Published in final edited form as:

Exp Hematol. 2015 October ; 43(10): 891–900.e4. doi:10.1016/j.exphem.2015.06.302.

Microfluidic assessment of functional culture-derived platelets in human thrombi under flow

Viraj Kamat⁺, Ryan W. Muthard⁺, Ruizhi Li, and Scott L. Diamond^{*}

Institute for Medicine and Engineering, Department of Chemical and Biomolecular Engineering, 1024 Vagelos Research Laboratory, University of Pennsylvania, Philadelphia, PA. 19104, USA

Abstract

Despite their clinical significance, human platelets are not amenable to genetic manipulation, thus forcing a reliance on mouse models. Culture derived platelets (CDPs) from human peripheral blood CD34⁺ cells can be genetically altered and may eventually be used for transfusions. Using microfluidics, the time-dependent incorporation of CD41⁺CD42⁺ CDPs into clots was measured using only 54,000 CDP doped into 27 μ L of human whole blood perfused over collagen at a wall shear rate of 100 s⁻¹. Using fluorescently labeled human platelets (instead of CDPs) doped between 0.25 and 2 % of total platelets, incorporation was highly quantitative and allowed monitoring of anti- α IIb β 3 antagonism that occurred after collagen adhesion. CDPs were only 15 % as efficient as human platelets in their incorporation into human thrombi under flow, although both cell types were equally antagonized with α IIb β 3 inhibition. Transient transfection allowed the monitoring of GFP⁺ human CDP incorporation into clots. This assay quantifies genetically-altered CDP function under flow.

Keywords

microfluidics; *in vitro* platelets; modified platelets; thrombopoiesis

INTRODUCTION

Platelets generated *in vitro* from stem cells can potentially be used for clinical transfusions¹. To this effect, several groups have developed protocols to produce human platelets *in vitro* from embryonic², iPS (induced pluripotent stem)^{3,4} and CD34⁺ cells^{5,6}. Additionally, culture-derived megakaryocytes and platelets can be used as *ex vivo* surrogates to identify

^{*}Corresponding Author. Tel: 1-215-573-5702, sld@seas.upenn.edu.

⁺Denotes equal contribution

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author contributions: V.K.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; R.W.M.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; R.L.: Collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.L.D.: Conception and design, manuscript writing, financial support, final approval of manuscript

Conflict-of-interest disclosure: The authors declare no competing financial interests.

novel proteins that play a role in hemostasis or thrombosis⁷ and rectify innate genetic defects in platelet production or function⁸. Current culture techniques have produced platelets with varying quality. Cultured derived platelets (CDPs) generated from some methods have shown comparable function to blood platelets.^{3,5,9} However, other techniques have produced CDPs that respond poorly to agonists such as ADP and thrombin^{1,10,11}, a result of platelets getting activated in the culture or during the purification process. Flow cytometry is the current gold standard for defining the yield and quality of CDPs based on their similarity in surface marker expression and scatter properties to blood platelets. Pre-activated platelets, megakaryocyte membrane particles and other non-functional platelet like particles (PLPs) may get classified as functional platelets by flow cytometry^{1,12}. Some groups have demonstrated that CDPs can incorporate into mouse thrombi and respond to inhibitors of platelet function²⁻⁴. However, inherent differences between human and mouse platelets, poor quantification capabilities, and the time consuming nature of these methods limit their use for studying *in vitro* derived platelets.

Microfluidic models of human thrombosis have been well-established as tools to study blood coagulation and platelet function¹³⁻¹⁷. Microfluidic systems require extremely low blood volumes (<240 μ L) and enable precise control of flow conditions over biomimetic surface-patterned prothrombotic proteins. Observation of clotting in real time is possible through epifluorescent imaging of clotting events that develop under flow in transparent channels¹⁸. In our study, we aimed to develop a rapid and economical tool to evaluate CDP function in a human thrombus under flow. Using microfluidics, we devised an assay that quantifies the function of CDPs by examining their incorporation into *in vitro* human blood clots (Figure 1A). Human CD34⁺ hematopoietic stem cells were differentiated into megakaryocytes and platelets *in vitro* and then doped into whole blood. This mixture was then perfused into an 8-channel microfluidic device over surface patterned type I fibrillar collagen. The incorporation of cultured platelets was quantified by analysis of doped platelet areas within the thrombi formed under flow. By using this method, we demonstrated that CDPs participate in thrombus formation under well-defined flow conditions, though at significantly lower rates as compared to the participation of freshly isolated blood platelets. We detected changes in the clot incorporation of CDPs subjected to $\alpha_{IIb}\beta_3$ inhibition at various stages of clot buildup. Finally we showed that genetically engineered CDPs incorporated into growing thrombi, thereby providing a novel tool to assess transient gene expression in platelets.

MATERIALS AND METHODS

Platelet Production from human peripheral blood CD34⁺ cells

Peripheral blood (PB) CD34⁺ cells were obtained from Fred Hutchinson Cancer Research Center (Seattle, WA, USA). Cells were thawed and washed in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, 1% fetal bovine serum) and cultured for 4 days at 39°C in hematopoietic stem/progenitor cell expansion medium¹⁹ comprising StemSpan SFEM (StemCell Technologies; Vancouver, BC, Canada), 1 $\times$ penicillin/streptomycin, SR1 (0.75 μ M) (Cellagen Technologies; San Diego, CA, USA), human LDL (40 μ g/mL) (StemCell Technologies) and the following

human cytokines (R&D systems; Minneapolis, MN, USA): TPO (20 ng/mL), IL-6 (10 ng/mL), IL-3 (10 ng/mL), Flt3 ligand (50 ng/mL) and SCF (100 ng/mL). On day 4, cells were placed in megakaryocyte differentiation medium⁶ containing 80% IMDM (Gibco, Life Technologies; Grand Island, NY, USA), 20% BIT9500 serum substitute (StemCell Technologies), 1× penicillin/streptomycin, human LDL (40 µg/ml), 0.55 mM β-mercaptoethanol and the following human cytokines (R&D systems): SCF (1 ng/mL), TPO (30 ng/mL), IL-9 (13.5 ng/mL) and IL-6 (7.5 ng/mL). Medium was replaced on day 7 and 10. GM6001 (25 µM) (Calbiochem, EMD Milipore; Billerica, MA, USA.) was added to the culture on day 12. Cultures were placed on an orbital shaker at 60 rpm on day 13 as previously described⁵. Platelets were harvested from the cell culture on day 14. Cells were cultured at 39°C (day 0–4) or 37°C (day 4–14) in 5% CO₂.

Isolation of Blood Platelets and Culture Derived Platelets

Following approval from the Internal Review Board and informed consent, blood was collected from healthy volunteers in 10% (%v/v) citrate and centrifuged at 200g for 14 min to obtain platelet rich plasma (PRP). PRP was incubated with platelet specific antibodies (0.125 µg/ml) and/or ReoPro[®] (100 µg/ml) (a generous gift from Dr. Thomas Diacovo, Dept. of Pediatrics, Columbia University) for 20 min. Tyrode's buffer containing PGE1 (1 µM) (Sigma-Aldrich; St. Louis, MO, USA) and apyrase (1 U/mL) (Sigma-Aldrich) was added to the PRP and the mixture was spun at 1200g for 14 min to obtain a platelet pellet which was then re-suspended in Tyrode's buffer to obtain a washed platelet suspension.

Day 14 cell culture with 10% (%v/v) citrate, PGE1 (1 µM) and apyrase (1 U/mL) was centrifuged at 100g for 10 min to remove megakaryocytes. The supernatant was gently collected and centrifuged for 1200g for 14 min to obtain a platelet pellet. The pellet was re-suspended in Tyrode's buffer and incubated with platelet specific antibodies (0.125 µg/ml) and/or ReoPro[®] (100 µg/ml) for 20 min. Tyrode's buffer containing PGE1 (50 nM) and apyrase (1U/mL) was added to the platelet suspension and the mixture was spun at 1200g for 14 min to obtain purified CDPs.

Platelet Doping Assay

Whole blood inhibited with 100 µM FPR-chloromethylketone (PPACK, Haematologic Technologies, Essex Junction, VT, USA) was obtained from healthy volunteers. Platelet counts for whole blood and isolated platelets were obtained using counting beads (SpheroTech; Lake Forest, IL, USA) and monoclonal antibody (CD41 & CD42b) labeling followed by flow cytometry. Samples were mixed with 5000 beads and the platelet count per sample was evaluated by: Platelet events/Bead events × 5000. Culture derived platelets were defined as CD41⁺ and CD42b⁺ events that had scatter properties similar to blood platelets. The isolated platelet solution volume was adjusted using Tyrode's buffer based on the desired % platelet doping (0.25% – 2%) in whole blood. Platelet solution was added to whole blood at a final concentration of 10% (%v/v). Prior to addition the platelet solution was recalcified to 10 mM Ca²⁺. For example, for 1% platelet doping and a whole blood count of 200,000 platelets/µL, 12 µL of recalcified isolated platelet solution containing 216,000 platelets (200,000 platelets/uL × 108 µL × 1%) was added to 108 µL of whole blood.

Microfluidic Flow Assay

Microfluidic devices were fabricated in polydimethylsiloxane (PDMS, Sylgard 184, Ellsworth Adhesives, Germantown, WI, USA) according to previously described techniques^{13,20}. The 8-channel microfluidic device is fed by 8 wells with perfusion by withdrawal from a single outlet into a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA). The channels (250 μm by 60 μm) run perpendicularly over a 250 μm wide strip of patterned equine fibrillar collagen type I (Chronopar, Chronolog). The microfluidic device was mounted on an inverted microscope (IX81, Olympus, Center Valley, PA). The micro-patterned 250 μm $\times$ 250 μm collagen patch in each microfluidic channel was imaged with a 20 $\times$ objective lens equipped with a CCD camera (ORCA-ER, Hamamatsu, Bridgewater, NJ) in 1 min 38 sec intervals. All channels were blocked with 0.5% bovine serum albumin (BSA) in HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.4) 30 min prior to experimentation. Five minutes prior to initiation of the flow assay, whole blood was treated with monoclonal fluorescently conjugated non-function blocking 0.125 $\mu\text{g}/\text{ml}$ anti-CD61 antibody (Abd Serotec) or 0.125 $\mu\text{g}/\text{ml}$ Alexa Fluor 647 anti-CD41 (Abd Serotec) for flow experiments involving transfected CDPs. Single platelets in the platelet solution were labeled with 0.125 $\mu\text{g}/\text{ml}$ Alexa Fluor® 647 anti-CD41 monoclonal antibody (Abd Serotec) and 0.125 $\mu\text{g}/\text{ml}$ PE anti-CD42b (Biolegend) prior to doping into whole blood. All microfluidic experiments were completed within 30 min of phlebotomy.

Image Analysis

Image processing was completed in ImageJ software (ImageJ; NIH, Bethesda, MD, USA) by a custom MATLAB script (MathWorks, Natick, MA, USA). Fluorescent images were background-corrected and threshold levels of fluorescence were set for CD41⁺ or CD42⁺ fluorescent doped whole blood platelets or CDPs in each microfluidic channel. Images were then converted to binary masks and the product of the CD41⁺ and CD42⁺ binary masks resulted in CD41⁺ & CD42⁺ platelet-like particles (Figure 1B). Finally, a size filter (1.1–5.1 μm) was applied to eliminate non-platelet particles and proplatelet fragments. Single platelet number and single platelet area were obtained from the 'Analyze Particles' toolbox in ImageJ. Total clot fluorescence was measured in an area comprising of 70% centre zone of the 250 μm $\times$ 250 μm collagen surface due to the slightly non-uniform shear profiles along the side of the microfluidic channel²⁰.

Flow Cytometry

Flow cytometry data was collected with the C6 flow cytometer (BD Accuri, BD Biosciences, San Jose, CA, USA) and analyzed with CFlow Plus software (BD Accuri). Cells and platelets were stained with the following monoclonal antibodies: Alexa 488® anti-CD61 (Abd Serotec, Raleigh, NC, USA), FITC anti-CD41 (Biolegend; San Diego, CA, USA), PE/Cy7 anti-CD34 (Biolegend), Alexa Fluor® 647 anti-CD41 (Abd Serotec), PE anti-CD42b (Biolegend) and APC anti-p-selectin (Biolegend). To induce α -granule release, platelets were incubated with thrombin (100 nM) (Haematologic Technologies, Essex Junction, VT, USA), ADP (100 μM) (Sigma-Aldrich) or convulxin (100ng/mL) (Pentapharm; Basel, Switzerland) for 10 min prior to analysis.

Transfection

A total of 4 million cells from Day 10 of culture were transfected with 2 μ g pmaxGFP vectors in a single cuvette using the Nucleofactor™ II device (Amata, Lonza, Walkersville, MD, USA) and the Human CD34 Cell Nucleofactor™ Kit (Lonza). On Day 14, purified CDPs from the transfected cultures were analyzed for GFP expression by flow cytometry.

Platelet Spreading assay

Isolated platelet solutions were incubated on fibrinogen coated glass slides (100 μ g/mL) for 30 min, then washed twice with PBS (containing 0.5% BSA) and then fixed with 4% formaldehyde for 20 min. The samples were then washed twice and treated with 0.1% Triton X-100 for 5 min. After additional wash steps, the samples were incubated with anti-CD41 and phalloidin 488 (Abcam, Cambridge, UK) for 20 min. The platelets were then washed and the samples were mounted on an inverted microscope (IX81, Olympus, Center Valley, PA) and visualized under differential interference contrast (DIC) microscopy at 60 $\times$.

Statistical analysis

Student's two tailed t-test was used for all statistical analysis. A p-value < 0.05 was considered statistically significant.

RESULTS

Platelet doping assay development

We first optimized the platelet doping protocol using washed platelets isolated from healthy donors. Washed platelets were doped into whole blood at 10% final volume (1:9, washed platelets:whole blood) and the mixture was perfused over a type I collagen at 100 s⁻¹ in the microfluidic device. Time lapse fluorescent images of the blood clots were processed to obtain the doped platelet area in the total clot. Three independent filters were applied to classify a particle as a doped platelet: GPIIb⁺ (CD41⁺), GPIb⁺ (CD42b⁺), platelet diameter (1.1 – 5.1 μ m) (Figure 1B). These criteria ensured that larger cells, microparticles, and GPIIb⁺ & GPIb⁻ (CD41⁺ & CD42b⁻) particles were excluded from our analysis. Omitting GPIIb⁺ & GPIb⁻ is critical as previous groups have shown that these platelet-like particles are not characteristic of functional platelets¹².

To assess the range and sensitivity of the assay, we added different quantities (0.25% – 2%) of washed platelets to whole blood, with additions expressed as a percentage of the whole blood platelet count. Doped platelets incorporated into the growing thrombus (Supplementary Figure 1A) and the differences in % addition were visualized (Figure 2A and B). With subsequent image processing and analysis, we identified differences in doped platelet area and doped platelet number over the time course of the experiment (Figure 2C, Supplementary Figure 1B). As expected, doped platelet area in the clot was highly correlated to % platelet addition across the tested range ($R^2=0.9931$) (Figure 2D). The sensitivity of this assay allows for detection in changes in doped platelet area within 0.5%–2% platelet addition to whole blood. For the rest of the study, isolated platelets were added to whole blood within the 0.5% to 2% range. The difference in doped platelet addition had no significant effects on total clot buildup, which is primarily comprised of whole blood

platelets (Supplementary Figure 1C). Hence, only doped platelet areas and numbers were considered when results are reported.

Inhibition of doped platelet function

We next investigated whether our assay could quantify changes in platelet function upon *ex vivo* treatment with anti-platelet therapies. Whole blood derived platelets (WBPs) were treated with ReoPro[®], a monoclonal antibody which selectively inhibits $\alpha_{IIb}\beta_3$ integrin function, to examine whether anti-platelet agents can reduce doped platelet incorporation into the developing thrombus. The CD41 marker criterion was not used in processing the images since the antibody binding site would interfere with the drug. Since all CD42⁺ WBPs also displayed CD41 (Supplementary Figure 3B), our analysis was not affected. ReoPro[®] (100 μ g/mL) treated platelets showed greater than 50% decrease in doped platelet area after 2.84 min of flow (Figure 2E), whereas ReoPro[®] did not have an effect on the growth of the total clot as measured by total clot fluorescence intensities (Figure 2F, **ns**). These results indicate that our assay is capable of detecting inhibition of doped platelet function.

Generation of platelets *in vitro* from human PB CD34⁺ cells

Human peripheral blood CD34⁺ cells were differentiated into megakaryocytes and platelets using a 14 day protocol similar to a previously described method¹². Progenitor cells were expanded for 4 days followed by differentiation in megakaryocyte conditions for an additional 10 days (Figure 3A). A day prior to harvesting platelets, cultures were placed on an orbital shaker as shear force has been previously shown to increase platelet shedding^{5,21–23}. Platelets were defined by flow cytometry as events that had similar size (forward scatter) and surface (CD41 and CD42b) marker expression as blood platelets (Figure 3B, Supplementary Figure 2). While all CD42b⁺ CDPs expressed CD41, only a fraction of CD41⁺ CDPs expressed CD42b as shown previously (Supplementary Figure 3)¹². Using this protocol, we generated approximately 60 CD41⁺CD42b⁺ platelets and 30 CD41⁺CD42b⁺ megakaryocytes on Day 14 for every CD34⁺ cells seeded on Day 0 (Figure 3C). CDPs responded to thrombin stimulation (Figure 3D, Supplementary Figure 4A) and spread on fibrinogen-coated glass slides (Figure 3E). However, CDPs exhibited 60% lower change in p-selectin expression upon thrombin stimulation, as compared to WBP p-selectin expression (Figure 3D). Additionally, the CDPs showed reduced response to platelets agonists ADP and convulxin (Supplementary Figure 4C and D). Furthermore, a majority (~75%) of CDPs displayed Annexin V suggesting pre-activation or apoptosis during culture or purification (Figure 3F, Supplementary Figure 4B).

Incorporation of culture derived platelets into human blood clots

To test the ability of CDPs to incorporate into human thrombi, CDPs were doped into whole blood and the mixture was subsequently perfused into an 8-channel microfluidic device with surface patterned fibrillar type I collagen. Similar to whole blood platelets, CDPs incorporated into the clot at early time points and their numbers increased with overall clot growth (Figure 4A). When doped into whole blood at the same concentration, CDPs incorporated at ~15% efficiency compared to WBPs ($P < 0.001$) (Figure 4B). These results verified our flow cytometry observations which suggest that ~75% of the CDPs may be non-

functional. Similar to previous reports¹², the average measured diameter of a CDP that incorporated into the thrombus was about 35% greater than a WBP (Supplemental Figure 5, **P<0.001**). CDPs treated with ReoPro[®] showed reduced clot incorporation compared to an untreated control (Figure 4C). At 8.3 min, we measured a 50% reduction in ReoPro[®] treated CDP area. This percent reduction was similar to the WBPs treated with ReoPro[®]. These results show that our assay is a novel tool capable of detecting cultured derived platelet function.

Transgenic CDPs incorporate into growing clots

We next investigated whether our method could serve as a screening platform for modified platelets. Cells from Day 10 cultures were transfected with GFP plasmid and the purified CDPs from this culture were doped into whole blood for microfluidic testing. The incorporation of GFP⁺ CDPs was visualized and quantified (Figure 5A and B). GFP⁺ CDPs incorporated at constant rate of ~25% over the entire duration of the flow experiment and showed good correlation with % GFP⁺ CDPs as measured by flow cytometry (Figure 5B and C).

DISCUSSION

We have developed an *in vitro* microfluidic assay which rapidly assesses CDP function and quantifies incorporation of CDPs in human thrombi formed under flow. To our knowledge, this is the first study showing successful incorporation of CDPs into a human thrombus. CDPs incorporated into clots at significantly lower numbers compared to WBP incorporation into clots. Additionally, CDPs responded to anti-platelet agents at various stages of clot build-up. Furthermore, we demonstrated that CDPs are amenable to genetic manipulation by GFP plasmid transfection; single GFP⁺ CDPs can then be tracked under flow in our microfluidic assay. Overall, we provide (1) a new standard for the testing of CDP quality under well-defined flow conditions and (2) a novel system for characterizing and screening genetically altered human platelets.

Our microfluidic assay, which recapitulates CDP incorporation into human thrombi, requires a low quantity of cultured material and tests platelet function under physiologically relevant conditions. We believe our method has several advantages over observing human CDP incorporation during murine vascular injury models. The relevance of these mouse models to human bleeding is questionable due to several differences between mouse and human hemostasis^{24,25}. The use of NOD/SCID macrophage depleted mice to study human CDP incorporation³ may deem the system non-physiological and cause improper quantification due to the inclusion of platelet like particles. Furthermore, the photochemical, thermal, and mechanical mechanisms of laser ablation injuries have not been clearly elucidated²⁶. Laser injuries may cause excessive tissue damage which can affect platelet adhesion²⁶. Recent studies have also questioned the mechanisms by which ferric chloride (FeCl₃) application affects exposed blood vessels²⁷. Microfluidics is an ideal platform for CDP testing as channel design, anticoagulation, flow rates, prothrombotic protein concentration and composition can be altered to mimic the specific biochemical and biophysical conditions in which CDPs must function^{16,28,29}. The high throughput nature of our human microfluidic

model of thrombosis allows us to obtain 4 clotting events from 108 μL of whole blood and ~216,000 CDPs at a 1% doping concentration, an 100-fold reduction in input material compared to a mouse model which requires $\sim 2 \times 10^7$ CDPs/mouse (based on 2 mL blood volume, 10^6 platelets/ μL).

While platelets derived in culture from stem cells hold the promise of overcoming platelet shortages for transfusion medicine, they cannot be used clinically without adequate and exhaustive testing. Prior studies show that *in vitro* produced platelets respond weakly to prothrombotic stimuli suggesting that they may be pre-activated in culture^{1,10,11}. In our studies, a very small fraction of the CDPs activated in response to thrombin, ADP or convulxin when assessed by flow cytometry. Additionally, ~75% of the CDPs stained positive for Annexin V indicating that major of the platelets produced may have undergone apoptosis. Interestingly, CDPs incorporated into clots at 15% of the rate of donor-derived platelet incorporation, indicating a good correlation with the flow cytometry results. The quality of the CDPs produced can be highly dependent on the culture protocol. In our studies, ~38% of CD41+ CDPs expressed CD42b. CDPs generated using other techniques^{3,5}, with higher CD42b expression, may shower better incorporation in our flow assay. Our method for platelet function testing provides an environment that is significantly more physiologic and rigorous than flow cytometry. In addition, our assay facilitates platelet function testing while allowing for real-time visualization and quantification of CDP incorporation into a growing thrombus. CDPs in our assay must adhere to collagen, establish platelet-platelet contacts in the growing thrombus under increasing shear stress, and respond to solute agonists such as ADP and thromboxane A_2 . In our studies we used an initial wall shear rate of 100 s^{-1} because clots formed at 100 s^{-1} in our microfluidic devices are less prone to partial or complete embolism downstream of the collagen patch as compared to clots formed at 1000 s^{-1} . Thus CDP function is more reliably measured at 100 s^{-1} . Additional work is required to characterize CDP capture via tethering through glycoprotein Ib (GPIb binding) at arterial shear rates in our microfluidic assays. Human embryonic stem (hES) cells and induced pluripotent stem (iPS) cells provide a potential source for large scale platelet production. The development of novel tools and techniques has significantly increased the yield of platelets that can be produced *in vitro*^{4,30}. Future studies using our method could shed new light on the functionality of platelets from these sources, thereby bringing us closer to a new era of transfusion medicine.

Several large scale “platelet-omic” studies have been conducted to advance our current knowledge of platelet biology and to identify new gene products that may regulate platelet function^{31–33}. The anucleate nature of platelets creates a major roadblock in the functional characterization of these genes. To this effect, several groups have developed knock out zebrafish and mouse models for platelet functional characterization *in vivo*⁷. Xenotransplantation models using humanized mice transplanted with lentiviral transduced human CD34⁺ HSCs have enabled modified human platelets studies in an *in vivo* setting³⁴. In addition to the limitations of these models with regards to either interspecies differences or low levels of gene delivery/knockdown, these methods are impractical for rapid screening of a large number of gene products due to the expensive, laborious and time-consuming nature of these approaches. With our method, we can rapidly screen 8 separate CDP samples

on a single device and test devices in triplicate to screen a total of 24 CDP samples. We showed that platelets derived from cells transfected with GFP plasmid incorporated successfully into a growing thrombus. Furthermore, we can accurately quantify the change in platelet function as demonstrated by our ReoPro[®] inhibition studies. These results demonstrate the feasibility of using our method as a screening platform for genetically engineered platelets. While we demonstrate a low cost and simple method for downstream assessment of modified platelet biology, there is still a need for improving the efficiency of genetic modification of progenitor cells. Transduction of cells on Day 3 with a GFP expressing lentiviral vector led to poor transduction efficiency (~16%) and an ~10-fold reduction in Day 14 platelet yield (data not shown). Transduction protocols will need to be optimized to assess the effect of gene knockdown in our assay. Recent advances with the CRISPR-Cas9 system has allowed for simple and highly efficacious genetic alterations in several cell types^{35,36}. Mandal et al. have successfully demonstrated the feasibility of this method in human CD34⁺ progenitor cells, thereby offering novel method to produce modified platelets³⁷. Creating iPS cell lines lacking platelets specific genes would pave the way for creating a homogenous population of cultured platelets devoid of a gene of interest. Combining these strategies with our method for testing human blood will help assess the relevance of proteins such as talin³⁸, kindlin³⁹, clec-2⁴⁰, which have been shown to affect platelet function in mice. The next step would be to increase genetic screening efforts to decipher novel genes which can further advance our understanding of platelet biology and lead to the development of novel anti-platelet therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Institutes of Health (R01 HL-103419 to S.L.D.). We thank Paul Gadue and Deborah L. French for their comments and advice.

REFERENCES

1. Lambert MP, Sullivan SK, Fuentes R, French DL, Poncz M. Challenges and promises for the development of donor-independent platelet transfusions. *Blood*. 2013; 121:3319–3324. [PubMed: 23321255]
2. Lu S-J, et al. Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice. *Cell Res*. 2011; 21:530–545. [PubMed: 21221130]
3. Feng Q, et al. Scalable generation of universal platelets from human induced pluripotent stem cells. *Stem cell reports*. 2014; 3:817–831. [PubMed: 25418726]
4. Nakamura S, et al. Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells. *Cell Stem Cell*. 2014; 14:535–548. [PubMed: 24529595]
5. Panuganti S, Schlinker AC, Lindholm PF, Papoutsakis ET, Miller WM. Three-stage ex vivo expansion of high-ploidy megakaryocytic cells: toward large-scale platelet production. *Tissue Eng. Part A*. 2013; 19:998–1014. [PubMed: 23190353]
6. Pineault N, Robert A, Cortin V, Boyer L. Ex vivo differentiation of cord blood stem cells into megakaryocytes and platelets. *Methods Mol. Biol*. 2013; 946:205–224. [PubMed: 23179834]

7. Thijs T, Deckmyn H, Broos K. Model systems of genetically modified platelets. *Blood*. 2012; 119:1634–1642. [PubMed: 22180441]
8. Sullivan SK, et al. High-level transgene expression in induced pluripotent stem cell – derived megakaryocytes : correction of Glanzmann thrombasthenia. *Blood*. 2014; 123:753–757. [PubMed: 24335497]
9. Choi ES, Nichol JL, Hokom MM, Hornkohl AC, Hunt P. Platelets generated in vitro from proplatelet-displaying human megakaryocytes are functional. *Blood*. 1995; 85:402–413. [PubMed: 7529062]
10. Pallotta I, Lovett M, Kaplan DL, Balduini A. Three-dimensional system for the in vitro study of megakaryocytes and functional platelet production using silk-based vascular tubes. *Tissue Eng. Part C. Methods*. 2011; 17:1223–1232. [PubMed: 21895494]
11. Sullenbarger B, Bahng JH, Gruner R, Kotov N, Lasky LC. Prolonged continuous in vitro human platelet production using three-dimensional scaffolds. *Exp. Hematol*. 2009; 37:101–110. [PubMed: 19013002]
12. Robert A, Boyer L, Pineault N. Glycoprotein Iba receptor instability is associated with loss of quality in platelets produced in culture. *Stem Cells Dev*. 2011; 20:379–390. [PubMed: 20504152]
13. Maloney SF, Brass LF, Diamond SL. P2Y12 or P2Y1 inhibitors reduce platelet deposition in a microfluidic model of thrombosis while apyrase lacks efficacy under flow conditions. *Integr. Biol. (Camb)*. 2010; 2:183–192. [PubMed: 20473398]
14. Hansen RR, et al. High content evaluation of shear dependent platelet function in a microfluidic flow assay. *Ann. Biomed. Eng*. 2013; 41:250–262. [PubMed: 23001359]
15. Li R, Fries S, Li X, Grosser T, Diamond SL. Microfluidic assay of platelet deposition on collagen by perfusion of whole blood from healthy individuals taking aspirin. *Clin. Chem*. 2013; 59:1195–1204. [PubMed: 23592503]
16. Colace TV, Diamond SL. Direct observation of von Willebrand factor elongation and fiber formation on collagen during acute whole blood exposure to pathological flow. *Arterioscler. Thromb. Vasc. Biol*. 2013; 33:105–113. [PubMed: 23104847]
17. Muthard RW, Diamond SL. Blood clots are rapidly assembled hemodynamic sensors: flow arrest triggers intraluminal thrombus contraction. *Arterioscler. Thromb. Vasc. Biol*. 2012; 32:2938–2945. [PubMed: 23087356]
18. De Witt SM, et al. Identification of platelet function defects by multi-parameter assessment of thrombus formation. *Nat. Commun*. 2014; 5:4257. [PubMed: 25027852]
19. Boitano AE, et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science*. 2010; 329:1345–1348. [PubMed: 20688981]
20. Neeves KB, et al. Microfluidic focal thrombosis model for measuring murine platelet deposition and stability: PAR4 signaling enhances shear-resistance of platelet aggregates. *J. Thromb. Haemost*. 2008; 6:2193–2201. [PubMed: 18983510]
21. Dunois-Lardé C, et al. Exposure of human megakaryocytes to high shear rates accelerates platelet production. *Blood*. 2009; 114:1875–1883. [PubMed: 19525480]
22. Nakagawa Y, et al. Two differential flows in a bioreactor promoted platelet generation from human pluripotent stem cell-derived megakaryocytes. *Exp. Hematol*. 2013; 41:742–748. [PubMed: 23618622]
23. Jiang J, Woulfe DS, Papoutsakis ET. Shear enhances thrombopoiesis and formation of microparticles that induce megakaryocytic differentiation of stem cells. *Blood*. 2014; 124:2094–2103. [PubMed: 24948658]
24. Schmitt A, Guichard J, Massé JM, Debili N, Cramer EM. Of mice and men: comparison of the ultrastructure of megakaryocytes and platelets. *Exp. Hematol*. 2001; 29:1295–1302. [PubMed: 11698125]
25. Ware J. Dysfunctional platelet membrane receptors: from humans to mice. *Thromb. Haemost*. 2004; 92:478–485. [PubMed: 15351843]
26. Neeves KB. Down in a hole: A new laser ablation model of hemostasis. *J. Thromb. Haemost*. 2014
27. Barr JD, Chauhan AK, Schaeffer GV, Hansen JK, Motto DG. Red blood cells mediate the onset of thrombosis in the ferric chloride murine model. *Blood*. 2013; 121:3733–3741. [PubMed: 23343833]

28. Onasoga-Jarvis AA, et al. Thrombin generation and fibrin formation under flow on biomimetic tissue factor-rich surfaces. *J. Thromb. Haemost.* 2014; 12:373–382. [PubMed: 24345079]
29. Colace TV, Fogarty PF, Panckeri KA, Li R, Diamond SL. Microfluidic assay of hemophilic blood clotting: distinct deficits in platelet and fibrin deposition at low factor levels. *J. Thromb. Haemost.* 2014; 12:147–158. [PubMed: 24261634]
30. Thon JN, et al. Platelet bioreactor-on-a-chip. *Blood.* 2014; 124:1857–1867. [PubMed: 25606631]
31. Goodall AH, et al. Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function. *Blood.* 2010; 116:4646–4656. [PubMed: 20833976]
32. Gnatenko DV, et al. Transcript profiling of human platelets using microarray and serial analysis of gene expression. *Blood.* 2003; 101:2285–2293. [PubMed: 12433680]
33. Jones CI, et al. A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. *Blood.* 2009; 114:1405–1416. [PubMed: 19429868]
34. Lavenu-Bombled C, et al. Glycoprotein Iba1 promoter drives megakaryocytic lineage-restricted expression after hematopoietic stem cell transduction using a self-inactivating lentiviral vector. *Stem Cells.* 2007; 25:1571–1577. [PubMed: 17379771]
35. Cong L, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013; 339:819–823. [PubMed: 23287718]
36. Ding Q, et al. Enhanced efficiency of human pluripotent stem cell genome editing through replacing TALENs with CRISPRs. *Cell Stem Cell.* 2013; 12:393–394. [PubMed: 23561441]
37. Mandal PK, et al. Efficient Ablation of Genes in Human Hematopoietic Stem and Effector Cells using CRISPR/Cas9. *Cell Stem Cell.* 2014; 15:643–652. [PubMed: 25517468]
38. Petrich BG, et al. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. *J. Exp. Med.* 2007; 204:3103–3111. [PubMed: 18086863]
39. Moser M, Nieswandt B, Ussar S, Pozgajova M, Fässler R. Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat. Med.* 2008; 14:325–330. [PubMed: 18278053]
40. Suzuki-Inoue K, et al. Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J. Biol. Chem.* 2010; 285:24494–24507. [PubMed: 20525685]

Highlights

- A microfluidic assay to evaluate culture-derived platelet (CDP) function under flow is proposed
- Assay utilizes 10-fold fewer CDPs compared to mouse laser injury models
- CDPs incorporate into human thrombi at significantly lower rates compared to human platelets
- Assay can quantify transient gene expression in CDPs under flow

Figure 1.

Microfluidic assay for detecting clot incorporation of culture derived platelets. **(A)** Human CD34⁺ hematopoietic cells were differentiated into megakaryocytes and platelets in vitro. On Day 14, CDPs were purified from the culture and labeled with monoclonal antibodies against CD41 and CD42b. The CDPs were then doped into whole blood that was obtained from healthy adult donors, at 0.25% –2% of the whole blood platelet count. The mixture was perfused into a microfluidic device over surface patterned type I fibrillar collagen, and the incorporation of cultured platelets was quantified by analysis of doped platelet areas in the clot. To detect the clot incorporation of modified CDPs, progenitor cells were transfected with GFP plasmid on Day 10 of culture. **(B)** Raw images obtained from the microfluidic

assay were converted into binary format in ImageJ. The images were then processed to isolate particles that fell within the platelet size gate (1.1– 5.1 μm) and finally cross-matched for CD41 and CD42b expression. The total area of all platelet-like particles that were positive for both CD41 and CD42b was used to assess clot incorporation of doped platelets.

Figure 2.

Platelet incorporation assay development. Washed platelets were doped into whole blood at numbers which were within 0.25% – 2% of the whole blood platelet count. The mixture was perfused into a microfluidic device over surface patterned type I fibrillar collagen at an initial wall shear rate of 100 s⁻¹ and the incorporation of doped platelets in to the growing thrombus was quantified by analysis of platelet areas in the clot. Doped platelets were labeled with fluorescently conjugated monoclonal antibodies against platelet marker CD41 and CD42b. Whole blood was labeled with a monoclonal antibody against CD61. Processed

images at 8.3 min showing clot incorporation of washed platelets for doping concentration of 0.5% (**A**) and 2% (**B**). Image processing was used to determine the total area of incorporated doped platelets at all time points. (**C**) The doped platelet area in the thrombus for the various doping concentrations is shown. $N = 4 - 6$. Data are represented as mean $\pm$ SEM. For all doping concentrations, $p < 0.09$ for $t = 1.38$ min and $p < 0.05$ for $t = 4.15$ min and 5.5 min. (**D**) The fold change in doped platelet area at 8.3 min for various doping concentrations is shown. $N = 4 - 6$. Data are represented as mean $\pm$ SEM. * $p < 0.05$. (**E**) Washed platelets were treated with 100 μ g/mL ReoPro prior to doping into whole blood. % reduction in doped platelet area compared to untreated control due to treatment with ReoPro is shown. $N = 3$. Data are represented as mean $\pm$ SEM. $P < 0.003$ for $t = 2.38$ min (**F**) Total clot fluorescence intensity in the absence or presence of Reopro. $N = 3$. Data are represented as mean $\pm$ SEM. $P > 0.14$ for $t = 2.38$ min

Figure 3.

Platelet production in vitro. **(A)** Schematic of 14-day differentiation protocol for production of platelets in vitro from mobilized peripheral blood CD34⁺ cells. CD34⁺ cells were expanded for 4 days followed by differentiation in megakaryocyte promoting conditions for 10 days. Platelets were purified from the cultures on Day 14. **(B)** Culture derived platelets were defined by CD41 and CD42b expression, and size (forward scatter). Flow cytometry criteria for classification and counting of culture derived platelets using whole blood derived platelet gates are shown. **(C)** Day 14 yield for megakaryocytes (n=6) and platelets (n=5) produced in culture. Yield is defined as the number of megakaryocytes or platelets in culture on Day 14 per CD34⁺ cell seeded on Day 0. Data are represented as mean $\pm$ SEM. **(D)** WBPs and CDPs were stimulated with 100 nM thrombin. The fold-change in p-selectin expression upon thrombin stimulation is shown. N = 4. Data are represented as mean $\pm$ SEM **(E)** Spreading of thrombin stimulated CDPs on fibrinogen coated glass slides. **(F)** Annexin V expression on unstimulated WBPs and CDPs is shown. N = 4. Data are represented as mean $\pm$ SEM * p < 0.05, ** p < 0.01.

Figure 4.

Incorporation of CDPs into a human thrombus. CDPs were doped into whole blood and the mixture was perfused into our microfluidic device at a shear rate of 100s⁻¹. **(A)** Representative time lapse images showing incorporation CDPs labeled with fluorescently conjugated monoclonal antibodies against platelet marker CD41 and CD42b. **(B)** CDPs and WBPs were doped at the same concentration into whole blood. The incorporation of each population was measured by determining the doped platelets numbers in the clot at various time points. Comparison of doped platelet clot incorporation, measured by the doped platelet

numbers from CDPs or WBPs is shown. $N = 4$. Data are represented as mean $\pm$ SEM. $P < 0.05$ for $t > 0$ min. (C) CDPs and WBPs were treated with 100 $\mu\text{g/mL}$ ReoPro prior to doping into whole blood. % Reduction in doped platelet area at 8.3 min compared to untreated control as a result of treatment with ReoPro is shown. The difference in % reduction between CDPs and WBPs treated with ReoPro is not statistically significant. $N = 3$ donors. Data are represented as mean $\pm$ SEM. $P > 0.3$

Figure 5.

Incorporation of transgenic CDPs into a human thrombus. **(A)** Platelets generated from cells transfected with GFP plasmid on Day 10 were doped into whole blood and analyzed in the microfluidic assay. The platelet areas for all doped platelets, as well as those expressing GFP were determined. Representative images of GFP⁺ (**left**) and total CDPs (**right**) are shown. **(B)** Incorporation of GFP⁺ CDPs compared to total CDPs over the time course of the experiment. $N = 7$. Data are represented as mean $\pm$ SEM. $P < 0.001$ for $t > 0$ min. **(C)** Representative flow cytometry data showing %CDPs expressing GFP.