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Microfluidic whole blood testing of platelet response to pharmacological agents

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Abstract

Platelets present a number of intracellular and transmembrane targets subject to pharmacological modulation, either for cardiovascular disease reduction or as an unintended drug response. Microfluidic devices allow human blood to clot on a defined surface under controlled hemodynamic and pharmacological conditions. The potencies of a number of antiplatelet and anti-cancer drugs have been tested with respect to platelet deposition on collagen under flow. Inhibitors of cyclooxygenase-1 (COX-1) reduce platelet deposition, either when added ex vivo to blood or ingested orally by patients prior to testing. Some individuals display a functional “aspirin-insensitivity” in microfluidic assay. When certain nonsteroidal anti-inflammatory drugs (NSAIDs) are taken orally, they block COX-1 acetylation by aspirin with concomitant reduction of aspirin efficacy against platelets in microfluidic assay. Both P2Y₁ and P2Y₁₂ inhibitors reduce platelet deposition under flow, as do NO donors and iloprost that target the guanylate cyclase and the prostacyclin receptor, respectively. In a microfluidic assay of 37 kinase inhibitors, dasatinib had potent antiplatelet activity, while bosutinib was less potent. Dasatinib and bosutinib have known profiles against numerous kinases, revealing overlapping and non-overlapping activities relevant to their unique actions against platelets. Also, dasatinib caused a marked and specific inhibition of GPVI signaling induced by convulxin, consistent with a dasatinib-associated bleeding risk. Microfluidic devices facilitate drug library screening, dose-response testing, and drug-drug interaction studies. Kinase inhibitors developed as anticancer agents may present anti-platelet activities that are detectable by microfluidic assay and potentially linked to bleeding risks.

INTRODUCTION

During clotting under flow, platelet signaling progresses in time and space. The local environment within a dense platelet deposit is quite different from that found in platelet rich plasma (PRP) or whole blood. Platelets are captured from flow, activate on various matrix ligands via GPVI, resulting in integrin activation, firm arrest, and granule release. Platelet

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Declaration of interest

The authors declare no conflicts off interest.

deposits formed from flowing blood may have a platelet density that is 50 to 200 times greater than that of PRP. The first layer of platelets depositing on collagen displays strong activation with released adenosine diphosphate (ADP) and synthesized thromboxane (TXA₂) helping to drive secondary deposition under flow conditions. In hemostatic clots, a core-shell hierarchy can be detected in vivo [1] and in vitro [2,3], where the platelets in the core are densely packed via retraction, P-selectin positive, and in proximity with thrombin and fibrin. In contrast, platelets in the shell region are less firmly attached, P-selectin negative, and less densely packed. Interestingly, apyrase, which degrades ADP in assays at PRP concentrations of platelets to prevent platelet activation, is ineffective under flow conditions because boundary layer concentrations of ADP and ATP within dense platelet deposits cannot be degraded fast enough by apyrase [4].

Using microfluidics, platelet function can be tested independent of thrombin and fibrin formation by use of whole blood collected in 1 μ M apixaban and/or 100 μ M PPACK (D-Phe-Pro-Arg chloromethylketone) to strongly inhibit Factors Xa (FXa), thrombin (FIIa), and FXIIa. Whole blood is essential for microfluidic studies since red blood cells drive platelets to the plasma layer immediately adjacent to the blood vessel surface. Reductions in hematocrit cause marked reductions in platelet deposition under flow [5]. For surfaces that facilitate platelet adhesion and activation, a number of purified proteins have been deployed in microfluidic studies, specifically fibrillar collagen, von Willebrand factor (VWF), laminin, etc. [6,7] In microfluidic devices, the flow rate through the system is typically controlled with a syringe pump which sets an initial or inlet wall shear rate of the experiment (venous wall shear rates of 100 to 200 s^{-1} ; arterial wall shear rates of 1000 to 2000 s^{-1}). [8] When a platelet deposit builds up in the device to partially occlude the channel, the local shear rates and shear stresses will increase dramatically, often to pathological levels ($> 5000 s^{-1}$). These high shear rates can drive embolism of the deposit, especially as full occlusion is approached in a flow channel. Colace et al. [9] developed an approach to use a syringe pump that allows flow to be diverted into a matched EDTA channel that lacks deposition while the other channel can progress to partial or full occlusion as a natural endpoint of the measurement. This pressure relief mode approximates a constant pressure drop situation which is representative of the in vivo hemodynamic setting. Use of hydrostatic pressure heads or pressure controllers can create a constant pressure drop configuration where the wall shear stress increases as the channel becomes filled with platelets and then decreases to zero at full occlusion. For microfluidic devices created with soft lithography, the width of the channel should exceed the height of the channel ($W/H > 3$) to help minimize corner and side-wall influences where the shear rates may be lower than the central portion of the rectangular channel. Additionally, extreme care should be taken to allow blood to flow in a minimally perturbed state before it reaches a defined region of the channel that presents a thrombogenic test surface. Commercial systems that involve coating the entire flow path of a blank device with a matrix protein such as collagen are problematic: blood will clot immediately in the inlet reservoir, thus interfering with any reliable measurement of platelet function under flow. Also, the length of the matrix coating in the direction of flow can influence the measurement since the first 250 microns of coating can capture platelets from the near wall plasma layer, but longer lengths (> 1 mm) are subject to boundary layer depletion of platelets from the plasma layer.[10]

For microfluidic measurements of drug efficacy in vivo or mechanisms of drug action, blood can be obtained from patients dosed with a drug or the pharmacological agent can be added to blood ex vivo. For exogenous addition of drug ex vivo, solvent controls should be tested to determine effects of ethanol or DMSO on platelet function with care taken to keep solvents at < 0.1 % by vol. The activity of known platelet targeting agents and the potential off-target effects of kinase inhibitors are discussed. For patients taking a drug, the pharmacokinetics of distribution, metabolism, and clearance should be considered to allow platelets to come in contact with the active agent. Drug-drug interactions present complex pharmacokinetic and pharmacodynamics interactions that can be monitored with microfluidic assay. For example, an investigation of the interaction of orally administered nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin (ASA) on human platelets is presented.

MATERIAL AND METHODS

Blood collection and IRB

Human blood was collected by venipuncture from healthy, non-smoking adult donors under IRB approval (Univ. Penn.) [11,12]. In some experiments, blood samples were drawn at $t = 0$ and 26-hr with ASA (325 mg) administration at 2 hr. In tests of NSAID-ASA interactions, donors ingested an NSAID (ibuprofen, 600 mg; naproxen, 500 mg; or celecoxib, 200 mg), followed 2-hr later with ASA (325 mg), with blood samples obtained for microfluidic testing at 26-hr post-NSAID administration.[13] For platelet microfluidic studies, up to 10 mL of whole blood was drawn into 100 μ M D-Phe-Pro-Arg-chloromethylketone (PPACK; Hematologic Technologies) to irreversibly inhibit FXIIa and thrombin as previously described [12, 13, 16].

PDMS devices, imaging, and thromboxane assay

An 8-channel poly(dimethylsiloxane) (PDMS, Sylgard 184, Ellsworth Adhesives) microfluidic device was used in constant flow mode (inlet wall shear rate constant) for whole blood perfusion through a rectangular channel (60 μ m high x 250 μ m wide). Platelet deposition on a 250- μ m long patch of patterned type 1 fibrillar collagen (Chronolog) was monitored with time by fluorescence microscopy, as previously described.[14,15] Glass slides were prepared for collagen micropatterning by functionalization with Sigmacote and coated with 5 μ L of fibrillar collagen and then blocked with 20 μ L bovine serum albumin (0.5 % BSA). Platelets were labeled with phycoerythrin-conjugated mouse anti-human CD61 antibody (0.125 μ g/mL; BD Biosciences). Thromboxane (TXA₂) is unstable and rapidly converts to the stable form TXB₂, which was measured in serum by mass spectrometry assay via methyloxamine derivatization, as described in Ref. [13].

RESULTS

COX-1 inhibition

Platelet activation results in calcium mobilization and activation of cyclooxygenase-1 (COX-1) which leads to the synthesis of TXA₂, a potent amplifier of platelet deposition under flow. Ex vivo addition of ASA to blood caused a dose dependent reduction of platelet

deposition on collagen (Fig. 1A). In quantifying platelet deposition under collagen (whole blood flow over type 1 fibrillar collagen at 200 s^{-1}), platelet deposition proceeds slowly over the first 50 sec and then increases to a faster rate that is sustained for over 300 sec. Although the early platelet deposition between 60 and 150 sec was largely ASA-insensitive, the deposition between 150 and 300 sec was particularly sensitive to addition of ASA ex vivo (Fig. 1B) with an IC_{50} of $10.5 \text{ }\mu\text{M}$ at 200 s^{-1} and $2.2 \text{ }\mu\text{M}$ at 1000 s^{-1} . [16] The thromboxane boundary layer concentration may be reduced at arterial flow conditions due to enhanced mass transfer, resulting in a lower IC_{50} at the higher shear. Similar results were observed for indomethacin to inhibit COX-1 [11].

By defining a self-normalized metric for a single blood sample, the ratio R of TXA_2 -dependent secondary deposition ($F' = F / t$ between 150 and 300 sec) to the primary deposition rate ($F' = F / t$ between 60 and 150 s) provides a measure of secondary deposition that is independent of any external standard or reference. For $R > 1$ secondary aggregation is prominent and ex-vivo ASA administration causes a dose-dependent reduction of the R -value (Fig. 1C). The potency of ASA ingestion can be observed in blood donors whose platelets display reduced deposition without ex vivo ASA addition and are no longer responsive to ex vivo ASA addition (Fig. 1D).

In a microfluidic study, only 2 of 28 healthy donors (no prior ASA consumption verified by mass spectrometric quantitation of COX-1 acetylation) had $R < 1$ in repeated testing under baseline conditions. Only 3 of 28 donors failed to respond ($R > 1$) to $500 \text{ }\mu\text{M}$ ASA added ex vivo. At 24-hr following ASA ingestion (325 mg dose), most healthy donors (21 of 28) displayed poor secondary aggregation ($R < 1$) (red and black symbol, Fig. 1E). The 7 individuals (blue symbol, 25% of total) that displayed functional aspirin-insensitivity in microfluidic assay ($R > 1$) at 24-hr post-ingestion were also insensitive to ex vivo ASA addition.

NSAID-ASA interactions

An important pharmacological issue with respect to prophylactic ASA consumption to reduce cardiovascular risk is the potential of NSAIDs to interfere with ASA acetylation of COX-1. The potential for various common NSAIDs to interfere with ASA acetylation of serine-529 of platelet COX-1 in vivo can be studied with microfluidics using platelets from blood donors taking oral doses of NSAIDs prior to taking ASA. [13] Normal donors had platelets that displayed an $R > 1$ indicative of secondary deposition in the flow assay while ASA consumption reduced the R -value to $R < 1$ indicative of in vivo action of ASA on platelet COX-1. Pretreatment with ibuprofen before ASA administration blocked the ability ASA to prevent secondary deposition in the microfluidic assay ($R > 1$) (Fig. 2A). In contrast, neither naproxen nor celecoxib prevents ASA from reducing platelet secondary aggregation ($R < 1$) (Fig. 2A). For naproxen pretreatment prior to ASA, however, the observed $R < 1$ metric was likely influenced by naproxen's long half-life in vivo and its continued presence at the time of assay at 26 hr after ingestion (naproxen does inhibit ASA acetylation in vivo). These results were fully consistent with measurements of serum TXB_2 levels and platelet aggregometry following 100 mM arachidonic acid induced activation.

P2Y inhibitors

When activated, platelets release ADP that potentiates clot buildup through activation of P2Y₁ and P2Y₁₂. Several P2Y₁₂ inhibitors are widely prescribed. For clopidogrel which is a prodrug that requires enzymatic transformation, antiplatelet activity may be reduced in patients that are poor metabolizers. Direct inhibition of P2Y₁ with MRS 2179 or direct inhibition of P2Y₁₂ with 2-MeSAMP caused a marked reduction of early and later stage platelet deposition under flow (Fig. 2B). Compared to ASA which has inhibitory action after 150 sec, P2Y inhibitors are more potent at earlier times likely because ADP does not require synthesis but only granule release, an early event in the clotting episode. Microfluidic assay using collagen surfaces and whole blood perfusion allowed the separate detection of GPVI signaling and subsequent secondary deposition driven by ADP release and thromboxane synthesis. Addition of exogenous ASA or direct P2Y₁ or P2Y₁₂ inhibitor to a patient sample prior to microfluidic assay allowed determination of an individual's drug insensitivity or detection of incomplete pharmacological inhibition.

Kinase inhibitors

Kinase inhibitors are widely used to treat various cancers. Unfortunately, bleeding risks may result due to the compound's activity against kinases within platelets. Kinase inhibitors have been well profiled against large portions of the human kinome, revealing that even highly specific inhibitors hit a number of different kinases. The human platelet proteome consists of 229 kinases and 73 phosphatases [17]. We tested 37 different kinase inhibitors (all well profiled against purified kinases in [18]) in whole blood assay of platelet function under flow. Two compounds, bosutinib and dasatinib significantly reduced platelet deposition on collagen at 450 sec of perfusion (Fig. 3A). Dasatinib was extremely potent in reducing platelet deposition on collagen at all times of the assay, while bosutinib had little potency in the first 150 sec but reduced secondary deposition between 200 and 600 sec (Fig. 3B). The early and potent action of dasatinib on platelet deposition in flow was fully consistent with its marked potency to block GPVI activation by convulxin in a calcium mobilization assay (Fig. 3B). A dose of 1 μ M dasatinib was selective for GPVI inhibition with little inhibitory activity on platelet calcium mobilization upon challenge with PAR1 or PAR4 agonist peptides, thromboxane mimetic (U46619), or ADP. Dasatinib and bosutinib have both overlapping and non-overlapping activities on various kinases (Fig. 3D). Notably, dasatinib is more potent than bosutinib in inhibiting (0% *residual activity* is full inhibition in [18]) the Src family kinases *lyn* (0.43 % dasatinib vs. 2.13 % bosutinib residual activity), *lyn* (1.96% vs. 29.4%), *fgr* (1.7 vs. 11.5%), and *Src* (3.52 vs. 6.1%). Interestingly, both dasatinib and bosutinib are relatively weak *syk* inhibitors (64.5% vs. 21.1%). The GPVI-inhibitory activity of dasatinib has been detected in other assays and dasatinib is associated with bleeding risks in cancer patients as well as inhibiting platelet production from megakaryocytes[19].

DISCUSSION

Microfluidic assays using whole blood with added drugs allow the study of drug action on platelets. While thrombin activity can be studied under flow conditions [20,21], the study of drugs that modulate platelet function can be studied directly with full thrombin inhibition. Microfluidic devices, through their deployment of defined surfaces and controlled flow

rates, allow the biology of blood function to reveal itself in space and time. Pharmacological agents can target precise platelet receptors or metabolic pathways which in turn have specific impacts on the performance of blood during clotting. In the present study, common medications that modulate arachidonic acid metabolism, purinergic receptors, and kinases have been studied in microfluidic experiments.

The interplay of various adhesive and activation mechanisms can become important under the various flow conditions found in microfluidic devices: at higher arterial levels of flow or at pathological levels of flow [22], von Willebrand factor (VWF) becomes an essential mediator of platelet function. The collagen surface allows evaluation of GPVI pathways and $\alpha_2\beta_1$ -mediated adhesion to collagen. Inhibition of $\alpha_{2b}\beta_3$ prevents continued platelet buildup on the first layer of collagen-adherent platelets [23]. Buildup of the deposit under flow is autocrine and dependent on platelet release of ADP to activate P2Y₁ and P2Y₁₂. At later stages the buildup of the clot is highly dependent on COX-1 dependent synthesis of thromboxane. For the ~20–25% of donors that display prominent secondary deposition on collagen under flow ($R>1$) in the presence of ingested or ex vivo ASA, the phenotype should be considered one of “high residual activity in flow assay under ASA” since COX-1 is still acetylated by ASA in these donors. Inhibitors of platelet targets (GPVI, $\alpha_{2b}\beta_3$, P2Y₁, P2Y₁₂, COX-1) or activators such as NO-donors or prostacyclin analogs [11,24] have a distinct effect on clotting under flow. Microfluidic devices are well suited for drug screening, dose-response, drug-drug interactions, patient-response, as well as off-target bleeding risks. While bedside platelet function testing remains somewhat uncommon, microfluidics may allow the future whole blood assay (without centrifugation of PRP) in the context of traumatic bleeding, validated prophylaxis prior to angiography, or non-responder identification in drug trials.

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Abbreviations:

ASA	aspirin
COX-1	cyclooxygenase-1
NSAID	nonsteroidal anti-inflammatory drugs
TXA₂	thromboxane A ₂

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Fig. 1. COX-1 inhibition and platelet function under flow.

Antagonism of thromboxane production with ex vivo ASA reduces platelet deposition on collagen ($t = 300$ sec) (A). Ex vivo ASA does not reduce the initial deposition rate (60 to 150 sec), but had dose-dependent activity on the secondary deposition rate (150 to 300 sec) (B). The R-ratio of secondary to primary deposition rate is a self-normalized metric that reveals the potency of ex vivo ASA where $R \sim 1$ at the IC_{50} of ASA of 10.38 μM (C). Normal blood is sensitive to ex vivo ASA, while blood from donors at 26-hr ASA ingestion displays less platelet deposition and an insensitivity to ex vivo ASA (D). For $n = 28$ donors tested, the R-ratio indicates that most donors ($R < 1$, red and black symbol) responded to ASA ingestion, while only 7 out of 28 donors (blue symbol) displayed ASA resistance following ASA ingestion (E). (FI, arbitrary fluorescence intensity units).

Fig. 2. NSAID-ASA interactions and P2Y inhibitors.

Ibuprofen ingestion prior to ASA ingestion interferes with the action of ASA on platelets under flow conditions, while naproxen and celecoxib sustain the inhibitory activity of ASA to reduce platelet deposition ($R < 1$) ($n = 7$ donors for each cohort) (**A**). Selective inhibitors of $P2Y_1$ (MRS 2179) and $P2Y_{12}$ (2-MeSAMP) are potent inhibitors of platelet deposition under flow at both early and late times of deposition (**B**). (Relative to Normal control or aspirin alone: * $p < 0.001$; ** $p < 0.05$; *** $p = 0.3/NS$).

Fig. 3. Screening of kinase inhibitors for antiplatelet activity under flow conditions.

A total of 37 kinase inhibitors were tested under flow and compared to vehicle control (A). Bosutinib (column 1) and dasatinib (column 13) were statistically significant inhibitors of platelet deposition. In comparison to normal blood, dasatinib inhibited early and late platelet deposition, while bosutinib inhibited deposition at later times (B). Dasatinib was a potent inhibitor of convulxin-induced calcium mobilization via GPVI (C). Dasatinib and bosutinib have been well profiled against kinases (used with permission, ref. [25]) and display differences in kinase specificity (D). (Error bars and shading: Standard Deviation).