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Contact activation of blood coagulation on a defined kaolin/collagen surface in a microfluidic assay

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Abstract

Generation of active Factor XII (FXIIa) triggers blood clotting on artificial surfaces and may also enhance intravascular thrombosis. We developed a patterned kaolin (0 to 0.3 $\mu\text{g}/\mu\text{m}^2$)/type 1 collagen fibril surface for controlled microfluidic clotting assays. Perfusion of whole blood (treated only with a low level of 4 $\mu\text{g}/\text{mL}$ of the XIIa inhibitor, corn trypsin inhibitor) drove platelet deposition followed by fibrin formation. At venous wall shear rate (100 s^{-1}), kaolin accelerated onset of fibrin formation by ~ 100 sec when compared to collagen alone (250 sec vs. 350 sec), with little effect on platelet deposition. Even with kaolin present, arterial wall shear rate (1000 s^{-1}) delayed and suppressed fibrin formation compared to venous wall shear rate. A comparison of surfaces for extrinsic activation (tissue factor TF/collagen) versus contact activation (kaolin/collagen) that each generated equal platelet deposition at 100 s^{-1} revealed: (1) TF surfaces promoted much faster fibrin onset (at 100 sec) and more endpoint fibrin at 600 sec at either 100 s^{-1} or 1000 s^{-1} , and (2) kaolin and TF surfaces had a similar sensitivity for reduced fibrin deposition at 1000 s^{-1} (compared to fibrin formed at 100 s^{-1}) despite differing coagulation triggers. Anti-platelet drugs inhibiting P2Y_1 , P2Y_{12} , cyclooxygenase-1 or activating IP-receptor or guanylate cyclase reduced platelet and fibrin deposition on kaolin/collagen. Since FXIIa or FXIa inhibition may offer safe antithrombotic therapy, especially for biomaterial thrombosis, these defined collagen/kaolin surfaces may prove useful in drug screening tests or in clinical diagnostic assays of blood under flow conditions.

Keywords

contact pathway; kaolin; microfluidics

1. Introduction

Contact pathway can be strongly triggered by negatively charged surfaces such as glass, kaolin and celite (1). Zymogen factor XII (FXII) is activated to FXIIa upon contacting with

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anionic surfaces and leads to a multistep cascade, whereby thrombin (FIIa) forms as a potent platelet activator and trigger of fibrin polymerization (2, 3). The pathophysiology of contact pathway is not fully elucidated. While tissue factor triggered extrinsic pathway prompts major response to vascular injury, contact pathway likely has a minor role in hemostasis since factor XII deficiency is not associated with a bleeding defect. However, recent experiments revealed that FXII-mediated fibrin formation is essential for thrombus stability in a mice model (4–6). In contrast, FXI-deficient (hemophilia C) patients display little spontaneous bleeding but at elevated risk of bleeding post-injury or post-operative, especially at sites with high fibrinolysis (7). It has been suggested that pharmacological inhibitors of FXIIa or FXIa may be drugs useful for limiting thrombosis with reduced risk of bleeding side effects (8–10).

Many studies of contact pathway have been conducted for the purpose of investigating unfavorable thrombosis on blood-contacting medical devices. Most of these studies mainly focus on the activation mechanism of FXII. The approach often eliminates blood flow and cellular components, which then allows contact activation in static tubes with plasma (11–13). However, flow and cellular constituents are both present in human blood vessels, fundamentally altering reaction dynamics as compared to a cell-free system under static conditions. Flow based studies designed to intentionally trigger and measure contact pathway are less common. Glass capillary flow reactor has been used to study plasma coagulation via artificial surface activation (14). Kaolin-activated thromboelastography (TEG) has been applied as a predictive test for post-operative bleeding to assess clotting factors (i.e. rate, strength, and stability) under non-flow condition (15, 16). Typically, citrate is used as an anticoagulant which allows recalcification immediately prior to an experiment. However, FXIIa can be formed under calcium-free conditions and the resting time in citrate is often an uncontrolled variable. Similar to the older practice of using “light heparinization” of blood ex vivo to study platelet function in the presence of triggered thrombin production (17), we use “light CTI” as a versatile tool to study contact pathway in the context of clotting ex vivo. CTI only inhibits β FXIIa and does not inhibit α FXIIa. Even at high CTI (40–100 μ g/mL), unactivated human blood will clot ex vivo after a period of time of 40 to 100 minutes (18). This clotting time becomes even shorter when the following three conditions are imposed: first, CTI is lowered to very low levels such as 4 μ g/mL; second, a platelet stimuli is used (collagen); and third, a potent contact activator is used (kaolin). While no perfect container exists to hold blood ex vivo prior its perfusion over a thrombotic patch, the use of low CTI at 4 μ g/mL and use of the blood within 20 min of phlebotomy allows controlled and repeatable ex vivo study of the contact pathway at a precise location of interrogation.

Microfluidic devices allow the study of thrombotic events by perfusion of whole blood over well-defined prothrombotic surfaces (19–22). Microfluidics enables precise control of flow condition and real-time observation of thrombus structure. In this paper, we describe a prothrombotic surface composed of collagen and kaolin that is capable of activating blood coagulation via the contact pathway, independent of tissue factor (TF). This surface also serves as a biologically important substrate for anchoring activated platelets and polymerized fibrin. Engagement of contact pathway was evaluated by dynamic accumulation of localized platelets and fibrin on the collagen/kaolin surface. This

microfluidic assay allowed a controlled study of the sensitivity of contact pathway function to wall shear rate.

2. Materials and Methods

2.1 Fluorescent labeling of kaolin particles

For imaging of kaolin on collagen, fluorescent labeling of kaolin particles was carried out in a two-step reaction (23). Kaolin was mixed with 3-mercaptopropyl-trimethoxysilane in 80% methanol (50 mL methanol/g kaolin) in a 3:1 mass ratio. The mixture was stirred at room temperature for 6 hr to activate kaolin surface by converting surface hydroxyl groups to thiol groups, filtered, and washed 3 times with 80% methanol. The residue was collected and vacuum-dried for 12 hr. Powdered kaolin was then dried at 80 °C for 5 hr. Labeling solution was prepared by adding 5 mg fluorescein-5-maleimide into 120 mL phosphate buffered saline (PBS). Activated kaolin (125 mg) along with 50 mL ethanol was mixed with labeling solution for 1 hr. Kaolin was centrifuged (5000 g, 1 min) and supernatant was discarded. The pellet was resuspended in 1 mL PBS buffer. Centrifugation and re-suspension were repeated several times until supernatant was clear. Fluorescent kaolin pellet was vacuum-dried (12 hr) and stored to avoid light and moisture.

2.2 PS/PC liposomes

Liposomes were prepared according to a previous reported technique (24). L- α -phosphatidylcholine (PC) and L- α -phosphatidylserine (PS) (Avanti Polar Lipids, Alabaster, AL) were vacuum-dried in an 80:20 molar ratio. The dried film was resuspended in 1 mL HEPES buffered saline (HBS) at 2.3 mg-lipid/mL. A size extruder generated <100 nm diameter liposomes.

2.3 Thrombin biosensor on platelet surface

Soluble thrombin was detected under flow conditions using a platelet-linked thrombin biosensor (25). A total of 4 μ L of anti-human CD61 antibody (5 mg/mL, Biolegend, San Diego, CA) was mixed with 8 μ L of 900 μ M DBCO-sulfo-NHS ester (Click Chemistry Tools, Scottsdale, AZ) in 28 μ L of HBS buffer. The mixture was incubated at room temperature for 30 min. A volume of 2.5 μ L of Tris-HCl (1M, pH 8) was then added to quench the DBCO linking of anti-human CD61. Diluted peptide thrombin sensitive peptide (4 μ L of 4 mM) was added into the reaction to initiate labeling reaction and incubated in the dark at room temperature for 4 hr. The thrombin sensor was then gel filtrated with P6-Gel beads (hydrated in HBS buffer) yielding approximately 100 μ L of platelet binding thrombin sensor (5 μ g/mL).

2.4 PDMS patterning and flow devices

The microfluidic patterning device and the 8-channel microfluidic flow device were fabricated with poly(dimethylsiloxane) (PDMS, Ellsworth Adhesives, Germantown, WI) as previous described (22). The protein patterning device has a single channel (250 μ m in width, 60 μ m in height) and two outlets at both ends of the channel allowing protein infusion for coating. The flow device has 8 cylindrical reservoirs connecting to 8 evenly spaced

channels that merge to a single outlet. Both devices have a vacuum groove that allows them to be reversibly vacuum bonded onto glass slides.

2.5 Kaolin/collagen and TF/collagen surfaces

Glass slides were rinsed with ethanol for 15 sec followed by DI water for 30 sec and were dried with compressed filtered air. The patterning device was vacuum bonded onto a cleaned glass slide. A volume of 5 μL of acid insoluble collagen type I (Chronolog Corp, Havertown, PA) followed by 20 μL bovine serum albumin (0.5% BSA in HBS) perfusion through the channel forming an immobilized thin matrix of well aligned collagen fibrils. Kaolin suspension (50 mg/mL HBS) was centrifuged briefly (500 g, 15sec) to remove aggregates and supernatant was mixed with prepared PS/PC liposomes in a 3:1 volume ratio. Kaolin surface concentration can be varied via changing the centrifugation time: 5 sec centrifugation gives highly packed kaolin surface; 30 sec centrifugation gives sparse kaolin deposition while 15 sec centrifugation gives a medium density of localized kaolin on collagen fibrils. A volume of 10 μL of kaolin/lipids suspension or Dade Innovin recombinant human tissue factor (50% in HBS, VWR Corp, Radnor, PA) was pulled through the channel and allowed to settle over collagen for at least 30 min before rinsing with 10 μL BSA to remove excess kaolin, TF or lipids.

2.6 Characterization of kaolin/collagen surface

For calibration, fluorescent kaolin was suspended in HBS buffer to five concentrations (0, 10, 20, 30, 40 mg/mL) and allowed to completely fill the main channel of the patterning devices and to settle overnight at 65°C forming five dried fluorescent kaolin (without collagen) films with surface concentrations from 0–2.4 $\text{pg}/\mu\text{m}^2$. Fluorescent intensity was measured by imaging. A fluorescent intensity vs. mass curve was then constructed (Supplemental Figure 1). Four fluorescent kaolin/collagen surfaces with zero, low, medium and high amount of kaolin were made. Their surface mass concentrations were extrapolated from the mass vs. fluorescent intensity curve. Surface coverage of kaolin/collagen surface was calculated with thresholding tool in imageJ (NIH). A calibration curve was made by relating surface concentration to surface coverage.

2.7 Blood collection and preparation for microfluidic assay

Blood was collected via venipuncture from health donors (who were free of alcohol and medication for 72 hr prior to experiments) into corn trypsin inhibitor (CTI, 4 $\mu\text{g}/\text{mL}$ WB, Haematologic Technologies, Essex Junction, VT). All donors were consent under approval of University of Pennsylvania Institutional Review Board. First 5 mL of blood was discarded to avoid tissue factor contamination. Blood was treated with anti-human CD61 antibody (BD Biosciences, San Jose, California) for platelet detection and Alexa Fluor 488 fluorescent fibrinogen (Life Technologies, Grand Island, NY) for observation of fibrin generation. All experiments were initiated within 5 min after venous phlebotomy. For antithrombotic therapy tests, platelet thrombin biosensor was added into blood in 1:9 ratio for the measurement of thrombin level. Anti-human CD41a antibody and Fluor 647 fluorescent fibrinogen were added for platelet and fibrin detection, respectively.

2.8 Microfluidic model for contact activation

The 8-channel flow device was vacuum bonded to a glass slide with its flow channels mounted perpendicularly to the patterned kaolin/collagen surface, forming eight evenly spaced $250\mu\text{m}\times 250\mu\text{m}$ prothrombotic patches (Figure 1). Blood was perfused over prothrombotic surfaces under either venous (100 s^{-1}) or arterial wall shear rates (1000 s^{-1}) controlled by a syringe pump (Harvard Apparatus PHD 2000, Holliston, MA) (Figure 1). A custom stage held 3 flow devices allowing up to 24 conditions to be imaged simultaneously in single experiment. Platelet accumulation, fibrin generation, and thrombin formation were monitored by 3-color imaging with a fluorescence microscope (IX81, Olympus America Inc., Center Valley, PA) with specified time intervals. Images were captured with a CCD camera (Hamamatsu, Bridgewater, NJ) and were analyzed with ImageJ (NIH). All images were background subtracted. The center 65% of the prothrombotic region was selected to avoid edge effects and fluorescent intensities of the selected region were recorded for analysis.

2.9 Constant flow mode and pressure relief mode

By changing the inlet condition of each well of the 8-channel device, thrombus could form under either constant flow mode or pressure relief mode (26). As a clot approaches channel occlusion, shear rates on the thrombus surface become very large under constant flow mode. Given the power of the syringe pump, a 60-micron thick clot can never block the channel in the constant flow rate mode. The pressure relief mode approaches a constant pressure drop driven flow allowing an occlusive clot to stop flow and divert flow to a relief channel.

Constant flow mode: CTI treated blood was perfused in all eight channels and inlet wall shear rate was maintained in all channels before full channel occlusion. *Pressure relief mode:* EDTA (8 mmol/L, ethylenediaminetetraacetic acid) treated blood was fed into every other channel to ablate platelet deposition and clotting. The matched EDTA channel allows clot formation to proceed at essentially constant pressure drop in the matched, active assay channel (no EDTA present).

2.10 Detection of thrombin activity with time in a tube clotting assay (no flow)

Citrated (1:9 WB) and CTI-treated ($4\mu\text{g/mL}$) whole blood was diluted 1:4 HBS buffer and recalcified to 10 mM final calcium concentration in 384-well plate ($65\mu\text{L/well}$) right before experiment. A thrombin specific fluorogenic substrate Boc-Asp(OBzl)-Pro-Arg-AMC ($10\mu\text{mol/L}$, peptide international) was added to detect thrombin generation in terms of fluorescence of released aminomethylcoumarin (AMC) (18, 27). Kaolin or recombinant TF was added into wells to trigger clotting. Fluorescence was measured with Thermo Fluoroskan in 15 sec time intervals for 1 hr. Fraction conversion of thrombin substrate f was calculated with following equation: $f(t) = [F(t) - F(0)] / [F_{\text{max}} - F(0)]$ where $F(t)$ is the instantaneous fluorescent reading in the well, F_{max} is the maximum readings in the well. $f(t)$ was calculated for each well and was averaged over all replicated wells. The initiation time T_i of thrombin generation was defined at the time point when 5% of the thrombin substrate was converted ($f=0.05$). A large burst in thrombin always occurs promptly after T_i .

2.11 Statistical analysis

Data were compared to controls using two-tail Student's t-test. P-value < 0.05 was considered statistical significant. For antithrombotic therapy tests, Bonferroni correction was performed since multiple statistical tests were being performed simultaneously.

3. Results

3.1 Kaolin surface concentration

Kaolin particles displayed flow-resistant adsorption to collagen (up to 1000 s^{-1}), an adsorption likely depending on electrostatic attraction between anionic kaolin surface and cationic regions on collagen fibrils. This kaolin adsorption occurred even with precoating of collagen with BSA. A calibration experiment demonstrated a linear dependency between kaolin surface concentration and % area surface coverage (Figure 2B) as expected for the thin collagen matrix. Figure 2C–F correspond to collagen surfaces with no, low, medium, and high level of fluorescent kaolin. Surface concentrations were determined from the calibration line and converted to surface ratio ($\mu\text{m}^2 \text{ kaolin}/\mu\text{m}^2 \text{ glass}$) using the specific surface area of kaolin particles of $16 \mu\text{m}^2/\mu\text{g-kaolin}$ (28, 29). Addition of PC/PS liposomes to the kaolin/collagen was designed to promote contact-triggered coagulation by providing an anionic lipid surface for prothrombinase formation (Factor Xa/Va) even in the absence of activated platelets. In well plate assay of thrombin generation, initiation of thrombin generation was accelerated when PC/PS ($2.3 \mu\text{g/mL}$ final concentration) was added into 5-fold diluted, $40 \mu\text{g/mL}$ CTI-treated, recalcified citrated PPP (Supplemental Figure 2). Using fluorescent annexin V binding assay, we also determined that PS/PC deposition was controlled by collagen and was not affected by kaolin (data not shown). To delineate the effect of PC/PS, same amount of liposomes were patterned onto all surfaces including kaolin free collagen surface. To demonstrate that kaolin could trigger contact activation in a well plate assay (no flow), citrated WB ($4 \mu\text{g/mL}$ CTI, 5-fold diluted) initiated thrombin generation at ~25 min after recalcification and addition of kaolin ($0.3\text{--}300 \mu\text{g/mL}$) shortened the time lag in a dose dependent manner (Supplemental Figure 3). The fastest thrombin generation was observed at about 10 min after recalcification.

3.2 Effect of kaolin surface concentrations on activity of contact pathway

In the microfluidic assay, highly concentrated kaolin ($> 0.3 \text{ pg}/\mu\text{m}^2$) blocked collagen fibrils and resulted in severe reduction in platelet deposition (data not shown) and was excluded from further experiments. Platelet deposition was not affected by medium or low kaolin concentration under either pressure relief or constant flow mode (Figure 3A–B). However, presence of medium level of kaolin ($0.12 \text{ pg}/\mu\text{m}^2$) accelerated onset of fibrin generation by over 100 sec and quantitatively promoted fibrin formation for both pressure relief and constant flow modes (Figure 3C–D). Depending on the donor, low level of kaolin ($0.03 \text{ pg}/\mu\text{m}^2$) could either enhance or had no effect on fibrin formation suggesting a modest inter-donor variation in response to the lowest dose kaolin (data not shown). All subsequent experiments used a medium level of kaolin, which always promoted fibrin formation under flow for all donors.

3.3. Effect of flow conditions on activity of contact pathway

Averaged results (Figure 4) showed that fibrin generation was favored at venous shear rate (100 s^{-1}) compare to arterial shear rate (1000 s^{-1}) for either constant flow or pressure relief mode. Under pressure relief mode, no significant difference was observed between the level of platelet deposition at arterial shear rate and at venous shear rate. Platelet deposition always preceded fibrin formation. At venous shear rate, fibrin generation was significantly more efficient under constant flow mode. However, at arterial shear rate, fibrin generation was diminished regardless of the flow modes. Under constant flow mode, reduced platelet deposition at arterial shear rate significantly delayed occlusion time. However, under pressure relief mode, there was no clear dependency of occlusion time on the degree of platelet deposition. Consistent with a previous study on TF bearing collagen surface, platelets tend to form plug at upstream of kaolin/collagen patches at venous shear rate (26). In contrast, a more homogenous platelet distribution with a heavy tail at the downstream region was observed at arterial shear rate (Supplemental Figure 4). It is possible that under pressure relief mode, full channel occlusion is affected more by the spatial distribution of platelet mass on collagen matrix.

3.4 Kaolin/collagen vs. TF/collagen surfaces

We compared the flow sensitivity of contact activation with that of extrinsic activation by TF. To avoid thrombi embolization as they grew in the presence of thrombin generation, all dynamic data was obtained under pressure relief mode. TF was more efficient in terms of stimulating thrombin formation in well plate (Supplemental Figure 5). A level of 2.3 pM TF induced faster thrombin generation than 0.78 mg/mL kaolin (comparable to concentration of kaolin suspension used for surface preparation). Under flow condition, platelet aggregation initiated slightly earlier on TF/collagen surface, but after the early phase (first 180 sec), platelet signal on kaolin/collagen and TF/collagen surface were statistically identical (Figure 5A–B and Figure 6). Fibrin formation was faster on TF/collagen surface at both venous and arterial shear rates. But at arterial shear rate, fibrin onset was substantially delayed and suppressed on both kaolin/collagen and TF/collagen surfaces (Figure 5C–D and Figure 6). Interestingly, at arterial shear rate, final fibrin fluorescent intensity was statistically identical on two surfaces (Figure 6).

3.5 Pharmacological effect of antithrombotic therapies

Concentrations of antithrombotic therapies were either based on the dose level resulting in 50%–70% reduction in platelet aggregation in the flow assay (data not shown) or doses from previous studies (21). MRS 2197 and 2-MeSAMP block P2-family P2Y_1 and P2Y_{12} receptors, respectively (30, 31). Both inhibitors showed robust inhibitory effect on platelet deposition (Figure 7A–B), which is consistent with previous observation in a similar microfluidic model lacking thrombin production (21). Aspirin inhibits thromboxane A_2 production by acetylating cyclo-oxygenase 1 (COX -1). 250 μM aspirin was required for significant reduction in platelet aggregation in this assay with thrombin generation. Thrombin and fibrin formation were also delayed with the presence of these three antiplatelet reagents. GSNO is a nitric oxide (NO) donor under physiological condition and had been known to inhibit platelet adhesion to collagen fibrils (32). Iloprost, as a

prostacyclin (PGI₂) analog, is an effective inhibitor of collagen-induced platelet aggregation (33, 34). Both reagents resulted in significant delay in platelet aggregation as well as reduction in fibrin accumulation (Fig. 8A–D). Thrombin level was lowered by both reagents but the effect of GSNO on thrombin was not statistical significant.

4. Discussion

A surface was designed to activate contact pathway and allow for platelet capture. The activity of contact activation in this microfluidic assay was evaluated by initiation time and dynamics of platelet and fibrin deposition on kaolin/collagen surface. Imaging fluorescent platelets at the collagen/kaolin surface is a measure of net adherent platelets. Distinct from measures of surface coverage, platelet retraction on its own does not alter the mass of platelets on surface and does not change the total fluorescence that is measured. In our prior studies with collagen alone (26), retraction of platelet mass has been followed with time, but no change in total surface fluorescence was detected due simply to retraction. Importantly, total fluorescence can change as the net result of platelet deposition and detachment, but the rates of these two distinct processes were not resolved individually. As seen previously under flow conditions with collagen surfaces (35), distinct mounds (inhomogeneous platelet patches) can grow on collagen that are elongated in the direction of flow. We have not quantified the morphology metrics of mounds formed on kaolin/collagen, but they are largely similar to those formed on collagen alone.

Collagen and platelets on their own can trigger contact pathway (36). However, Kaolin induced more efficient contact activation compare to collagen alone. Collagen stimulated platelets could enhance both coagulant activity and proteolytic cleavage of FXII and of FXI (37). The presence of a medium level of kaolin (0.12 pg/ μm^2) accelerated fibrin generation but did not interfere with platelet activation or platelet deposition on collagen. Fibrin generation was completely abolished by high dose of CTI (40 $\mu\text{g/mL}$) confirming the enhancement of fibrin generation by kaolin was FXIIa dependent (Supplemental Figure 6). This result is consistent with the defect in fibrin deposition when blood from a severe FXI deficient patient was perfused over collagen (38). This result with high CTI dosing is also consistent with a published comparison between FXII and FXI inhibition in a baboon model demonstrating the efficacy of antibodies targeting either FXI or FXII to inhibit fibrin and thrombus formation (39). The prothrombotic effect of kaolin/collagen surface can be affected by different flow conditions. In this study, initial wall shear rate (100 s^{-1} and 1000 s^{-1}) and flow mode (pressure relief and constant flow) were the two variables in flow condition. A previous reported COMSOL model showed that under constant flow mode, wall shear rate increases dramatically when thrombus approaches to full channel occlusion (26). At arterial shear rate, fibrin formation was suppressed under both flow modes. High shear rate probably caused enhanced dilution of coagulation factors and inhibition of fibrin assembly. Our results suggested that at low shear condition when platelet plug forms at upstream side of kaolin/collagen patch, pressure driven convective transport of activated coagulation factors and fibrin monomers could aid coagulation reactions while at high shear condition excessive fast convection severely disturbed coagulation reactions.

We conclude that surface-linked kaolin can activate contact pathway under flow conditions but is not nearly as potent as insoluble particles added to closed systems lacking flow. In the flow assay, TF/collagen surface triggered extrinsic pathway particularly well to allow fibrin generation, especially at venous shear rate. In well plate, TF was much more potent than kaolin in terms of stimulating thrombin generation. Compare to contact pathway, extrinsic pathway is also a much shorter reaction pathway leading to prothrombinase (FXa/Va), which may contribute to the stronger potency of TF than kaolin under either static or flow condition.

Antithrombotic reagents targeting P2Y₁, P2Y₁₂, cyclooxygenase-1 or activating IP-receptor or guanylate cyclase were tested in the microfluidic model. To explore occlusive thrombus growth under flow in the presence of thrombin, tests were conducted at arterial wall shear rate and under pressure relief mode. All tested anti-platelet agents showed inhibitory effect on platelet deposition. Under flow condition, platelets could aid coagulation reactions by helping localizing coagulation proteins and providing required phospholipid surface. Delay and reduction in thrombin and fibrin generation was observed as expected as platelet deposition was disturbed by antithrombotic reagents.

The role of FXII in coagulation has been investigated in several animal models. FXII-mediated fibrin formation contributes to thrombus stability in mouse models (4–6). Antibody targeting heavy chain of FXII and antibody blocking FXI activation by FXIIa both reduces thrombus growth in baboon arteriovenous shunt thrombosis model (39, 40). Knocking out FXIIa provides thromboprotection without increasing bleeding risk in an extracorporeal bypass system in rabbits under TF deficient condition wherein the thrombin generation is mainly driven by contact activation on non-physiological surface (41). The role of FXII in thrombus formation in human is however not elucidated. Individuals with complete deficiency (<10%) of FXII is protected from myocardial infarction, whereas mild FXII deficiency (10–50%) increases the risk of myocardial infarction (42) indicating the effect of FXIIa is complicated and yet to be established when thrombus formation is initiated by extrinsic pathway. It is revealed in a recent study that extrinsic pathway prompts the initial pathological thrombosis formation whereas FXIIa promotes thrombus stability in later phase (43) suggesting the complementary roles extrinsic and contact pathway played in pathological thrombosis. It is however should be noticed that prior studies showing XIIa potentiation often are initiated with TF. The presented microfluidic assay is free of TF. This is one of the first studies to explore the contact pathway under controlled flow and defined surface conditions in the presence of anti-platelet agents.

5. Conclusion

The primary goal of this study was to develop a microfluidic model that can be used to assess the activity of contact pathway under flow conditions. Our approach was to bind kaolin to collagen, thus forming a substrate that can simultaneously induce contact activation and platelet activation/binding. Perfusion of whole blood over the kaolin/collagen surface in microfluidic flow chamber allowed observation of thrombus structure, specifically platelets and fibrin formation on the surface. Instead of directly measuring the bulk level of FXIIa, we evaluated the activity of contact pathway by dynamic change of platelet and

fibrin, as they have the critical effects on thrombus structure. Fibrin formed distal of FXIIa was subject to reduced assembly at arterial flow conditions. We found that TF-triggered extrinsic pathway is more potent than kaolin initiated contact pathway in terms of stimulating thrombin formation under both static and low shear condition, but both surfaces showed a similar sensitivity to high shear rate despite different pathways they triggered. This microfluidic assay was also sensitive to inhibitory effect of antithrombotic therapies targeting P2Y₁, P2Y₁₂, cyclooxygenase-1 or activating IP-receptor or guanylate cyclase. The sensitivity of this microfluidic assay to antithrombotic drugs makes it a good candidate for potential drug screening tests and clinical diagnostic assays of antithrombotic therapy targeting contact pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

Biased microfluidic model activating contact pathway on a defined kaolin/collagen surface.

Alteration in flow condition (shear rate, transthorbus pressure) can affect activity of contact pathway.

Contact pathway is less efficient in prompting thrombin generation compare to extrinsic pathway.

This assay is sensitive to the inhibitory effect of multiple antithrombotic therapies.

Figure 1.

Experimental design. Collagen was patterned with a single channel device that was vacuum bonded on a glass slide (left, red dye to show flow path). After BSA-blocking, a mixture of kaolin and PS/PC liposome was pulled through the main channel and allowed to settle for 30 min, forming a 250- μm wide immobilized kaolin/collagen film (middle). The patterning device was then replaced by an 8-channel device that was mounted perpendicular to kaolin/collagen strip forming 8 evenly spaced 250 $\mu\text{m} \times 250 \mu\text{m}$ procoagulant zones. CTI (4 $\mu\text{g}/\text{mL}$) treated blood was perfused over kaolin/collagen surfaces in the presence of fluorescent conjugated platelet and fibrinogen labels (right). Flow was initiated within 5 min after venous phlebotomy and shear rate was controlled by a syringe pump. In some experiments, EDTA was added to blood in lanes 2, 4, 6, 8 to operate in a constant pressure drop mode.

Figure 2.

Determination of kaolin surface concentration. (A) Kaolin/collagen surface (with no PS/PC) was visualized using scanning electron microscopy (black bar represents 5 μm). White particles are kaolin and grey lines in the background are collagen fibrils. A calibration curve of fluorescent kaolin surface concentration was constructed (B). Surface with no (C), low (D), medium (E) and high level (F) of fluorescently labeled kaolin particles were visualized with fluorescent microscope. Surface concentrations were determined from (B).

Figure 3.

Dynamic change of platelet and fibrin fluorescent intensities on kaolin/collagen surface. CTI ($4\mu\text{g/mL}$) treated whole blood was perfused over kaolin/collagen surface at 100 s^{-1} . Platelet deposition ($\pm$ SD, shaded) for different kaolin concentrations are identical indicating kaolin is not interfering with platelet deposition under either pressure relief (A) or constant flow (B) mode. Medium level of kaolin accelerated the onset of fibrin formation under both pressure relief mode (C) and constant flow mode (D).

Figure 4.

Averaged platelet and fibrin signal on kaolin/collagen surface at three representative time points. Full channel occlusion times are included in an embedded table. Student's t-test was applied to compare the differences on platelet and fibrin signal and occlusion time under different flow conditions (*, p < 0.05; **, p < 0.01).

Figure 5.

CTI-treated whole blood (4 $\mu\text{g/mL}$) was perfused over kaolin/collagen or TF/collagen surface at either venous (100s^{-1}) or arterial (1000s^{-1}) shear rate under pressure relief mode. No significant difference was observed in platelet mass growth on two surfaces (A, B). TF significantly accelerated fibrin generation at low shear rate (C) but not at high shear rate (D) (black arrows indicate occlusion time).

Figure 6.

Averaged platelet and fibrin intensities on TF/collagen and kaolin/collagen surface at three representative time points. No significant difference on platelet aggregation was seen on two surfaces after the first 180 sec. TF induced faster fibrin generation. However, at arterial shear rate, endpoint fibrin level was not significantly different on two surfaces. Full channel occlusion time points are included in an embedded table. T test was applied to compare the platelet and fibrin signal and occlusion time on two surfaces (*, p<0.05; **, p<0.01).

Figure 7.

Anticoagulated blood (CTI, 4 μ g/mL) was treated with MRS (10 μ M), 2-MeSAMP (100 μ M) or ASA (250 μ M) right before experiment and perfused over kaolin/collagen surface at 1000s⁻¹ under constant pressure mode. Dynamic changes of platelet aggregation (A), fibrin formation (C) and thrombin generation (E) on kaolin/collagen surface are based on a representative experiment ($\pm$ STD, shaded). Averaged fluorescent intensities from three experiments for platelet (B), fibrin (D) and thrombin (F) are presented at three representative time points (*, p<0.05; **, p<0.01).

Figure 8.

Anticoagulated blood (CTI, 4 μ g/mL) was treated with Iloprost (5 nM) or GSNO (70 μ M) right before experiment and perfused over kaolin/collagen surface at 1000s⁻¹ under constant pressure mode. Representative dynamic changes of platelet (A), fibrin (C) and thrombin (E) on kaolin/collagen surface is based on a single experiment ($\pm$ STD, shaded). Averaged fluorescent intensities from three experiments for platelet (B), fibrin (D) and thrombin (F) are presented at three representative time points (*, p<0.05; **, p<0.01).