening.² Platelets generate contractile forces to allow clots to match the stiffness of the endothelium.³ Interactions between myosin II and actin filaments govern this contraction and are regulated by the activation of myosin light chain kinase through calcium/calmodulin and/or Rho kinase signaling.^{4,5} During contraction, force transmission ultimately occurs via talin and $\alpha_{IIb}\beta_3$, which binds platelets via fibrinogen and fibrin.^{6,7} Following clot retraction, the tight seal that is formed around the injured tissue significantly reduces clot permeability, consequently limiting the leakage of cells and plasma. Also, the permeability of occlusive clots is critical to thrombolytic therapy for acute myocardial infarction since permeation dictates penetration of plasminogen activators.⁸ The effects of local hemodynamics on clot contraction and permeability are poorly understood, yet highly relevant to thrombus growth, stability, or susceptibility to embolism, fibrinolysis, or bleeding.

Prior studies of clot permeability have used whole blood clots that do not achieve the 50 to 100-fold increase in platelet concentration on a surface that occurs under flow conditions.^{9,10} While clot contraction has been studied for decades using clots formed in test tubes, there exists a large gap in the fundamental understanding of mechanisms that initiate and control the response under hemodynamic conditions. In terms of force-loading of thrombotic structures, platelets respond with larger stall forces when exposed to stiffer fibrinogen-coated atomic force microscopy (AFM) cantilevers.³ Studies of whole clot contraction forces may not necessarily predict clot contraction dynamics under flow conditions.

We measured, for the first time, clot contractility and permeability under hemodynamic conditions. The presence of tissue factor caused a significant decrease in thrombus permeability. Unexpectedly, flow arrest caused enhancement of permeability for platelet deposits due to a triggered clot contraction and consequent collagen restructuring. To further examine platelet contraction after flow cessation, a rigid wall flow device was used. Platelet deposits were developed and antagonized using blebbistatin, a myosin II inhibitor and ADP/thromboxane receptor antagonists. Flow arrest caused an intracellular increase in Ca^{2+} that preceded contraction and was dependent on the autocrine signaling of ADP and TXA_2 . These studies provide new insight into the ability of platelets to sense local hemodynamic flow based on the convective-diffusive transport of autocrine signaling molecules.

MATERIALS AND METHODS

Reagents

The following reagents and instrumentation were obtained and stored according to manufacturers' instructions: polydimethylsiloxane (PDMS) (Ellsworth Adhesives); sigmacote, streptavidin, sulforhodamine 101 acid chloride (Texas Red), fluorescein isothiocyanate (FITC), and 2-MeSAMP (Sigma-Aldrich); human type-1 monomeric collagen (VitroCol, 3 mg/ml) (Advanced Biomatrix); equine tendon-derived type-1 fibrillar collagen (Chrono-log); biotinylated goat anti-collagen type I polyclonal antibody (Abcam); 0.05 μ m Fluoresbrite® microspheres (Polysciences Inc.); fluo4-NW (Life Technologies); blebbistatin (EMD Millipore); SQ 29,548 (Cayman Chemical); MRS-2179 (Tocris Bioscience); anti-fibrin antibody (gift from the M. Poncz, Children's Hospital of Philadelphia); L- α -phosphatidylserine (PS), L- α -phosphatidylcholine (PC), and biotinylated phosphatidylethanolamine (bPE) (Avanti Polar Lipids); analog pressure transducers (Honeywell Sensing and Control).

Blood Collection

All donors were reported as medication free for the previous 10 days and blood collection was in accordance with the University of Pennsylvania's IRB. Human blood from healthy volunteers was collected into 100 μ M Phe-Pro-Arg-chloromethylketone (PPACK, Haematologic Technologies Inc.) or 40 μ g/ml corn trypsin inhibitor (CTI, Haematologic Technologies Inc.). PPACK-treated whole blood was also treated with fluorescently conjugated anti-CD41 monoclonal antibody purchased (1 μ g/ml; Abd Serotec). PE-conjugated anti-CD61 (0.125 μ g/ml; Becton Dickinson Biosciences) and fluorescently conjugated anti-fibrin antibodies (0.5 μ g/ml) were added to CTI-treated blood as previously described.^{11,12}

Permeation device design and manufacture

PDMS was used to construct microfluidic devices following soft lithography protocols¹³ as previously described.^{14,15,16} The primary blood flow channel (250 μ m wide $\times$ 60 μ m high) was designed to create flow over a micropost scaffold region as illustrated in Fig. 1A. At this micropost scaffold junction, blood could continue along the primary channel or exit through the scaffold channel (50 μ m wide $\times$ 60 μ m high) to an outlet port maintained at P3 = atmospheric pressure or blocked so that P3 equals the pressure within the blood flow channel. Real-time pressure measurements were collected in LabVIEW (National Instruments) using 0-1 psig pressure transducers upstream (P2), downstream (P1) and exiting the scaffold region (P3) as shown in Fig. 1. Channel pressure was controlled using two constant volume syringe pumps (Harvard Apparatus). A syringe pump located upstream of the collagen scaffold delivered anti-coagulated whole blood at an independently controlled initial inlet wall shear rate (1130 s⁻¹), while a downstream pump perfused Ca²⁺ buffer (5 mM) at a rate set by a proportionate controller programmed in LabVIEW.

Microfluidic device for thrombus permeation

PDMS devices were sealed to Sigmacote-treated glass slides using vacuum-assisted bonding. Type I human monomeric collagen solution was polymerized at 2.4 mg/ml overnight using 8 parts collagen, 1 part 0.09 M NaOH, and 1 part 10 $\times$ PBS. Prior to loading the scaffold with collagen, all channels were incubated with 10% BSA for 30 min at room temperature. Following incubation, well mixed polymerized collagen solution was pipette on the upstream and downstream pressure ports and localized into the micropost scaffold region by pulling the solution (2.5 μ L) through the scaffold exit channel with syringe withdraw for $\sim$ 15 s. A fixed amount of collagen ($\sim$ 3.3 ng) was thus deposited on the micropost scaffold. Collagen solution remaining in the channel region (between P2 and P1) was removed by infusion of Ca²⁺ buffer (5 mM) from the blood inlet port prior to instillation of blood for the experiment. For experiments using collagen with linked tissue factor (TF), biotinylated and TF liposomes (20:79:1, PS/PC/bPE) were prepared as previously described^{15,16} following the method of Smith et al.¹⁷ Polymerized collagen (2.4 mg/ml) was mixed in a 10:1 ratio by volume with biotinylated anti-collagen (4 μ g/ml) and incubated at room temperature for 5 minutes. Streptavidin (10 μ g/ml) at a 1:10 volumetric ratio and TF liposomes at a 1:20 volumetric ratio with collagen were sequentially added and incubated for 5 min and 10 min, respectively. The fibrillar collagen/TF solution was then perfused through the micropost array region as described for fibrillar collagen.

Permeability Measurements

Whole blood was anti-coagulated with PPACK for perfusion over collagen or with CTI for perfusion over collagen/TF. Each whole blood perfusion was conducted at an inlet wall shear rate of 1130 s⁻¹ for 10 min. The transthrbus pressure drop was immediately set to the controlled value and side view images of the platelet or platelet/fibrin thrombus were

taken at the blood contact region of the collagen scaffold. Following thrombus development, an injection valve (Idex Health & Science) was manually switched to pulse Ca^{2+} buffer (5 mM) containing Texas Red or FITC dye ($\sim 25 \mu\text{L}$) without disruption of the flow. Real-time dye, platelet, and fibrin fluorescent intensities were imaged with an inverted microscope (IX81, Olympus America Inc.) using a CCD camera (ORCA-ER, Hamamatsu). Confocal images of platelet/fibrin thrombus were taken in $2\text{-}\mu\text{m}$ sections, under flow, with a disk-scanning unit (IX2, Olympus America). ImageJ software was used to analyze all images and develop 3-dimensional representations of thrombus.

Contraction Measurements

PPACK-treated whole blood with or without the addition of 50 nm fluorescent microspheres (10^{10} beads/ml blood) was perfused through the permeation device at 1130 s^{-1} . Platelet deposits were formed for 10 min under a constant transthrombus pressure drop (23.5 mm Hg). Whole blood flow was then switched without interruption to Ca^{2+} buffer (5 mM) flow. Following 4.5 min of buffer flow, both syringe pumps were stopped and the transthrombus pressure drop immediately approached zero. Platelet deposits were imaged in 15 sec intervals over 30 min.

Thrombus contraction studies were also performed in a parallel channel microfluidic device.¹⁴ Briefly, a microfluidic device was used to print a $250 \mu\text{m}$ wide strip of diluted fibrillar collagen lengthwise on a Sigmacote treated glass slide. The patterning device was removed and second device was positioned with ten parallel channels ($250 \mu\text{m}$ wide $\times$ $60 \mu\text{m}$ high) perpendicular to the collagen. All channels were pre-incubated with 0.5% BSA. Anti-coagulated whole blood (PPACK) was treated with or without an intracellular Ca^{2+} dye. Fluo4-NW (2.5 mM probenecid) was loaded into platelets by incubating 1 part dye with 4 parts blood for 45 min. Dye treated or untreated blood was then placed in the inlets of three channels. A syringe pumped allowed simultaneous perfusion of the three channels at an initial wall shear rate of 1160 s^{-1} . After the collagen patches ($250 \times 250 \mu\text{m}$) were covered with platelets, flow was immediately switched to Ca^{2+} buffer (5 mM, 0.01% DMSO) in the presence or absence of antagonist (blebbistatin or mixtures of SQ-29,548, MRS-2179, 2-MeSAMP). In some experiments, buffer flow was stopped (30-sec or 1-min) and then reestablished or completely stopped following 7 min of perfusion. Intracellular Ca^{2+} fluorescence and platelet deposit structures were imaged with a $20\times$ objective, in 15 sec intervals, for the duration of the experiment.

Finite Element Analysis

COMSOL Multiphysics software was used to numerically solve steady state pressure gradients, blood flow velocities and permeation velocities over complex geometries (collagen plus thrombus) with constant permeability in the PDMS permeation device. Blood ($\rho=1060 \text{ kg/m}^3$, $\mu=0.003 \text{ Pa}\cdot\text{s}$) and buffer ($\rho=1000 \text{ kg/m}^3$, $\mu=0.001 \text{ Pa}\cdot\text{s}$) were both modeled using laminar flow for solution of the Navier-Stokes equation ($\text{Re}=0.49$, $\text{Re}_{\text{element}}=0.25$). The collagen region was modeled using Darcy's law ($\nabla^2 P=0$) with entrance and exit pressures being coupled to the external laminar flow properties and permeability (κ) set to a previously determined value (eg. $\kappa = 1 \times 10^{-15} \text{ m}^2$).¹⁸ Flow rates of $13 \mu\text{L/min}$ for blood and buffer allowed the downstream resistance length to be modified to achieve $\Delta P=23.5 \text{ mm Hg}$ at the collagen scaffold interface.

Collagen, platelet, and platelet/fibrin permeability were each solved in COMSOL for the complex geometries of the collagen scaffold and developed thrombus. Calculating and comparing the permeability of collagen to previously reported values provided experimental validation of the device.^{18,19} Experimental pressure and calculated average velocity data were used as input parameters. The square difference between the experimental and

computational average velocity across the collagen scaffold were iteratively reduced ($<0.001 \mu\text{m}^2/\text{s}^2$) by varying the collagen permeability. With the solved collagen permeability, the process was repeated for the platelet or platelet/fibrin geometry on the collagen. The normalized dye concentration in the channel was used as transient input data into the model. Matching the transient pulse concentration at the collagen output with the experimental output validated the computational model. As expected, calculated values of κ were not dependent on ΔP .

Statistics

Two-tailed Student's *t*-tests were used to calculate all *P* values. Statistically significant differences were reported if $P < 0.05$.

RESULTS

Microfluidic device for measuring clot permeability and contractility

A microfluidic device was designed to allow pressure-driven transthrumbus permeation with simultaneous imaging of clot contractile dynamics under flow. The device has a blood and buffer inlet port, collagen scaffold, and three ports for pressure readings (P1, P2, P3) up to 50 mm-Hg (Fig. 1A and Fig. S1). The downstream buffer and upstream whole blood flows merge into a narrow channel to create resistance and control the lumen pressure at the collagen site corresponding to $(P2+P1)/2$. The pressure readings in these locations allowed the pressure drop across the collagen to be controlled, thus allowing computer simulation (Fig. 1B) of Darcy flow through the complex geometry assuming constant permeability of the collagen (Fig. 1C). Exposing a $250 \mu\text{m}$ long $\times$ $60 \mu\text{m}$ wide surface area of collagen ($\pm$ linked lipidated tissue factor) to whole blood flow provided a localized thrombotic site comparable to a previous device without permeation.¹⁴ The side view of collagen made it possible to image the permeation of dyes and the morphology of fibrin and platelet deposits as they contracted (Fig. 1D and E).

Stopping flow caused platelet and clot retraction

During permeability testing of platelet deposits (no thrombin), the interruption of blood flow resulted in a drastic contractile response of the newly formed clot (Fig. 2 and Video S1). This retraction unexpectedly enhanced permeability due to the reconfiguration of the supporting collagen and opening of flow paths along the sides of the collagen (Fig. 3). To investigate the contraction triggered by flow cessation, platelet deposits were formed for 10 min with embedded 50 nm fluorescent beads as fiduciary markers (inlet wall shear rate of 1130 s^{-1}). A wall shear rate of 1160 s^{-1} was used to reproduce rates found in capillaries where the pressure drops between the vessel lumen and interstitial space are known.^{20,21} This shear rate is also in the range of arterial flows. Following the thrombus formation for 10 min, the flow was switched without interruption to buffer for 4.5 min and then flow was completely stopped. Switching the flow from blood to buffer prevented the arrival of new platelets and release of fresh ADP or TXA_2 .^{22,23} Thrombus structure was mapped before and after stopping the flow (Fig. 2A). The upstream and downstream edge contraction rates were measured throughout the buffer flow period and after flow stoppage (Fig. 2B). Contraction rates 1 to 2 min after flow cessation significantly increased by 6.5-fold (upstream region) and 4.6-fold (downstream region) ($P < 0.05$), compared to the rate during buffer flow. Donor to donor variability ($n=3$ donors) for total clot contraction, 7 minutes after flow arrest, was $8.93 \pm 3.89 \mu\text{m}$ and $13.6 \pm 4.42 \mu\text{m}$ at the upstream and downstream positions respectively. The restructuring of the thrombus upstream and downstream edges by 2 min post flow cessation resulted in contractile trajectories of embedded beads towards the center of the thrombus mass (Fig. 2C and Supplemental Fig. S2C). Comparisons of the time dependent contraction data in the Y and Z direction at upstream and downstream

positions of the clot demonstrated increased contraction rates after flow cessation (Fig. 2D). Movement in the +Z-direction occurred rapidly and simultaneously in the upstream, downstream and middle positions due to an immediate rebound effect caused by a reduced pressure drop and reduced permeation when the flow was stopped (Fig. S2). Total bead distance in the Y and Z direction shows the rates at which the clot contracts in all three locations (Fig. S2). The ~1 min delay in contraction in the Y direction is one indicator of an active signaling mechanism that must be engaged following the cessation in flow.

Permeability of collagen, platelet deposits, and platelet-fibrin thrombus without flow interruption

To measure permeability without triggered thrombus contraction, dye tracer was pulsed immediately following whole blood flow without interruption of the flow. Inlet and outlet concentrations were measured with time to determine the permeation velocity at several physiologic pressure drops.^{20,21} Permeability was numerically calculated over the complex geometry (Fig. 3 A and B) by reducing the squared error between the experimental and simulation permeation velocity. We validated this approach by comparing our calculated collagen permeability ($\kappa_{\text{collagen}} = 1.98 \times 10^{-11} \pm 0.640 \times 10^{-11} \text{ cm}^2$) with previous literature values (Fig. 3C).^{18,19} Similarly, permeation velocity was measured across platelet deposits and platelet-fibrin thrombi formed on collagen or TF/collagen scaffolds, respectively. Simulations that accurately predicted the experimentally measured output of dye concentration over the course of these experiments allowed determination of the permeability of the thrombus (Fig. 3 A and B). The resulting permeabilities for platelet deposits ($\kappa_{\text{platelet}} = 5.45 \times 10^{-14} \pm 0.898 \times 10^{-14} \text{ cm}^2$) and platelet-fibrin thrombus ($\kappa_{\text{thrombus}} = 2.71 \times 10^{-14} \pm 0.377 \times 10^{-14} \text{ cm}^2$) formed after 10 min of flow quantify the resistance that each structure provides to resist bleeding. In the absence of thrombin, PPACK-treated whole blood formed a platelet mass that was >350-fold less permeable than collagen alone. The presence of thrombin and formation of fibrin provided a further 50% reduction in permeability under hemodynamic conditions. Comparing the measured platelet deposit permeability (without flow interruption) to that obtained following flow interruption ($\kappa_{\text{interrupted}} = 2.91 \times 10^{-13} \pm 0.745 \times 10^{-13} \text{ cm}^2$) demonstrated the impact that stopping the flow had on the ability of the platelet deposit to maintain hemostasis in our device. In addition to permeability measurements, we observed decreased platelet accumulation with increased pressure drop (13.8 vs. 23.4 mm Hg) across the intraluminal thrombus in PPACK-treated whole blood (Fig. S3), likely due to increased transthrumbus permeation of ADP and TXA₂ into the collagen.

Clot retraction in a rigid wall flow device with flow reduction or cessation

Clot retraction was also examined in a rigid, impermeable parallel-plate microfluidic device lacking transthrumbus permeation. Clot development in PPACK-treated whole blood was imaged on a 250 μm x 250 μm area of glass-supported fibrillar collagen at an initial wall shear rate of 1160 s^{-1} . Flow cessation resulted in a ~10 μm contraction (upstream region) towards the center of the thrombus as outlined in Fig. 4A. In comparison, by switching to 10 μM blebbistatin perfusion (without flow disruption) for 7 min before the flow cessation, clot retraction after flow cessation was reduced 90% to only ~1 μm (Fig. 4B). Blebbistatin is a well established platelet myosin IIA ATPase inhibitor.²⁴ In a scenario where flow was reduced from arterial (2000 s^{-1}) to venous (100 s^{-1}) shear rates, total contraction was similar to that observed with flow cessation. Also, the contraction was ~5 μm larger than stopping the flow following blebbistatin treatment (Figure S4). This result demonstrates the requirement of platelet myosin IIA activity in the triggered thrombus contraction following flow reduction or cessation.

We also measured clot retraction while stopping and restarting flow after a 30-sec or 1-min interruption (Fig. 4C). Statistical differences between complete cessation of blood or buffer and the 30-sec flow interruption became apparent after 1 min. Interrupting flow for 1 min took 5 min to diverge statistically from complete flow cessation in buffer. While neither of the temporary flow interruptions (30-sec or 1-min) were statistically different from each other, the longer 1-min delay provided enough time to engage the contraction mechanism before eventually being diminished by the return of flow. The technique of switching to a non-physiologic buffer and measuring contraction was validated by showing no contractile differences between stopping the flow in blood or stopping buffer flow after 7 min of rinsing.

Since flow cessation results in a dramatic change in both wall shear stress and wall shear rate on the thrombus structure, we investigated the role of soluble autocrine mediators whose concentration may change when flow is stopped. To explore the role of TXA₂, we added 1 μ M SQ-29,548, a potent TXA₂ receptor antagonist.²⁵ Additionally, the contribution of ADP was investigated by adding 10 μ M MRS-2179 and 50 μ M 2-MeSAMP, selective inhibitors of the P2Y₁ and P2Y₁₂ platelet receptors, respectively.²⁶ The dose response curves for these inhibitors have been well established and the final concentrations used under flow exceeded the experimentally determined IC₅₀ values to ensure complete inhibition.^{25,26} After 7 min of buffer/antagonist perfusion, flow was stopped and total contraction was measured with time (Fig. 4D). Both antagonists significantly reduced the total clot contraction as compared to buffer. ADP antagonists had the largest effect, reducing the total contraction nearly 75% over 7 min, whereas TXA₂ antagonist reduced the contraction by 44%. While uninhibited contraction can vary significantly between donors (~32-44%), the addition of antagonist results in consistent donor to donor percent reductions that vary <7% for ADP and <3% for TXA₂. These findings demonstrate ADP (from dense granules) and TXA₂ from activated cyclooxygenase-1 (COX1) were the mediators of the triggered contraction response upon flow cessation.

Intracellular calcium mobilization is triggered by ADP and TXA₂ after flow cessation

To explore the role of ADP and TXA₂ in the flow sensing by the thrombus, we loaded platelets in PPACK-treated whole blood with Ca²⁺ sensitive dye fluo-4. Prior to flow cessation, we treated the platelet deposits with buffer in the presence and absence of a triple cocktail of 1 μ M SQ-29,548, 10 μ M MRS-2179, and 50 μ M 2-MeSAMP. To minimize the measured intracellular Ca²⁺ fluctuations prior to flow cessation, initial contraction measurements were made 3 min after treatment and 3 min before flow arrest. The calcium signal increased as the platelet mass accumulated on the collagen between 0 and 4 min. When flow was switched to buffer at 4 min, there was no further deposition of platelets resulting in a slight decrease in signal. In the absence of inhibitors, flow cessation at 10 min caused an immediate and substantial mobilization of intracellular calcium (Fig. 5A) that was completely blocked by the triple inhibitor cocktail to antagonize ADP and TXA₂ (Fig. 5B). The calcium mobilization occurred within seconds following flow cessation and preceded the maximum platelet contraction rate which began 1 to 2 min after flow stoppage (Fig. S5).

The triggered contraction by the platelet mass, following flow cessation, is an active material response requiring myosin (Fig. 4C) and represents a flow sensing mechanism to impede contraction when flow is present. ADP and TXA₂ are the soluble mediators responsible for this response (Fig. 4D and Fig. 5B). Both of these agonists are highly sensitive to convective dilution which controls their concentrations in the boundary layer around the thrombus.²⁷ Since soluble species mediated Ca²⁺ mobilization and contraction upon flow cessation, the flow sensing involved a transport mechanism: flow dilutes soluble species and flow cessation allows a rapid accumulation of autocrine ADP and TXA₂ to trigger G_q signaling via platelet P2Y₁, P2Y₁₂, and TP receptors (Fig. 5C).

DISCUSSION

We report a novel hemodynamic sensing function of intraluminal blood clots. Flow impedes clot retraction. When flow stops, intraluminal clots retract more rapidly and to a greater extent. This hemodynamic sensing by platelets in thrombus involves a rapid mobilization of calcium that strictly requires released ADP and TXA₂. Myosin activity is also required for the triggered contraction upon flow cessation, indicating that the contraction is not a passive material response to reduced drag forces. This concept of a triggered, active response is also consistent with the ~1 min delay between flow cessation and enhanced contraction.

While platelet contraction of a clot is usually considered in an isotropic context where blood is clotted in a tube and then detached from the glass walls to allow contraction, intraluminal thrombus formation is fundamentally different. In an isotropic assay, platelets have random orientation within a fibrin network whereas platelets depositing under flow conditions spread on collagen and then form many more platelet-platelet interactions due to high platelet concentration in the thrombus. In the thrombus formed under flow, the structure is less isotropic and spread platelets would be expected to exert greater forces along their axis of spreading and lesser forces perpendicular to the plane of spreading. Furthermore, clotted whole blood in a tube contains incompressible red blood cells at the prevailing hematocrit, while thrombus formed under arterial flow are greatly enriched in platelets (50-100X platelet rich plasma levels) and substantially depleted of RBC.

Despite recent advances in microfluidic devices and intravital mouse microscopy, few tools are available to control and study the effects of thrombus permeation under flow conditions. Recent studies examining angiogenesis demonstrated microfluidic devices that hold promise for studying *in vitro* clot permeability under flow conditions.^{19,28} However, these designs lacked the potential to produce controllable pressure drops and shear rates relevant to thrombosis and hemostasis. In the present study, we designed a microfluidic device to develop whole blood clots under physiologic flow and to investigate transthrombus permeation in the presence of a controlled pressure drop. The microfluidic device allowed the first reported *in vitro* clot permeability for clots formed under flow. While a measurement for the permeability of a contracted clot could not be obtained due to the accompanied structural changes in the absence of endothelium, it is expected to be less than that of a non-retracted platelet-fibrin deposit. Our measurement for these deposits represents a quantitatively important upper bound of the contracted clot permeability and the associated inner clot transport of ADP and TXA₂. Interestingly, the permeability of healthy rabbit aortic wall is on the order of 10⁻¹⁴ cm²,²⁹ which is quite similar to our measurement of a platelet-fibrin thrombus. This suggests that a platelet-rich intraluminal thrombus has a permeability that is well matched to the surrounding intact endothelium. In addition to matching rigidity,³ an intraluminal thrombus may match permeability to the surrounding vessel wall. Under flow conditions, we propose flow sensing helps the spread platelet(s) maintain hemostatic function by balancing the contractile apparatus with the applied flow to limit platelet contraction since contraction would potentially create gaps for leakage or alter nearby endothelial function.

In a quantitatively more intense example of hemostasis, a blunt impact that compresses a vessel without rupturing the vessel would be expected to cause more extensive endothelial denudation. This situation is perhaps most analogous to the experimental configuration developed in this study. When blood flow is maintained in such an injured vessel, the flow impedes clot contraction because wound closure would not be needed. Also occlusion might be prevented since clot stabilization via contraction is impeded by flow. Reduced ADP/TXA₂ transport may also facilitate the formation of a dense inner thrombus core,³⁰ while the outer domains of the clot remain loose and friable due to flow sensing. Throughout our

studies with multiple donors, this was repeatedly verified by the constant but relatively low contraction rate under flow (Fig. 2B and Fig. S6). As vessel injury becomes severe enough to cause vessel rupture with blood leaving the vascular space, blood pools around the puncture/rupture/severed site. This results in more isotropic clotting of whole blood, which can exert isotropic contraction on the surrounding tissue to facilitate wound closure and consequently hemostasis (Fig. S7). In this situation, the pooled blood around a leaking vessel is not subjected to substantial hemodynamic flow to dilute ADP/TXA₂ and thus impair platelet actinomyosin-mediated contraction. Additional highly diffusible platelet activators may also play a role in this observed contraction and provide an area of future study.

To further investigate the novel flow sensing abilities of platelets we examined their ability to contract following 30 seconds or 1 minute interruptions in flow. Contraction rates initially followed previous experiments but were drastically dampened upon the return of flow (Fig. S8). This result suggests that the quasi-steady state that platelet deposits reach under hemodynamic forces preserves their ability to rapidly contract in response to flow arrest. Flow sensing by a thrombus balances the need for wound closure and hemostasis against the risk of intraluminal occlusive thrombosis with dense contracted and lytic-resistant structures.³¹ In cases of normal hemostasis, this mechanism would allow clots to contract gradually from their interior towards their exterior as hemodynamic flow is reduced. The extent to which stress present in the thrombus plays a role in signaling (i.e. mechanotransduction) remains a subject of future study. These experiments clearly demonstrate the ability of platelet deposits to rapidly assemble into a hemodynamic sensor and contract upon the arrest of flow.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Microfluidic device to independently control blood flow and transthrbus pressure drop

COMSOL was used to determine the pressure throughout the microfluidic device (A) including the collagen scaffold region (B), where $L=250 \mu\text{m}$. Human type I polymerized collagen was localized in the scaffold region (C). Following 10 min of CTI-whole blood flow at 1130 s^{-1} , platelets (red), fibrin (green) and their overlap (yellow) form a thrombus on the collagen (D). A confocal image of platelets (red) and fibrin (green) shows the 3D structure the microfluidic device (E).

Figure 2. Flow arrest triggers clot contraction

A thrombus formed in the absence of thrombin and presence of fluorescent 50 nm beads was rinsed with Ca^{2+} buffer for 4.5 min before the cessation of flow caused a rapid contraction. The outline of a pre-retracted thrombus ($t' = -1$ min) shows the inward retraction of the thrombus following flow stoppage ($t' = 0$ to 2 min) (A). Contraction rate of the upstream and downstream sections of the thrombus were measured before and after flow arrest ($n = 3$ donors) (B). Trajectories of the 50 nm beads represent the contractile response of the thrombus at upstream (red, $n = 6$), middle (blue, $n = 3$), and downstream (green, $n = 6$) locations (C). Stopping the flow caused a significant increase in contraction rate in the Y and Z directions. To quantify these rates, the times before (0-2 min) and after flow cessation (0-1 min, 1-2 min) were monitored for bead velocity in the three sections of the thrombus (D). Downstream contraction rate in Y direction is shown as absolute value for contraction toward the middle region. *, $P < 0.01$; error bars indicate mean $\pm$ SD.

Figure 3. Clot permeability

Thrombi formed under $1130 s^{-1}$ were pulsed with fluorescent dye at controlled pressure drops. The normalized input and output fluorescent intensities, along with a numerically predicted output from COMSOL were measured over time for clots formed in the absence (A) and presence of TF (B). Thrombus formed without thrombin reduced the residence time of the permeated dye by 30 s compared to platelet/fibrin thrombus. COMSOL simulations were used to numerically calculate permeability over collagen (n=9), platelet (n=6, 5 donors), platelet/fibrin (n=4, 4 donors), and platelet/flow interrupted (n=2, 2 donors) geometries at constant pressure drops (C). †, $P < 0.01$; error bars indicate mean $\pm$ SD.

Figure 4. Contraction after flow arrest requires myosin and released ADP/TXA₂

A parallel channel microfluidic device was used to develop thrombus in PPACK, at 1160 s⁻¹. Following the formation of stable thrombi, Ca²⁺ or antagonist buffer was used to rinse the surface for 7 min before stopping flow. Upstream contractions were observed in five sections (blue dashed line) over ten min. Stopping flow without a buffer rinse (A) and with a 10 μM blebbistatin rinse (B) show clot retraction compared to a trace (pink line) before flow cessation. Total clot contraction measured over time compares the effects of blebbistatin and intermediate flow stopping with stopping flow with and without a buffer rinse (C). TXA₂ and ADP antagonist significantly reduced total contraction after flow cessation as compared

to buffer (D). n=3 events at 5 discrete points for each time-point indicated, using 5 separate donors; error bars indicate mean $\pm$ SD.

Figure 5. Flow cessation triggers platelet intracellular calcium mobilization via ADP/TXA₂ autocrine signaling

Platelets in PPACK whole blood were loaded with Ca^{2+} dye and perfused over collagen at 1160 s^{-1} . Following thrombus formation, whole blood perfusion was switched to Ca^{2+} or antagonist buffer for six minutes prior to stopping flow. Intracellular Ca^{2+} was measured via fluorescent intensity over time for either a Ca^{2+} buffer (A) or ADP and TXA₂ antagonist rinse (B). $n=3$ events using 2 donors, error bars indicate mean $\pm$ SD. A schematic illustrates the flow sensing ability of platelets via convective removal of ADP/TXA₂ (C). Shear (τ) acting on the exterior of aggregated platelets accompanied by the strain placed on interconnected $\alpha\text{IIb}\beta_3$ may signal the inhibition of contraction or dense granule (δ) release.