Multiscale prediction of patient-specific platelet function under flow

Matthew H. Flamm, Thomas V. Colace, Manash S. Chatterjee, Huiyan Jing, Songtao Zhou, Daniel Jaeger, Lawrence F. Brass, Talid Sinno, and Scott L. Diamond

Introduction

During thrombotic or hemostatic episodes, platelets bind collagen and release ADP and thromboxane A2, recruiting additional platelets to a growing deposit that distorts the flow field. Prediction of clotting function under hemodynamic conditions for a patient’s platelet phenotype remains a challenge. A platelet signaling phenotype was obtained for 3 healthy donors using pairwise agonist scanning, in which calcium dye–loaded platelets were exposed to pairwise combinations of ADP, U46619, and convulxin to activate the P2Y1/P2Y12, TP, and GPVI receptors, respectively, with and without the prostacyclin receptor agonist iloprost. A neural network model was trained on each donor’s pairwise agonist scanning experiment and then embedded into a multiscale Monte Carlo simulation of donor-specific platelet deposition under flow. The simulations were compared directly with microfluidic experiments of whole blood flowing over collagen at 200 and 1000/s wall shear rate. The simulations predicted the ranked order of drug sensitivity for indomethacin, aspirin, MRS-2179 (a P2Y1 inhibitor), and iloprost. Consistent with measurement and simulation, one donor displayed larger clots and another presented with indomethacin resistance (revealing a novel heterozygote TP-V241G mutation). In silico representations of a subject’s platelet phenotype allowed prediction of blood function under flow, essential for identifying patient-specific risks, drug responses, and novel genotypes. (Blood. 2012;120(1):190-198)
(MRS-2179), or with a PGI2 receptor (IP receptor) activator (iloprost). NN models of platelet calcium signaling were then embedded in a multiscale model that simulated platelet deposition to collagen. The multiscale model accounted for changes in blood flow as the platelet deposit grew into the channel, the motion of platelets within the blood flow, and the release of soluble platelet agonists (ADP and TXA2). This allowed for multiscale prediction of donor-specific clotting events under flow and pharmacologic modulation, a fundamental step in describing how a heart attack would progress based on a patient’s unique platelet phenotype.

Methods

Blood collection

Blood was drawn from healthy male volunteers (who self-reported to be free of medication or alcohol consumption in the prior week) into H-D-Phe-Pro-Arg-chloromethylketone (100μM PPACK; Calbiochem) to inhibit thrombin production, all at normal extracellular calcium concentration. Donors provided informed consent in accordance with University of Pennsylvania institutional review board approval and the Declaration of Helsinki.

PAS of platelet calcium signaling

Platelet calcium mobilization in response to agonist stimulation was measured in a high-throughput assay, as described previously.22 Diluted PPACK-treated platelet-rich plasma (12% by volume) was treated with indomethacin (28μM) to eliminate autocrine signaling via thromboxane and loaded with Fluo-4NW dye (Invitrogen) for 30 minutes before activation in a 384-well plate assay with all pairwise mixtures of ADP, U46619, and convulxin using a FlexStation III (Molecular Devices) at final concentrations of 0, 0.1, 1, and 10 μM (EC50 levels for ADP, 1μM; for U46619, 1μM; and for convulxin, 5nM). Each combination of agonists was tested in the presence or absence of iloprost (1μM). All wells contained normal extracellular calcium. NN training was also carried out as described previously.22 The NN training set consisted of the concentrations of the 4 molecular inputs (ADP, U46619, CVX, and iloprost) and the resulting calcium transients for the 74 conditions. A unique NN was trained for each donor (see supplemental Methods, available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Microfluidic phenotyping of platelet

The microfluidic devices were fabricated in poly(dimethylsiloxane) as described previously23 with 8 parallel channels (250 μm wide by 60 μm high) that converged to a common outlet. The channels ran perpendicularly over a 250-μm-long stripe of patterned equine fibrillar collagen type I
(Chrono-par; Chrono-log), allowing 8 separate platelet deposition events per device to be imaged simultaneously by epifluorescence microscopy (magnification, 4x; 620 nm EX/700 nm EM) every 15 seconds (ORCA-ER CCD camera; Hamamatsu).

Before blood perfusion, channels were blocked with 0.5% BSA in HEPES-buffered saline (20mM HEPES and 160mM NaCl, pH 7.5) for 30 minutes. Blood was treated with vehicle (0.01% final concentration of DMSO) or various concentrations of indomethacin (Tocris Bioscience) or acetylsalicylic acid (aspirin; Sigma-Aldrich) 40 minutes before perfusion or with iloprost or MRS-2179 (Tocris Bioscience) for 5 minutes before perfusion. Alexa Fluor 647–conjugated anti-CD41 (clone PM6/248; AbD Serotec) for 3 minutes before perfusion. Samples were perfused at a wall shear rate of 200/s (2 μL/min per channel; PHD2000 Harvard Syringe pump) for 8 minutes.

**Sequencing of the TP gene**

DNA was isolated from whole blood using the FlexiGene DNA kit following the manufacturer’s instructions (QIAGEN) and subjected to PCR amplification using primers against TP exons 2, 3, and 4 with sequences kindly provided by Andrew Mumford (Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol, United Kingdom).24 PCR products were either sequenced directly or subcloned for sequencing by the University of Pennsylvania DNA-sequencing facility.

**Calcium measurements in HEK cells expressing wild-type TP or TP-V241G**

The wild-type human TXA2 receptor cDNA (TXA2R clone ID TXA2R0000) was obtained from the University of Missouri cDNA Resource Center. The cDNA construct is cloned into pcDNA3.1+ (Invitrogen) at EcoRI (5’) and XhoI (3’) for expression under a CMV promoter. The variant V241G TXA2R was generated from this wild-type template by site-directed mutagenesis using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer’s instructions. The mutated clone was sequenced and verified in the University of Pennsylvania DNA-sequencing facility. Equivalent transient expression after lipofection of plasmid for wild-type TP and TP-V241G was verified by flow cytometry using anti–human TP Ab (Cayman Chemical) at 21%–22% transfection. Lipofected HEK-293T (4 μg of plasmid with 10 μL of lipofectamine 2000 well per well of a 6-well plate) were cultured for 1 day and then reseeded in 96-well plates with clear bottoms (COSTAR 3603) and cultured 2 days before the calcium measurements. The cells were washed once using HEPES-buffered saline and incubated with the fluorescent Ca2+ indicator Fluo-4NW (Invitrogen) at room temperature for 30 minutes. Fluorescence using Molecular Devices Flex Station III after stimulation with U46619 was normalized to the fluorescence before stimulation (F/F0) and then background subtracted by the signal from untransfected HEK.

**Multiscale simulation of platelet deposition under flow**

The multiscale model consisted of 4 submodels: lattice kinetic Monte Carlo (LKMC), NN, finite element method (FEM), and lattice Boltzmann (LB). The LKMC submodel tracked individual platelet motion in the fluid and platelet binding/unbinding to the surface. LKMC is a stochastic method that solves for the motion of particles within fluid and can incorporate cellular binding rates. The main input into a LKMC model is the rate for all events, solves for the motion of particles within flow and can incorporate cellular binding events. The NN submodel computed the transient concentration in this case, motion and binding events. The main input into a LKMC model is the rate for all events, solves for the motion of particles within flow and can incorporate cellular binding events. The NN submodel provided the binding rates. The FEM submodel computed the transient concentration in this case, motion and binding events. The NN submodel provided the binding rates. The LKMC is a stochastic method that accounts for receptor and ligand copy numbers, single bond kinetics, and the integrated calcium concentration, $\xi(t)$, for $\alpha_\beta_3$ integrin activation, as follows:

$$F(\xi) = \alpha + (1 - \alpha) \frac{\xi^n}{\xi^n + \xi_0^n},$$

where $n$ controls the sharpness of the response, and $\xi_0$ is the critical value for 50% activation. In the present study, the values of $n = 0.75$ and $\xi_0 = 9$μM-sec were used for both $\alpha_\beta_2$ and $\alpha_\beta_3$ integrin activation. The quantity $\alpha$ represents the basal level of integrin activation and was set to 0.001 in all simulations so that 0.001 $\leq F(t) < 1$.

The overall rate of attachment of a platelet to collagen is defined by

$$\Gamma_{\text{coll}} = k_{\text{coll}} \Gamma_{\text{coll}} F(\xi),$$

where $k_{\text{coll}}$ is the attachment rate of a fully activated platelet ($F = 1$) and accounts for receptor and ligand copy numbers, single bond kinetics, and the effects of VWF. Similarly, the rate of attachment between 2 platelets via fibrinogen, $\Gamma_{\text{fibr}}$, depends on the activation states of both platelets. To model the 2-body interaction, the rate of binding between platelets $i$ and $j$ is given by the geometric mean of integrin activation

$$\Gamma_{\text{fibr}} = k_{\text{fibr}} \frac{1}{\sqrt{F_i F_j}} F(\xi),$$

where $F(\xi)^{-1}$ is a metric of the number of bonds that must be broken, $\gamma_i$ is the local shear rate around platelet $i$, and $\gamma_j$ is the characteristic shear rate required to initiate accelerated bond breakage. Similarly, the detachment rate between 2 bound platelets is given by

$$\Gamma_{\text{det}} = k_{\text{det}} \frac{1}{\sqrt{F_i F_j}} F(\xi)^{-1} \exp \left( \frac{\gamma_i + \gamma_j}{2\gamma_i} \right),$$

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The concentration fields, $C_j(x,y)$, ($j = \text{ADP}$ and $\text{TXA}_2$) were determined by FEM solution of the convection-diffusion-reaction equation,

$$\frac{\partial C}{\partial t} + \nabla \cdot (C \nabla F) = D \nabla^2 C + R_c,$$

where $D$ is the Brownian diffusion coefficient of $\text{ADP}$ or $\text{TXA}_2$, $R_c$ is the volumetric rate of release or generation of $\text{ADP}$ or $\text{TXA}_2$, and $\nabla$ is the velocity field (obtained from LB). The platelet release rate of soluble species $\text{ADP}$ and $\text{TXA}_2$ depends on the internal activation state, $\xi$. We assume that each platelet only releases $\text{ADP}$ and $\text{TXA}_2$ if its integral calcium, $\xi(t)$ is larger than the critical threshold, $\xi_0 = 9$μM-sec.
at which a platelet reaches $t_{50}$ is denoted as $t_{\text{release}}$. For $t \leq t_{\text{release}}$, the rate of release of ADP or TXA$_2$ is modeled by an exponential decay of the form

$$R_j(t) = \frac{M_j}{\tau_j} \exp \left( -\frac{t - t_{\text{release}}}{\tau_j} \right),$$

where $M_j$ is the total amount of releasable ADP or TXA$_2$ in a platelet and $\tau_j$ is the characteristic time constant of release. Once activated, each platelet releases $1 \times 10^{-8}$ nmol of ADP with a time constant of 5 seconds, and $4 \times 10^{-10}$ nmol of TXA$_2$ with a time constant of 100 seconds. All parameters are defined in supplemental Table 1 and were held constant across all donors and drug conditions. To simulate individual donors, a donor-specific NN was embedded in the multiscale model. To simulate the effect of drug treatment, signals were either inhibited or stimulated at the NN level. COX-1 inhibition was modeled by inhibiting TXA$_2$ signaling. P2Y$_1$ inhibition was modeled by inhibiting ADP signaling. Iloprost was a direct input to the NN (see supplemental Methods).

Results

PAS and platelet calcium phenotyping

For each of 3 donors, 74 calcium responses were measured in quadruplicate on a 384-well plate (Figure 2A). The trained NN models predicted these calcium responses accurately within 5% at all times (Figure 2A and supplemental Figure 2). The measured synergies for 63 conditions, containing 2 or 3 inputs (agonists with or without iloprost), provide a “synergy vector” that is a measurement of a subject’s phenotype (Figure 2B). We found previously that the synergy vector is a unique and repeatable fingerprint of the subject. The NN training resulted in accurate prediction of this measured synergy vector for each donor (Figure 2B-C), with the synergy scores highly correlated ($R = 0.9543$) between experiment and simulation (Figure 2C).

Multiscale simulation of platelet function under flow

Multiscale simulations predicted the density of platelets adherent to the surface, platelet activation states, and the spatiotemporal dynamics of ADP and TXA$_2$ release, morphology of the growing aggregate, and the distribution of shear along the solid-fluid boundary (shown in Figure 3 for Donor 1; see supplemental Video 1). Initially, platelets only adhered to the collagen surface until the adherent platelets became sufficiently activated to release ADP and TXA$_2$. The released ADP and TXA$_2$ formed a boundary layer extending up to 10 $\mu$m from the platelet deposit, which was sufficient to recruit platelets in the near-wall region. Within the boundary layer, concentrations of up to 10 $\mu$M ADP and 0.1 $\mu$M...
TXA$_2$ were observed. Concentrations of ADP and TXA$_2$ and the boundary layer thickness were consistent with earlier continuum models that imposed a thrombus growth rate and geometry. TXA$_2$ concentrations were found to be subphysiologic ($<0.00067\mu$M or $<0.1 \times EC_{50}$) until a sufficient platelet mass accumulated at the surface after approximately 250 seconds. Boundary-layer ADP concentrations were within the effective dynamic range (0.1-10$\mu$M or 0.1-10 $\times EC_{50}$) throughout the simulation. The ADP concentration profile had spatial and temporal fluctuations of 2-3 orders in magnitude, whereas the TXA$_2$ concentration profile had less than an order of magnitude of spatial fluctuations and no temporal fluctuations. Spatial fluctuations in the TXA$_2$ profile were created by an inhomogeneous distribution of platelet density along the surface. The strong temporal and spatial fluctuations in the concentration of ADP were predominately driven by the short release time (5 seconds), whereas the longer release time of TXA$_2$ (100 seconds) smoothed fluctuations. Platelets interacting with collagen became the most activated because of the sustained calcium mobilization in collagen signaling, whereas platelets that were stimulated by only ADP and TXA$_2$ had only a transient increase in intracellular calcium. The platelets participating in the initial platelet-collagen interaction experienced a fluid shear close to the initial wall shear rate of 200/s. The shear rate along the solid-fluid boundary became highly heterogeneous during the simulation, with areas of high shear rate (5- to 10-fold increase above 200/s) at the projections into the flow and areas of near zero shear rate in the valleys between platelet aggregates. Therefore, within a single simulation (and the corresponding microfluidic experiments), a large range of shear rates are sampled by platelets, even in the presence of a constant inlet wall shear rate. Low shear recirculation regions of up to 10 $\mu$m were formed within the valleys of the platelet deposit. At 500 seconds, the platelet deposit was characterized by platelet clusters approximately 30 $\mu$m in size, which is fully consistent with microfluidic measurements of platelet cluster size on collagen at this shear rate.

The multiscale model relies on 21 parameters (supplemental Table 1) held constant in all simulations, the first 14 of which are well-measured or accurately estimated physical and biologic constants ($\mu$blood, $\mu$fibrinogen, etc) and the remaining 7 parameters ($n$, $\xi_0$, $v_{\text{katt}}$, $k_{\text{collagen}}$, $k_{\text{fibrinogen}}$, $k_{\text{phosphatidylyserine}}$, and $\gamma_i$) were constrained by the dynamical responses measured for the 4 conditions across 3 donors (Figure 4), allowing a coarse-grained description of receptor-mediated adhesion. Because $\alpha_{\text{IIb}}\beta_3$, P-selectin, and phosphatidylyserine exposure (measured with PAC1, anti–P-selectin, and Annexin V, respectively) were uniform across the 3 donors used in this study in response to strong activation with convulxin (supplemental Figure 3), it was reasonable to treat these 7 coarse-grained parameters as uniform across the set of donors.

**Multiscale simulation predicts donor-specific platelet deposition under flow**

Using microfluidic devices, real-time platelet deposition from PPACK-treated whole blood flowing over fibrillar collagen (250 $\mu$m $\times$ 250 $\mu$m patch) was measured for 500 seconds at inlet wall shear rate of 200/s for 3 separate donors under untreated conditions or in the presence of aspirin, indomethacin, MRS-2179, or iloprost (Figure 4A and supplemental Video 2). In the 2D simulations, the overall extent of platelet deposition was measured by the number of platelets on a patch with surface area of 750 $\mu$m$^2$ (a 250 $\mu$m $\times$ 3 $\mu$m slice; Figure 4B-C) over 500 seconds. In the 2D simulations, a total of 100-300 platelets were deposited on the collagen surface at 500 seconds for the control condition (corresponding to 8000-25 000 platelets on a 250 $\mu$m $\times$ 250 $\mu$m area), which compares very well with the maximal deposition measured in the microfluidic experiment (approximately 20 000
platelets at $F_l = 3000$ in Figure 4A). Several platelet-signaling pathways were examined using drug treatments (Figure 1A). For both experiment and simulation, all donors were the most sensitive to iloprost and the least sensitive to removal of TXA$_2$ via indomethacin or aspirin treatment (TXA$_2$ = 0 in the simulation). The effect of removing ADP-induced calcium mobilization via MRS-2179 treatment (ADP = 0 in the simulation) was between that of iloprost and that of removing TXA$_2$. Blood from Donor 1 was observed to produce significantly larger clots at 500 seconds than blood from Donors 2 and 3 under control conditions ($P < .001$; Figure 4A), a trait exactly predicted in multiscale modeling (Figure 4B-D). In both experiment and simulation, Donors 1 and 2 exhibited significant sensitivity ($P < .01$) to the removal of TXA$_2$ (indomethacin or aspirin in the experiment, TXA$_2$ = 0 in the simulation) at 500 seconds, whereas Donor 3 was insensitive to indomethacin or aspirin. For Donors 1 and 2, the effect of removing TXA$_2$ via COX-1 inhibition occurred after approximately 250 seconds in the experiment, which corresponded well to the time at which physiologically active levels of TXA$_2$ were predicted to occur in the boundary layer. In both simulation and experiment, the effect of ADP removal or iloprost treatment occurred after 100 seconds, the time at which propagation of the platelet aggregate away from the clot surface began. The SD of the simulation (because of stochasticity in the LKMC method) was comparable to that observed with the microfluidic chamber replicates.

To test the predictive accuracy of the simulation under hemodynamic conditions of arterial thrombosis, we conducted experiments and simulations with an initial arterial wall shear rate of 1000/s. A total of 48 clotting events (over 3 separate days) were conducted at either 200/s or 1000/s for Donors 2 and 3 (Donor 1 refused continued participation in the 3-day study). The multiscale model predicted a significant decrease ($P < .0001$, $n = 24$) in platelet deposition, which was also observed in the microfluidic experiment (supplemental Figure 4). In the simulation, shear rates range from the initial wall shear rate of 1000/s to more than 3000/s at the peaks of the thrombus that perturbed into the flow field.

**Donor 3 platelet phenotype reveals a heterozygotic mutation in the TP receptor**

In stark contrast to Donors 1 and 2, Donor 3 was insensitive under flow conditions to the COX-1 inhibitors aspirin and indomethacin (Figure 4A,C), a trait predicted exactly in the multiscale modeling (Figure 4D) and fully consistent with an insensitivity to the thromboxane mimetic U46619 (Figure 5A). Platelet aggregation in response to 2µM U46619 was also abnormal for Donor 3 relative to a healthy donor (Figure 5B). After sequencing the 4 exons of the TXA2R gene of Donor 3 that were amplified using the PCR primers of Mumford et al., a heterozygous T $\rightarrow$ G mutation was found at position 11 921 in Exon 2 (National Center for Biotechnology Information reference number NG_013363.1; Figure 5C, supplemental Figure 5, and supplemental Methods), resulting in the identification of a novel heterozygote TP-V241G mutation in Donor 3. Because Gq signaling through ADP was normal in Donor 3 (supplemental Figure 6) and the TP receptor level in Donor 3 was also normal relative to Donors 1 and 2 (supplemental Figure 7), we conclude that the V241G mutation was the cause of abnormal coupling of TPs to G$_q$ and impaired calcium mobilization. Using transient expression of wild-type human TXA2R (TP) or mutated TXA2R (TP-V241G) in lipofected HEK cells (approximately 21% transfection efficiency and equivalent expression for both plasmids; supplemental Figure 8 and supplemental Methods), we detected an impaired calcium response to U46619 in Flou-4NW–loaded HEK expressing the mutated TP receptor relative to responses observed in cells transfected with the wild-type TP receptor (Figure 5D-E).
in the presence of pharmacologic mediators, ideally in a patient-specific and disease-relevant context (ie containing hemodynamics). Because platelet activation is defined by calcium mobilization in this model, it was expected that drug treatment through IP activation, COX1 inhibition, and P2Y1 antagonism in the model would lead to decreased aggregation. However, the multiscale simulation accurately predicted the ranking of pharmacologic sensitivity of blood under flow although the only “knowledge” of drug treatment was through the NN. The effect of COX1 inhibition in Donors 1 and 2 was observed in the microfluidic assay after ~200 seconds of perfusion. This onset of action was also apparent in the simulation and is consistent with aggregometry results showing action of COX1 inhibitors at the later stages after primary aggregation.56 Prediction of individual aggregate sizes corresponded well with experimental observations and such predictions would not be possible with continuum models. In addition, Donor 1 produced significantly larger platelet masses in the control condition than the other donors (P < .001). The difference between donors was captured from the high-dimensional calcium data (concentrations of 3 agonists and 1 inhibitor) predicted by the NN model. Iloprost treatment, however, resulted in the same level of reduced deposition in all 3 donors in both simulation and experiment. Thus, the model is robust to changes in platelet signaling as captured in the NN for each unique donor and in response to multiple drug treatments. Furthermore, PAS and microfluidic assay for platelet phenotyping resulted in identification of a new TP-V241G mutation in Donor 3, the functional impact of which was quantitatively captured in an in silico model of blood from Donor 3 (Figure 4D).

The main mechanism of P2Y12 inhibition is through an increase in the levels of cAMP.37 We investigated the effect of an increase in cAMP by stimulating the IP receptor via iloprost in this study. cAMP levels have been shown to strongly correlate with platelet activation regardless of whether the increase of cAMP was because of P2Y12 inhibition or IP receptor stimulation.38 Our results indicate that cAMP elevation is more effective than COX-1 or P2Y1 inhibition under these conditions.

The multiscale and patient-specific modeling presented here extends earlier modeling efforts of platelet function and/or coagulation20,21,39-42 by including a highly robust description of intracellular platelet signaling through GPVI, P2Y1, TP, and IP. To our knowledge, our NN/multiscale model is the first such approach to make donor-specific predictions of platelet function under flow in the presence of both released ADP and thromboxane and pharmacologic modulators of clinical relevance. However, the model reported here does not account for thrombin production (and all measurements were obtained in 100μM PPACK-treated whole blood). This simplification enables the input of NN phenotyping, consideration of multiple platelet therapies, and direct quantitative comparison to experimental results from microfluidic systems. A continuum coagulation model similar to the Leiderman-Fogelson model21 can be adapted to a discrete platelet model as in Xu et al42 although a computational price is paid for the addition of approximately 50 coupled partial and ordinary differential equations (compared with 2 partial differential equations here). The simulations in Figure 4 required 192 simulations (3 donors × 4 conditions × 16 repeats) each requiring up to 8 hours on Lonestar at the Texas Advanced Computational Center) with a significant computational burden associated with FEM calculation of ADP and TXA2 concentration fields. Whereas it is not intractable to include the effects of thrombin production, the scope of such investigation is
bleeding traits will have significant impact in stratifying patients according to risk. The multiscale model used in the present study also makes feasible patient-specific prediction of platelet deposition and drug response in more complex in vivo geometries such as stenosis, aneurysms, stented vessels, valves, bifurcations, or vessel rupture (for prediction of bleeding risks) or in geometries encountered in mechanical biomedical devices.

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Authorship

Contribution: M.H.F. developed all of the multiscale simulations; T.C. conducted the microfluidic studies and aggreometry; M.S.C. conducted the PAS and NN training; H.J. and S.Z. conducted the experiments shown in Figure 5C through E; D.J. conducted the flow cytometry; L.F.B. advised on platelet signal transduction; T.S. advised on multiscale simulation code development; and S.L.D. directed the study and wrote the manuscript with contributions from all authors.

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References


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