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Platelet Activation Via Shear Stress Exposure Induces A Differing Pattern Of Biomarkers Of Activation Versus Biochemical Agonists

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Abstract

Background.—Implantable cardiovascular therapeutic devices, while hemodynamically effective, remain limited by thrombosis. A driver of device-associated thrombosis is shear-mediated platelet activation (SMPA). Underlying mechanisms of SMPA, as well as useful biomarkers able to detect and discriminate mechanical versus biochemical platelet activation, are poorly defined. We hypothesized that SMPA induces a differing pattern of biomarkers compared with biochemical agonists.

Methods.—Gel-filtered human platelets were subjected to mechanical activation via either uniform constant or dynamic shear; or to biochemical activation by ADP, TRAP-6, thrombin, collagen, epinephrine or arachidonic acid. Markers of platelet activation (P-selectin, integrin α IIb β 3 activation) and apoptosis (mitochondrial membrane potential, caspase 3 activation, and phosphatidylserine externalization) were examined using flow cytometry. Platelet procoagulant activity was detected by chromogenic assay measuring thrombin generation. Contribution of platelet calcium flux in SMPA was tested employing calcium chelators, EDTA and BAPTA-AM.

Results.—Platelet exposure to continuous shear stress, but not biochemical agonists, resulted in a dramatic increase of PSE and procoagulant activity, while no integrin α IIb β 3 activation and P-selectin exposure occurred. SMPA was associated with dissipation of mitochondrial membrane potential, but no caspase 3 activation was observed. Shear-mediated PSE was significantly

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AUTHOR CONTRIBUTIONS

Yana Roka-Moiia designed and performed experiments, analyzed and interpreted data, wrote the manuscript; Ryan Walk, Daniel E. Palomares, and Annalisa Dimasi performed experiments; Kaitlyn Ammann and Joseph E. Italiano participated in discussions and manuscript preparation; Jawaad Sheriff and Danny Bluestein provided acetylated prothrombin, developed the PAS assay protocol, participated in discussions and manuscript preparation; Marvin J. Slepian designed research, interpreted data, and wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

decreased by chelation of extracellular calcium, while intracellular calcium depletion had no significant effect. In contrast, biochemical agonists ADP, TRAP-6, arachidonic acid, and thrombin were potent inducers of α IIB β 3 activation and/or P-selectin exposure. This differing pattern of biomarkers seen for SMPA for continuous uniform shear was replicated in platelets exposed to dynamic shear stress via circulation through a VAD-propelled circulatory loop.

Conclusions.—Elevated shear stress, but not biochemical agonists, induce a differing pattern of platelet biomarkers – with enhanced PSE and thrombin generation on the platelet surface. This differential biomarker phenotype of SMPA offers the potential for early detection and discrimination from that mediated by biochemical agonists.

Keywords

shear-mediated platelet activation; shear stress; cardiovascular therapeutic device; phosphatidylserine externalization; biomarker

INTRODUCTION

Cardiovascular disease remains the leading cause of morbidity and mortality in the United States and worldwide¹. The treatment of cardiovascular disease today increasingly relies on the use of a wide variety of cardiovascular therapeutic devices (CTDs) directly implanted in arterial blood flow. Current CTDs include mechanical circulatory support devices – ventricular assist devices (VADs) and the total artificial heart – for heart failure²; percutaneous heart valves and repair systems for aortic stenosis and mitral regurgitation^{3,4}; and stents for obstructive atherosclerotic arterial disease⁵. Despite the hemodynamic efficacy of these devices, they remain limited by device-related thrombosis^{6–8}. CTD thrombosis is associated with a range of devastating consequences including, device malfunction, regional ischemia, distal thromboembolic events, e.g. stroke, and an overall increase in patient morbidity and mortality. An unmet need exists in defining blood-based biomarkers able to identify CTD-mediated thrombosis.

A central driver of CTD thrombosis is shear-mediated platelet activation (SMPA)^{8–10}. This occurs as non-physiologic arterial flows impart supraphysiologic shear stress to overflowing platelets coursing through flow paths and local geometries of implant devices^{11–13}. Traditionally, SMPA has been described in relation to shear-driven platelet interactions with damaged vascular endothelial surfaces. There, elevated shear stresses induce conformational changes to von Willebrand factor (vWF) allowing its interaction with subendothelial proteins and platelet glycoprotein GPIb¹⁴. Shear-mediated binding of vWF to GPIb, not shear itself, initializes rapid platelet adhesion, activation and subsequent aggregation, leading to thrombus formation^{14–16}. In this process, vWF acts as both a shear “sensor” and agonist of SMPA on subendothelial surfaces. In contrast, CTD-related SMPA in free-flowing blood has largely been regarded as occurring via the direct interaction and impact of shear forces on platelets in the “free flow,” at a point where the threshold of shear force-time exposure is exceeded^{17,18}.

Our group has focused on extending the understanding of SMPA in the free flow. We have developed a numerical model of SMPA able to differentiate and value the impact of specific

hemodynamic characteristics of shear stress (i.e. magnitude, rate, and exposure time) on platelet activation^{13,19}. We determined that platelets accumulate shear stress “damage” over time, with the extent of SMPA strongly correlating with the “history” of shear exposure²⁰. Further, we have shown that high frequency components of CTD-related dynamic shear profiles are primary drivers of SMPA, yielding platelet activation even after ultra-short term shear exposure²¹. This work collectively suggests that significant mechanistic differences exist in SMPA compared with activation mediated by biochemical agonists and adhesive proteins.

This differential platelet behavior has been confirmed by recent studies examining inhibition of SMPA by antiplatelet agents targeting major receptor-driven pathways of platelet activation. Agents such as aspirin, eptifibatide and dipyridamole are commonly prescribed to mitigate shear-driven consequences of progressive atherosclerotic disease, or to limit SMPA for patients implanted with CTDs^{10,22}. Despite widespread clinical use of these agents, platelet activation and CTD thrombosis continues to occur, with devastating consequences, including device thrombosis, thromboembolic stroke, VAD pump stop and death^{23,24}. Our recent systematic analysis of antiplatelet agents has shown that, in fact, they have little to no efficacy in limiting SMPA. As such, these observations further confirm that SMPA mechanistically differs from conventional biochemical platelet activation.

In the clinical setting of CTD use, it is often necessary to differentiate thrombotic events driven by the implanted device versus events driven by biochemical mediators – e.g. in the setting of simultaneous infection or inflammation. In that context, to date, limited methods exist to facilitate this differentiation. Recent clinical studies monitoring platelet activation during CTD support have demonstrated that conventional biomarkers of platelet activation show only minor, inconsistent, or no changes, and ultimately do not correlate with coagulation tests and clinical outcomes^{25,26}.

We hypothesized that conventional biomarkers of platelet activation currently applied in clinical settings have limited ability under high shear conditions to: 1) detect early excessive levels of platelet activation, and 2) differentiate shear versus biochemical platelet activation. In the present study, we tested and compared molecular markers of activation driven by hypershear stress versus biochemical agonist exposure. We demonstrate that under high shear neither P-selectin nor $\alpha\text{IIb}\beta 3$ integrin activation reveal ongoing SMPA, being poor biomarkers. Rather, platelet annexin V binding, indicating phosphatidylserine externalization (PSE), and thrombin generation on the platelet surface are increased with SMPA, not only positively correlating with the magnitude of shear stress exposure, but also being increased even when low shear stress is applied. Further, we reveal that shear-mediated PSE is not associated with caspase 3 activation, but is significantly reduced (but not abolished) via extracellular calcium chelation. PSE, as a potential biomarker of SMPA phenotype was further validated with platelet exposure to dynamic hypershear in an *ex vivo* circulatory loop model utilizing a clinical VAD.

METHODS

Blood collection and platelet isolation

Blood was obtained from healthy volunteers, who were free of medications affecting platelet function for the prior two weeks. The study protocol #1810013264 “Optimization Cardiovascular & Mechanical Circulatory Support (MCS) Devices Thrombogenicity for Eliminating Anticoagulation” was approved by the IRB of the University of Arizona. Written informed consent was received from participants prior to inclusion in the study. Blood was drawn via venipuncture using a 21-gauge needle, and anticoagulated with acid citrate dextrose solution (85 mM trisodium citrate, 78 mM citric acid, 111 mM glucose), blood : ACD ratio – 10 : 1. Platelet-rich plasma (PRP) was obtained by blood centrifugation at 300g for 15 min at room temperature. Gel-filtered platelets (GFP) were isolated from PRP via gel-filtration as previously described²⁷. Platelet fractions were stored and handled at room temperature if not otherwise indicated. Platelet count was quantified with Z1 Coulter Particle Counter (Beckman Coulter Inc., Indianapolis, IN). Platelet viability in PRP and GFP was monitored using CellTiter-Glo[®] luminescent cell viability assay (Promega Corporation, Madison, WI).

Platelet aggregation and activation induced by biochemical agonists

Platelet aggregation was assessed employing the Platelet Aggregation Profiler PAP-8E (BioData Corporation, Horsham, PA). Prior to aggregation testing, PRP and GFP samples were diluted with modified Tyrode’s buffer to platelet count of 250,000 and 150,000 platelets/ μ L, respectively, and recalcified with 1 mM CaCl_2 . Then, 3 mg/ml human fibrinogen (Sigma-Aldrich, St. Louis, MO) was added to GFP (except thrombin-induced platelet aggregation). To initiate platelet aggregation, the following concentrations of biochemical agonists were added: 10 μ M ADP (Sigma-Aldrich, St. Louis, MO), 32 μ M Thrombin Receptor Activating Peptide 6 (TRAP-6, Roche Diagnostics GmbH, Mannheim, Germany), 10 μ g/mL collagen type I from calf skin (MP Biomedicals, Solon, OH), 1 mM arachidonic acid (BioData, Horsham, PA), 10 μ g/mL epinephrine (Sigma-Aldrich, St. Louis, MO), and 1 U/mL thrombin (Sigma-Aldrich, St. Louis, MO). Aggregation was recorded for 5 minutes at 37°C with 1000 rpm stirring.

To induce platelet biochemical activation, 100 μ L of recalcified GFP (20,000 platelets/ μ L, 2.5 mM CaCl_2) was treated with the aforementioned biochemical agonists and incubated undisturbed for 10 minutes at room temperature. No fibrinogen was added to the reaction well to avoid platelet aggregation.

Platelet activation by uniform and dynamic shear stress

GFP were exposed to uniform continuous shear stress utilizing a Hemodynamic Shearing Device (HSD) – a computer-controlled, modified cone-plate-Couette viscometer allowing application of uniform continuous shear stress of defined magnitude (30, 50 and 70 dynes/ cm^2)¹³. The shear stress conditions employed here were chosen based on our previous numerical studies of VAD hemodynamics, as compared with physiological levels of shear existing within normal circulatory conditions²⁸. Prior to an HSD run, a GFP aliquot was diluted with modified Tyrode’s buffer to a count of 20,000 platelets/ μ L and recalcified

(2.5 mM CaCl_2). Sheared platelet samples were collected after 0 and 10 minutes of shear exposure and processed immediately.

To apply dynamic shear stress, a VAD-driven circulatory loop was assembled incorporating an axial, continuous flow VAD, the HeartAssist V (MicroMed Technologies Inc., USA), 1/2" diameter non-thrombogenic Tygon[®] tubing, and 90°-angulated Teflon[®] connector mimicking the VAD outflow graft-aorta anastomotic angle. The HeartAssist V, while not in clinical use at present in the U.S. (though with human use outside the U.S.), was employed in our study as a reference VAD. Our previous numerical study using DTE indicated that the dynamic shear pattern of the HeartAssist V showed significant similarity to that of the FDA-approved Heart Mate II (HMII), though the HMII revealed a greater thrombogenic potential leading to significantly higher stress accumulations and platelet activation levels²⁹. Recalcified GFP (20,000 platelets/ μL , 2.5 mM CaCl_2) were recirculated through the loop under VAD-operating speed 8000 rpm at room temperature. Platelet samples were collected at 0, 2, 5, 10, 30, and 60-minute time points.

As a positive control for SMPA, platelets were activated by sonication (10 W for 10 s, Branson Sonifier 150 with microprobe, Branson, MO)³⁰.

Platelet calcium depletion

To evaluate the effect of calcium depletion on SMPA, GFP were treated with calcium chelators binding extracellular (EDTA) or intracellular (BAPTA-AM) calcium pools in platelets. Chosen concentrations of calcium chelators were shown to inhibit platelet membrane scrambling in previous studies. Specifically, non-recalcified GFP (20,000 platelets/ μL) were treated with 1 mM EDTA (Sigma-Aldrich, St. Louis, MO) for 10 min^{31,32} and subjected to shear stress of 30 and 70 dynes/ cm^2 in the HSD or sonication. Then, activated GFP were recalcified with 2.5 mM CaCl_2 and processed to detect annexin V binding as described below. Alternatively, non-recalcified GFP (100,000 platelets/ μL) were loaded with 20 μM BAPTA-AM (Cayman Chemical, Ann Arbor, MI) for 30 min with minimal agitation on orbital shaker at room temperature³³. Residual solution-phase BAPTA was washed via second gel-filtration of BAPTA-treated GFP. Then, BAPTA-loaded GFP was diluted and recalcified to undergo shear exposure in the HSD or sonication as described above.

Flow cytometry detection of platelet activation markers, mitochondrial membrane potential, and caspase-3 activation

Flow cytometry detection of platelet activation markers was performed following published recommendations^{34,35}. Briefly, 100 μL of recalcified GFP (20,000 platelets/ μL , 2.5 mM CaCl_2) activated with shear stress or biochemical agonists were stained with fluorescein-conjugated antibodies anti-CD41-APC (1:200, clone MEM-06, Thermo Scientific, Rockford, IL), anti-CD62P-PE (1:100, clone Psel.KO2.3, eBioscience, San Diego, CA), antiCD41/CD61-FITC (1:100, clone PAC-1, BioLegend, San Diego, CA) and annexin V-FITC (1:20, eBioscience, San Diego, CA) for 30 minutes at room temperature in the dark. Shear-stimulated and sonicated platelets were then fixed with 3.5% PFA for 20 minutes, to avoid dissipation of shear-mediated alterations of platelet morphology. Stained

and fixed platelet samples were washed by centrifugation (2300g, 5 min), and resuspended in 1 mL of 0.05 M phosphate buffer (pH – 7.4).

To detect alterations of platelet mitochondrial membrane potential, 100 uL of recalcified GFP (20,000 platelets/uL, 2.5 mM CaCl₂) activated with shear stress or biochemical agonists were incubated with the membrane permeabilizing mitochondrial probe 3,3'-Diethyloxycarbocyanine iodide (DiOC₆(3), Sigma-Aldrich, St. Louis, MO) at a final concentration of 7.2 nM and anti-CD41-APC (1:200) for 30 minutes at room temperature^{36,37}. Then, GFP aliquots were diluted with 1 mL of 0.05 M phosphate buffer (pH – 7.4) for flow cytometry analysis.

To track platelet caspase-3 activation, 100 uL of activated GFP (20,000 platelets/uL, 2.5 mM CaCl₂) were stained with membrane permeabilizing derivative of caspase-3 inhibitor FITC-Asp-Glu-Val-Asp-fluoromethyl ketone (FITC-DEVD-FMK, BioVision, Milpitas, CA) at final concentration 6 µM and anti-CD41-APC (1:200) for 30 min at 37°C³⁸. Then, GFP aliquots were diluted with 1 mL of 0.05 M phosphate buffer (pH – 7.4) for flow cytometry analysis.

Flow cytometry was conducted on FACSCanto II (BD Biosciences, San Jose, CA). Single platelets were distinguished from their aggregates and microvesicles based on their forward/side scatter characteristics, and 10,000 events were captured. Marker-positive platelets were identified within the CD41-positive platelet population based on their fluorescence intensity as compared with non-activated platelet group. In figures, flow cytometry data are reported as 1) typical peak diagrams “Fluorescence Intensity/Count” representing fluorescence intensity distribution within platelet population for every biochemical agonist and shear stress level tested, and 2) as bar graphs summarizing data obtained in as a series of experiments.

Assessment of Platelet Procoagulant Activity via Thrombin Generation

Platelet procoagulant activity was quantified using the chromogenic Platelet Activation State (PAS) assay³⁹. Following activation with biochemical agonists, shear stress or sonication, 100 uL of recalcified GFP (5,000 platelets/µL, 5 mM CaCl₂) were incubated with 200 nM acetylated prothrombin, 100 pM factor Xa (Enzyme Research Laboratories, South Bend, IN) in 20 mM HEPES buffer, pH 7.4, containing 130 mM NaCl and 0.1% BSA at 37°C for 10 minutes. Then, 10 uL of each GFP sample were tested for thrombin activity in microplate wells, containing 0.3 mM Chromozym TH (Roche Diagnostics GmbH, Mannheim, Germany), 3 mM EDTA. Kinetic changes of light absorbance ($\lambda = 405$ nm) were recorded for 7 minutes using a microplate reader Versa MAX (Molecular Devices Corp., Sunnyvale, CA). The rate of thrombin generation was calculated as a slope of kinetic curve (1/min) using SoftMax Pro6 software (Molecular Devices Corp., Sunnyvale, CA).

Statistics

Results from 6 to 10 independent experiments with different donors were summarized in plots. All flow cytometry and PAS assay samples were run in duplicate. The data were statistically analyzed using one-way analysis of variance (ANOVA) from GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Averages are reported as the mean $\pm$

margin of error. The level of statistical significance is indicated on figures as $p < 0.05$ (*) and $p < 0.01$ (**).

RESULTS

With the overall goal of determining if definable differences in biomarker expression exist for platelets activated via differing activation stimuli, we examined and compared the patterns of biomarkers expressed on platelets following exposure to shear stress (mechanical activation) and soluble agonists (biochemical activation). To separate platelets from other blood cells and plasma proteins, platelets were isolated from fresh blood via gel-filtration. Gel filtration procedure did not affect platelet viability as indicated by equal level of platelet ATP in PRP and GFP (Figure S1.A). Similarly, ATP level remained constant over GFP storage time up to 6 hours, as our experiments were conducted (Figure S1.B). To induce biochemical activation, platelets were stimulated by ADP, epinephrine, collagen, TRAP-6, thrombin, or arachidonic acid. Agonists' effective concentrations were chosen based upon their ability to induce platelet aggregation in PRP and GFP (Supp. Info. Figure S2). Mechanical platelet activation was achieved by platelet exposure to *uniform shear* in the HSD. Alternatively, platelets were subjected to *dynamic shear* stress in a VAD-propelled circulatory loop, emulating flow conditions within CTD-supported circulation. As essential markers of platelet activation, the following were detected: P-selectin, a marker of α -granule secretion; integrin α IIb β 3 activation; annexin V binding, a marker of PSE; and thrombin generation, a marker of platelet procoagulant activity. Then, the underlying mechanisms of shear-mediated alterations of platelet function were evaluated. Specifically, we tested if platelet exposure to shear stress promotes platelet a pro-apoptotic phenotype, i.e. mitochondrial membrane depolarization and caspase-3 activation, or is associated with alterations of calcium metabolism.

P-selectin exposure occurs following platelet activation with biochemical activators but not with shear stress

P-selectin (CD62P), an internal protein of platelet α -granule membranes, appears on the platelet surface following granule secretion as a result of fusion of α -granules with the external platelet plasma membrane during exocytosis⁴⁰. In our study, P-selectin exposure was detected by flow cytometry. Platelets revealing high fluorescence intensity (> 3000 AU) were gated as P-selectin positive population P6. A right-side shift of a fluorescence intensity peak along with the increase of P-selectin positive platelet number (% of platelets) indicates the increase of P-selectin exposure.

We found that all biochemical agonists induced P-selectin exposure, however, the extent of platelet activation notably differed between agonists tested. As shown in Figure S3, platelet stimulation with ADP and arachidonic acid resulted in prominent platelet activation, indicated by $46.8 \pm 3.9\%$ and $61.7 \pm 12.1\%$ of platelets presenting P-selectin on their surface. Following stimulation with thrombin and its mimetic TRAP-6, clear majority of platelets appeared as P-selectin positive. Epinephrine typically considered as a weak platelet agonist induced low P-selectin exposure, the number of CD62P-positive cells were as low as $18.8 \pm 1.9\%$. The narrow fluorescence distribution observed for TRAP-6, thrombin,

and arachidonic acid-stimulated platelets (Figure S3, peak diagrams “Fluorescence Intensity/Count”) suggested a uniform response of the platelet population to the biochemical stimuli with massive α -granule exocytosis.

In contrast to biochemical stimulation, platelets subjected to shear stress expressed low levels of P-selectin on their surface. As compared with intact platelets, the number of P-selectin positive platelets was not significantly elevated following 30 dynes/cm² and 50 dynes/cm² shear exposure and achieved only $18.8 \pm 4.2\%$ after exposure to 70 dynes/cm² (supraphysiologic shear) (Figure 1F). Sonicated platelets, a positive control for mechanical platelet activation⁴¹, similarly showed low level of P-selectin exposure on their surface (Figure 1F). The distribution of fluorescence intensity in the P-selectin positive population after SMPA is represented by wide low-pitched fluorescence peaks reported for every shear stress condition and sonication, indicating a very low, non-uniform activation response from the overall platelet population despite uniform shear stress applied (Figure 1A–E).

Biochemical agonists, but not shear stress, promote platelet integrin α IIB β 3 activation while shear stress yields a downregulation of α IIB β 3 surface expression.

Integrin α IIB β 3 activation is a vital step essential for platelet-platelet aggregation and thrombus formation. Integrin activation occurs as a result of conformational changes of the cytoplasmic domains of the glycoprotein complex, resulting in a dramatic increase of adhesivity to the protein ligands fibrinogen and vWF, with subsequent platelet aggregation⁴². To measure the extent of α IIB β 3 activation, we quantified and compared the number of all α IIB β 3-positive platelets with the number of platelets presenting the activated form of the integrin on their surface. Within the parental CD41-positive platelet population, CD41/CD61-positive cells were distinguished based on their fluorescence intensity (> 4000 AU) and defined as presenting activated α IIB β 3 (Subpopulation P3) (Figure S4 and 2).

We found that α IIB β 3 activation occurred only following platelet stimulation by some biochemical agonists but not others. As shown in Figure S4, the number of platelets presenting activated α IIB β 3 in ADP- and thrombin-treated cell groups was increased 4.2-fold and 9.6-fold, respectively, as compared to intact platelets. Taken alone, collagen, TRAP-6, arachidonic acid, and epinephrine failed to promote α IIB β 3 activation, since platelet count and fluorescence intensity remained unaltered or not significantly elevated as compared with baseline levels reported for intact platelets (Figure S4).

In contrast to biochemical stimuli, platelet exposure to shear stress did not promote α IIB β 3 activation, as the number and fluorescence intensity of CD41/CD61-positive platelets was not increased after 30, 50 or 70 dynes/cm² shear stress exposure (Figure 2A–D, and F). Even following sonication, which induces significant mechanical platelet activation, only $5.6 \pm 3.8\%$ of platelets expressed an activated form of α IIB β 3 (Figure 2E&F).

Phosphatidylserine externalization and thrombin generation on the platelet surface are induced by shear stress but not biochemical activation

Reorganization of the platelet membrane lipid bilayer and externalization of anionic phospholipids are late events of platelet activation, requiring a high, sustained intracellular calcium concentration^{43,44}. These anionic clusters, mainly composed of phosphatidylserine

and phosphatidylethanolamine, provide a surface for assembling coagulation factors, for facilitation of local thrombin generation on the platelet membrane. To detect PSE, in our study, the annexin V binding assay was employed. This assay is based on high affinity binding of protein annexin V to phosphatidylserine on platelet surface⁴⁵. Within the platelet population, two sub-populations were distinguished: a high annexin V sub-population P3 (fluorescence intensity > 4000 AU) and a low annexin V sub-population P4 (fluorescence intensity 500 – 4000 AU) (Figures 3 and 4).

Platelet activation by most biochemical agonists alone did not facilitate platelet PSE. Arachidonic acid was the only biochemical agonist shown to promote annexin V binding when all platelets became procoagulant. The number of platelets in the low and high annexin V binding sub-populations reached $38.8 \pm 4.6\%$ and $52.1 \pm 6.8\%$, respectively, versus $2.0 \pm 1.2\%$ and $0.4 \pm 0.3\%$ in the control group (Figure 3G & H). However, annexin V binding was not detected following platelet stimulation with ADP, epinephrine, or TRAP-6 (Figure 3B, C, & E). Collagen and thrombin induced a minor barely significant increase in the number of annexin V-positive platelets and their fluorescence (Figure 3D, F, & H).

In contrast, platelets subjected to uniform shear stress demonstrated prominent annexin V binding, indicating significant PSE on the platelet surface. As shown in Figure 4, the number of annexin V-positive platelets was evidently elevated after SMPA with a positive relationship to shear stress magnitude ($24.7 \pm 5.1\%$, $31.2 \pm 10.6\%$, and $46.6 \pm 12.0\%$ following 30, 50, 70 dynes/cm² shear, respectively, vs. $0.6 \pm 0.3\%$ in the control group, ANOVA: $p < 0.01$). It is worth emphasizing that significant intensification of annexin V binding (as shown by an increase of annexin V-positive platelet count) was observed even after platelet activation by relatively low shear stress (30 dynes/cm²) applied uniformly to the platelet population (Figure 4F). The distribution of fluorescence intensity within shear-treated platelet groups appeared as high narrow peaks (Figure 4B–D), indicating a uniformity of PSE response to shear stress. The fluorescence intensity tended to increase with the shear magnitude, although the significant change was shown only after platelet exposure to high shear stress (Figure 4G, “70 dynes/cm²” vs. “Intact platelets”, ANOVA $p < 0.01$). Sonication, as positive control for mechanical activation, indeed resulted in dramatic augmentation of annexin V binding, with $46.2 \pm 13.2\%$ of cells exposing phosphatidylserine on their surface (Figure 4E & F).

As indicated via the PAS assay, platelets stimulated with the majority of biochemical agonists did not catalyze thrombin generation (Figure 5A). Only arachidonic acid stimulation resulted in a distinct rate of thrombin generation on the platelet surface. SMPA however promoted notable procoagulant activity. Platelet shear exposure induced thrombin generation on the platelet surface (Figure 5B). The rate of thrombin generation was found to strongly correlate with the shear stress magnitude applied, reaching $67.2 \pm 5.8 \text{ min}^{-1}$ after 70 dynes/cm² shear.

Biomarker phenotype of elevated, constant shear-mediated platelet activation was similarly observed for elevated dynamic shear stress exposure in VAD circulatory flow loops

To compare platelet function alterations following SMPA induced by uniform shear stress exposure in the HSD with actual dynamic shear conditions existing within VAD-supported circulation, GFP were recirculated through a VAD-operated circulatory loop system. Serial timed samples were collected to record the dynamics of platelet activation. Platelet P-selectin exposure, as a representative *marker of platelet biochemical activation*, versus annexin V binding and platelet procoagulant activity, as *markers of SMPA*, were simultaneously evaluated. We found that platelets exposed to dynamic shear stress in the VAD-operated loops showed exponential augmentation of annexin V binding over time (Figure 6A). After 30 min- and 60 min-shear exposure, the number of annexin V-positive platelets was significantly elevated reaching $2.9 \pm 1.0\%$ and $7.0 \pm 0.7\%$ of the total population, as compared with $0.4 \pm 0.1\%$ in non-sheared platelets. The procoagulant activity of platelets exposed to shear stress in the VAD-operated loops was also drastically increased (Figure 6B). The rate of thrombin generation was approximately 7-fold increased after 60 min shear exposure to dynamic shear as compared with baseline level (ANOVA, $p < 0.05$). Simultaneously, no significant P-selectin exposure was detected in platelets despite 60 min of dynamic shear exposure (Figure 6C).

Platelet exposure to shear stress and biochemical agonists led to a decrease of mitochondrial membrane potential

To detect alterations of mitochondrial membrane potential following platelet activation with shear stress or biochemical agonists, we applied the lipophilic mitochondrial probe DiOC₆(3). This green fluorescing dye penetrates platelet membranes and accumulates in the mitochondrial matrix. Mitochondrial membrane depolarization results in a decrease of DiOC₆(3) accumulation and a corresponding dissipation of the fluorescent signal^{36,46}. We showed that platelet exposure to continuous, elevated shear stress resulted in a significant decrease in mitochondrial membrane potential. Thus, the number of DiOC₆(3)-positive platelets dropped to $76.0 \pm 5.1\%$ and $75.45 \pm 7.0\%$, after 50 and 70 dynes/cm² shear stress, respectively, as compared to non-sheared control (Figure 7A, ANOVA: $P < 0.01$ vs. "Intact platelets"). The DiOC₆(3) fluorescence intensity of sheared platelets tended to decrease gradually, with the increase of shear stress magnitude reaching as low as 0.55 ± 0.16 of the control value following 70 dynes/cm² shear (Figure 7B, ANOVA: $P < 0.01$ vs. "Intact platelets"). Sonication, as a positive control for platelet mechanical activation, led to a major decrease of DiOC₆(3)-positive platelet number, while fluorescence intensity decreased insignificantly. Platelet exposure to ADP and thrombin, taken as illustrative biochemical agonists, did not result in a significant change of DiOC₆(3)-positive platelet number, while significantly decreasing their fluorescence intensity (Figure 7A&B). Thus, DiOC₆(3) fluorescence intensity of ADP- and thrombin-activated platelets was 0.57 ± 0.33 and 0.49 ± 0.13 of the control value, respectively.

Shear-mediated platelet activation did not result in caspase-3 activation while sonication and biochemical agonists promote moderate caspase activation

Caspase-3 has been long recognized as a major executive caspase essential for PSE and membrane fragmentation associated with platelet apoptosis^{47–50}. To identify if activation of caspase-3 occurs during SMPA, we used FITC-conjugated tetrapeptide DEVD-FMK that penetrates platelet membranes and specifically binds to the active form of caspase-3^{46,48,51}. We showed that platelet exposure to continuous shear stress of the magnitudes tested did not result in caspase-3 activation. In Figure 8A, representative curves of FITC-DEVD-FMK fluorescence distribution in activated platelet population are shown. No fluorescence shift was noticed for platelets exposed to 70 dynes/cm² shear stress as compared with the fluorescence peak of control platelet group (Figure 8A, red vs. blue peak, correspondingly). Similarly, no significant changes in fluorescence intensity of sheared platelets were reported across all donors (Figure 8B), yet a tendency of a slight increase of fluorescence intensity with the increase of shear magnitude was noticed. Platelet sonication indeed resulted in a significant augmentation of caspase-3 activation, as indicated by notable right-side shift of FITC-DEVD-FMK fluorescence (Figure 8A, grey peak) and more than a two-fold increase of MFI (Figure 8B). Interestingly, platelet stimulation with biochemical agonists ADP and thrombin was also associated with an evident augmentation of FITC-DEVD-FMK fluorescence as indicated by a right-side shift of the fluorescence intensity peaks and corresponding 2.079- and 2.345-fold increase of MFI values in ADP- and thrombin-stimulated platelet groups (Figure 8A&B).

Chelation of platelet extracellular but not intracellular calcium inhibited phosphatidylserine externalization mediated by shear stress

Elevation in cytosolic calcium concentration via Ca²⁺ release from intracellular depots or Ca²⁺ influx from extracellular media is a critical downstream event occurring as a result of platelet activation⁵². The extent of platelet stimulation largely predefines the amplitude of cytosolic calcium rise, which then coordinates the type of platelet response. Calcium release from intracellular depots is sufficient to initiate platelet adhesion, shape change, integrin activation, and granule secretion, while platelet membrane scrambling and procoagulant activity requires platelet influx through plasma membrane calcium channels⁵³. In our study, to evaluate the contribution of extracellular and intracellular calcium pools in shear-mediated PSE, two calcium chelators were utilized. For intracellular calcium depletion, platelets were preloaded with BAPTA-AM and then exposed to shear stress in the presence of physiologic calcium concentration. For extracellular calcium depletion, EDTA was introduced in platelet media, and no exogenous calcium was added during platelet exposure to shear stress. We found that intracellular calcium chelation had no significant effect on shear-mediated PSE. Thus, the number of annexin V positive platelets in the BAPTA-treated group increased with the shear stress magnitude similar to control group (Figure 9A), reaching $9.8 \pm 1.7\%$ and $31.9 \pm 6.8\%$ after 30 and 70 dynes/cm², respectively. However, extracellular calcium chelation with EDTA resulted in a significant decrease of shear-mediated PSE, as indicated by a two-fold decrease of annexin V positive platelet number after platelet exposure to 70 dynes/cm² shear (Figure 9B, 70 dyn/cm²: “Control” vs. “-Ca²⁺ extra”, ANOVA: $p < 0.01$). Interestingly, PSE mediated by sonication was not significantly affected by any of the applied calcium chelation strategies, with resultant prominent annexin V binding on the

platelet surface in the presence of EDTA and BAPTA-AM being comparable to control level (Figure 9A&B).

DISCUSSION

The worldwide use of cardiovascular therapeutic devices continues to grow exponentially. Sadly, these devices remain limited by continued, unpredictable device-related thrombosis. Being able to define and discriminate whether platelet activation and resultant thrombosis is due to flow, i.e. SMPA, or rather due to biochemical factors, offers clinical diagnostic utility. Despite this need, limited data exists directly comparing both forms of activation under controlled conditions. Here, we clearly demonstrate that a distinct and differing pattern of readily detectable biomarkers exists between mechanical activation versus biochemical activation. In essence, a differentiating phenotype of SMPA is identifiable, which differs from that due to biochemical agonist activation.

Platelet activation due to shear stress has long been recognized as a triggering element essential for initiation and progression of arterial thrombosis. The traditional view is that shear-mediated binding of vWF to platelet GPIb facilitates platelet adhesion, inducing activation of integrin α IIb β 3, with subsequent platelet aggregation through α IIb β 3-fibrinogen interactions^{14,16}. However, according to this concept, platelet activation and aggregation mediated by shear stress is not sufficient to maintain stability of growing thrombus, and becomes irreversible only if platelets are co-stimulated with biochemical agonists (i.e. ADP, TXA₂, epinephrine, and thrombin) presented at the site of injury⁵⁴. Operating through fast-acting GPCR, biochemical agonists amplify platelet activation, inducing platelet granule secretion, supporting permanent α IIb β 3 activation and irreversible platelet aggregation⁵⁵. In contrast, under CTD-supported circulation, hypershear stress indeed is a primary mechanical force, directly inducing platelet activation^{13,17,18,56}. Shear-mediated blood damage also results in ADP leakage from damaged blood cells^{57,58}, activation of the coagulation cascade and rapid thrombin generation⁵⁹, all contributing to CTD-related thrombosis via paracrine activation of circulating platelets with these biochemical stimuli.

Identification of SMPA *in vitro* and *in vivo* to date has largely relied on measurement of biomarkers of biochemical platelet activation – platelet-specific proteins expressed on the membrane surface after activation, (e.g. activated form of integrin α IIb β 3, P-selectin, CD40R) or released via platelet granule secretion (i.e. platelet factor 4, β -thromboglobulin, CD40L)⁸. However, clinical attempts to monitor platelet activation in VAD-implemented patients have demonstrated that conventional biomarkers failed to differentiate platelet activation under the high shear conditions of CTD-supported circulation, from biochemical stimuli, showing no correlation with coagulation tests and clinical outcomes^{10,25,26}. Recent reports by Leytin's group showed that platelet exposure to hypershear stress *in vitro* promoted apoptosis-like events, i.e. expression of pro-apoptotic proteins, mitochondrial damage, membrane depolarization, shrinkage of their size, and fragmentation^{48,50}, suggesting that other biomarkers distinguishing shear-mediated changes of the platelet phenotype from biochemically-driven events could be defined.

Our experimental approach using GFP, a platelet suspension free from other blood cells and plasma components, allowed us to dissect shear-mediated alterations of platelet function apart from their cross-activation by biochemical stimuli. To emulate shear conditions of normal and CTD-supported circulation, GFP were exposed to shear in an HSD, a cone-plate-Couette viscometer designed to generate precisely controlled and uniformly distributed shear stress to all platelets¹³. This uniform shear system, an “idealized” alternative to experimental uncontrolled shear systems, allows us to observe the phenotypic range of platelet activation responses generated when every platelet experiences equal shear stress of a defined magnitude. Therefore, it is very likely that under such idealized conditions, as we have employed here, that a characteristic biomarker pattern of a platelet phenotypical response to shear stimulation would be revealed and amplified. Four well established markers of platelet activation were chosen to “sort out” their distribution between biochemical versus mechanical activation – P-selectin exposure and α IIB β 3 activation as those are routinely employed in the clinic^{9,26}, versus annexin V binding, as alternative marker of PSE often utilized to identify platelet procoagulant activity and apoptotic response^{48,54}, and thrombin generation, via the PAS or other assays^{39,60,61}.

We found that platelet activation by uniform shear stress induced prominent procoagulant activity, as indicated by both an increase of PSE and an accelerated rate of thrombin generation. The number of annexin V-positive platelets and thrombin generation rate grew in parallel with the magnitude of shear stress applied. Notably, even low shear stress (30 dynes/cm²), when applied uniformly to all platelets within the flow field, induced a significant increase in PSE and the thrombin generation rate. The last observation suggests that platelet membrane reorganization and procoagulant surface exposure occurs, and is detectable, in early stages of SMPA when platelets experience moderate shear stress, being typical of dynamic flow conditions of VAD-supported circulation *in vivo*.

Interestingly, neither P-selectin nor α IIB β 3 activation – conventional biomarkers of platelet activation – were able to validate ongoing SMPA. Even with sub-pathological shear stress exposure, platelet P-selectin exposure was mild and uneven. The limited capability of P-selectin in detecting platelet activation under continuous or pulsatile shear stress conditions was also reported in other *in vitro* studies^{48,62}. Recent clinical study suggested that long-term VAD support is associated with an acquired defect of α -granule secretion⁶³ which could also contribute to low levels of platelet P-selectin after SMPA. Our findings demonstrate that SMPA does not facilitate integrin α IIB β 3 activation. Yet, evidence supporting shear-mediated α IIB β 3 activation could be found in the literature. As such, Wu’s group noted that whole blood exposure to high shear stress *in vitro* resulted in α IIB β 3 activation, simultaneously causing its shedding from platelet surface⁶⁴. We suggest that integrin activation reported in that study might result not from SMPA itself, but rather from platelet cross-activation by biochemical agonists, i.e. ADP released from red blood cell damaged by shear stress, or from thrombin generation on the platelet surface following SMPA in whole blood. Here, we demonstrated that unlike shear stress both biochemical agonists, ADP and thrombin, promote high-level α IIB β 3 activation. In the clinic, long-term MCS was not associated with either integrin activation or alteration of its platelet surface expression^{10,26,65}. Recent findings of Wu’s group indeed reported the decrease of platelet

α IIB β 3 surface expression in VAD-supported patients with an accompanying increased risk of bleeding^{66,67}.

Our studies showed that a similar pattern of biomarker expression was observed following dynamic shear stress exposure, generated via flow through VAD-propelled circulatory flow loops, as with *in vitro* HSD exposure. This observed similarity of molecular marker patterns for platelet activation under dynamic and uniform shear stress conditions further supports the finding of a characteristic biomarker phenotype as an indicator of SMPA. To our knowledge, annexin V binding has not been routinely applied to monitor platelet activation in CTD implanted patients in the clinical setting. Recent reports indeed note that increased levels of platelet reactive oxygen species and apoptotic markers, including mitochondrial damage, caspase activation, and annexin V binding (as indicated by MFI), are associated with non-surgical bleeding events during long-term VAD support⁶⁷, underscoring the significant potential for alternative biomarkers in evaluating the risk of VAD-related adverse events. Further, a pilot study examining cf-LVAD-implanted patients has recently shown that the PAS assay, i.e. thrombin generation, is clinically useful in identifying patients with increased risk of device-related thrombosis⁶⁰. This observation was further confirmed by our group in a recent larger clinical study examining the PAS assay as marker or predictor of VAD thrombosis in 68 cf-LVAD patients implanted with either the HeartMate II, HeartMate 3, or HeartWare HVAD⁶⁸.

Analyzing the competence of biochemical agonists to induce platelet activation, we have found that all agonists promote a similar pattern of molecular biomarkers appearing on the platelet surface. The biomarker pattern of biochemical activation differs from the one identified for SMPA. As such, all biochemical agonists induce rapid platelet aggregation and notable P-selectin exposure yet fail to induce platelet PSE and procoagulant activity. It is known that platelet activation by soluble agonists, i.e. ADP, thrombin, epinephrine, is driven by fast-acting GPCRs⁵⁵. Due to amplification mechanisms underlying GPCR signaling, soluble agonists facilitate rapid changes in platelet physiology even when platelets are exposed to very low concentrations of biochemical stimuli. In our study, ADP and thrombin promote high levels of P-selectin exposure and integrin α IIB β 3 activation. While platelet stimulation by TRAP-6 and epinephrine resulted only in P-selectin exposure, with no α IIB β 3 activation detected. Observed variability in the platelet biochemical activation response reflects the diversity of GPCR types involved in its activation. Both powerful agonists, ADP and thrombin, simultaneously evoke multiple types of GPCRs (P2Y1 and P2Y12 for ADP, PAR1 and PAR4 for thrombin), while TRAP-6 and epinephrine activate only one GPCR type (PAR1 and α_2 -adrenergic receptor, correspondingly)⁵⁵. Our observations and finding of others⁶⁹ suggest that such cooperative signaling involving two or more GPCR types is essential to promote integrin activation as the later downstream activation event. However, neither ADP nor thrombin were capable of inducing platelet PSE or facilitating thrombin generation on the platelet surface, suggesting that additional co-stimulatory support, other than GPCR stimulation, is required to promote platelet membrane phospholipid scrambling.

Taken as an agonist of the TXA₂-evoked pathway, arachidonic acid is the only biochemical agent simultaneously provoking high-amplitude platelet aggregation, P-selectin exposure,

and profound PSE. The exclusive ability of arachidonic acid to induce such eclectic platelet responses could be explained by its heterogenous effect on cell functions. In platelets, arachidonic acid stimulates a dramatic increase in intracellular calcium concentration, through simultaneous Ca^{2+} release from intracellular stores and its influx through plasma membrane channels⁷⁰. Recently it was also shown that sub-millimolar concentrations of arachidonic acid induced platelet pro-apoptotic events, i.e. cleavage of procaspase 3, mitochondrial damage, and annexin V binding⁷¹. In cancer cells, an elevation of intracellular arachidonic acid level is also associated with the increase of mitochondria permeability, caspase 3 activation, PSE, following chromatin fragmentation, and rapid cell death^{72,73}.

In order to further tease out the underlying mechanism of shear-mediated PSE, we have tested two hypotheses: 1) shear-mediated PSE is a downstream event following mitochondrial membrane potential collapse and caspase-3 activation associated with platelet apoptosis; or 2) shear-mediated PSE is driven by calcium influx associated with platelet hyper-activation. We found that platelet exposure to continuous shear stress in the HSD in presence of physiological calcium concentration led to dissipation of mitochondrial membrane potential. Thus, the number and fluorescence intensity of platelets accumulating the mitochondrial probe DiOC₆(3) decreased with the level of applied shear stress (Figure 7). However, no significant activation of caspase-3, a primary apoptosis executor, was detected even after 70 dynes/cm² shear, the highest shear stress magnitude tested in our study (Figure 8). Platelet exposure to sonication, applied as a high-magnitude mechanical activation, indeed resulted in a notable decrease of mitochondrial membrane potential and nearly two-fold increase of active caspase-3 fluorescence. Taken together, these observations suggest that membrane scrambling caused by low and intermediate shear stress conditions tested in our study is not a downstream result of platelet proapoptotic alterations, while PSE mediated by rigorous extreme mechanical activation, i.e. sonication, might be associated with or be promoted by mitochondrial potential dissipation and caspase-3 activation. Our observations are in agreement with previous studies showing that only extremely high continuous shear stress of > 200 dynes/cm² facilitated platelet caspase activation and related PSE^{48,50}, while PSE induced by lower shear (i.e. rate below 6000 s⁻¹ or levels < 240 dynes/cm²) was only slightly inhibited by a pan-caspase inhibitor and thus was considered to be independent from platelet apoptosis⁵¹.

Although PSE is widely recognized as the "eat me" signal disclosed by cells undergoing apoptosis, in platelets, pro-apoptotic events could occur without any evidence of membrane phospholipid scrambling. Thus, weak biochemical agonists ADP, epinephrine, and thromboxane analog U46619 induce dissipation of membrane mitochondrial potential and gelsolin cleavage but not PSE on platelets⁷⁴. PSE induced by strong biochemical agonists, e.g. collagen and thrombin, requires co-stimulation by low shear stress exposure, and also was shown to occur independently from platelet apoptosis, secretion, and aggregation pathways⁵¹. Likewise, in our study, platelet activation with biochemical agonists ADP and thrombin alone led to a modest decrease of mitochondrial membrane potential, elevation of caspase-3 activity, granule secretion, and integrin activation, without evident annexin V binding indicating platelet PSE (Figure 7, 8 and 3B, F & H).

An alternative mechanism of platelet membrane scrambling, which is Ca^{2+} -induced, and energy- and apoptosis-independent, has been recently described to play a major role in platelet procoagulant activity and microparticle generation essential for hemostasis and thrombosis. Specifically, Ca^{2+} -activated ion channel and scramblase TMEM16F (anoctamin 6) has been reported as the main regulator and executor of PSE resulting from rapid, massive influx of Ca^{2+} following platelet hyperactivation via a cocktail of strong biochemical agonists, e.g. collagen + thrombin, or synthetic ionophores^{75–77}. Analyzing the sensitivity of shear-mediated PSE to platelet Ca^{2+} fluctuations, we found that depletion of platelet intracellular calcium with BAPTA-AM had no significant effect on shear-mediated membrane scrambling if physiological calcium concentration was present in the media (Figure 9A). However, when extracellular calcium was chelated with EDTA and no exogenous calcium was added, shear-mediated PSE was notably decreased (but not completely abolished) as indicated by a significant decrease of annexin V binding following shear exposure of EDTA-treated platelets (Figure 9B, 70 dyn/cm²: Control vs $-\text{Ca}^{2+}$ extra, ANOVA: $p < 0.01$). Interestingly, annexin V binding induced by sonication was not susceptible to chelation of the intra- or extracellular platelet calcium pool.

Summarizing our findings, we conclude that shear-mediated PSE largely depends on extracellular calcium entry, while intracellular calcium depletion does not appear to be crucial for shear-mediated membrane scrambling. Whether Ca^{2+} -activated TMEM16F scramblase is directly involved in shear mediated lipid scrambling, will require further study. Yet, even in the presence of exogenous EDTA, modest PSE was detected following shear stress exposure, indicating that an additional mechanism, other than a Ca -dependent mechanism, might contribute to shear-mediated loss of platelet membrane bilayer symmetry. As such, Kholmukhamedov et al. reported that shear-dependent binding of vWF to GPIb-IX could potentiate calcium- and caspase-independent formation of procoagulant platelets⁷⁸. Since GFP free from plasma proteins were used in our study, we believe that the contribution of this mechanism would be minor. On the other hand, we have previously demonstrated that shear stress exposure results in a significant increase in platelet membrane lateral fluidity⁷⁹. Therefore, we can not exclude that an increase in membrane fluidity might also be contributory to an increased susceptibility to lipid scrambling, thus facilitating Ca -dependent alteration of membrane symmetry.

Limitations.

Our study was conducted specifically with gel-filtered platelets in an attempt to segregate mechanical platelet activation by shear stress from paracrine cross-activation via biochemical agents (ADP or thrombin) inevitably released during exposure in whole blood or plasma containing platelet samples to shear stress^{57–59}. Therefore, our findings differ from other *in vitro* studies^{80,81} where platelet-containing samples, other than GFP, e.g. platelet-rich plasma or whole blood, were used. Specifically, prominent P-selectin exposure indicating α -granule secretion as well as $\alpha\text{IIb}\beta 3$ activation claimed to be mediated by shear stress might actually occur as a result of biochemical platelet activation by natural agonists – thrombin, ADP or hemoglobin, as shown elsewhere^{57–59}. Therefore in one of this studies, this shear-mediated integrin $\alpha\text{IIb}\beta 3$ activation was diminished by heparin anticoagulation preventing shear-mediated thrombin generation⁸⁰. Similarly, while we largely exclude vWF

mechanisms in this study, focusing on free flow mechanical activation in GFP, we cannot fully exclude a minor contribution of residual vWF or fragments to the observed platelet phenotypic changes.

Conclusion.

Under exposure to both constant and dynamic elevated shear stress, platelets express a characteristic pattern of biomarker expression, which differs from that induced by biochemical agonists. We observed this under both *in vitro* HSD, as well as with VAD circulatory flow conditions. Thus, PSE, as a marker composite of a shear phenotype, offers unique features: 1) *high sensitivity* to shear stress magnitude and application time; 2) *high selectivity* to SMPA versus biochemical activation; 3) *inter-species conservation*: since PSE clusters are conserved across mammalian species, the annexin V binding might be successfully employed during pre-clinical testing of CTD thrombogenicity in a large animal model. Taken together, identification and validation of specific biomarkers, suggests the potential for discrimination of SMPA versus biochemical platelet activation, offering a tool worthy of further translational study. Further validation of these discriminative biomarkers may provide a means to guide therapeutic choice for prevention and treatment of devastating adverse thrombotic consequence in otherwise vital cardiovascular therapeutic implant devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY TABLE

- 1.** What is known on this topic:

No specific biomarkers exists to detect shear-mediated platelet activation within mechanical circulatory support; the operative mechanisms of platelet activation by shear stress is poorly defined.
- 2.** What this paper adds:

The effects of shear stress and biochemical agonists on human platelet activation were compared; shear stress, but not biochemical agonists, promote phosphatidylserine externalization and thrombin generation on platelets; shear-mediated membrane scrambling is external calcium dependent and caspase-3 independent.

Figure 1. High shear stress induces mild platelet P-selectin exposure on platelet surface. Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were exposed to shear stress utilizing HSD constant modes 30, 50 or 70 dynes/cm² for 10 min or sonication at room temperature. **A – E:** representative peak diagrams of fluorescence intensity distribution in platelets after shear stress or sonication. **F:** P-selectin positive platelet number. Data of 6 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs. Intact platelets by one-way ANOVA: ** - $p < 0.01$.

Figure 2. Uniform shear stress does not promote α IIB β 3 integrin activation.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were exposed to shear stress utilizing HSD constant modes 30, 50 or 70 dynes/cm² for 10 min at room temperature. **A – E:** representative peak diagrams of fluorescence intensity distribution in platelets after shear stress or sonication. **F:** Number of platelets presenting active form of α IIB β 3 integrin. Data of 6 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs. Intact platelets by one-way ANOVA: ** - $p < 0.01$.

Figure 3. Biochemical agonists other than arachidonic acid do not induce platelet phosphatidylserine externalization.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were incubated with agonists undisturbed for 10 min at room temperature. Here, in figures 4 and 6 phosphatidylserine externalization was visualized using annexin V (FITC-conjugate, eBioscience, San Diego, CA) binding. **A – G:** representative peak diagrams of fluorescence intensity distribution in platelets after activation with biochemical agonists. **F:** annexin V positive platelet number. Data of 6 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs. Intact platelets by one-way ANOVA: * - $p < 0.05$, ** - $p < 0.01$.

Figure 4. Uniform shear stress and sonication induce platelet phosphatidylserine externalization. The extent of phosphatidylserine externalization increases dose-dependently with the shear stress magnitude applied.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were exposed to shear stress utilizing HSD constant modes 30, 50 or 70 dynes/ cm^2 for 10 min or sonication at room temperature.

A – E: representative peak diagrams of fluorescence intensity distribution in platelets after shear stress or sonication. **F & G:** annexin V positive platelet number and their fluorescence intensity, respectively. Data of 8 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs. Intact platelets by one-way ANOVA: ** - $p < 0.01$.

Figure 5. Biochemical agonists, with the exception of arachidonic acid, do not significantly increase thrombin generation rate, in contrast, uniform shear stress increases thrombin generation rate at low and high magnitudes:

A – platelets activated by biochemical agonists, **B** – platelets activated by shear stress.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were incubated with biochemical agonists or exposed to shear stress in the HSD for 10 min at room temperature. Thrombin generation was detected using chromogenic PAS assay. In bar graphs, data of 4 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs. Intact platelets by one-way ANOVA: ** - $p < 0.01$.

Figure 6. Dynamic shear stress within a VAD-propelled circulatory loop induces time-dependent increase of phosphatidylserine externalization (A) and thrombin generation rate (B), but not P-selectin exposure (C) on platelet surface.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were recirculated through the VAD-propelled circulatory loop for 1 hour at room temperature, timing GFP samples were collected and processed immediately. In bar graphs, data of 4–6 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs “0” time point by one-way ANOVA: * - $p < 0.05$, ** - $p < 0.01$.

Figure 7. Uniform shear stress, sonication, and to less extent biochemical agonists induce a decrease of mitochondrial membrane potential.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were exposed to shear stress in the HSD or incubated with biochemical agonists for 10 min at room temperature. Mitochondrial membrane potential was detected using a mitochondrial probe DiOC₆(3) (Sigma-Aldrich, St. Louis, MO). **A & B:** number and fluorescence intensity of DiOC₆(3)-loaded platelets following exposure to shear stress and biochemical agonists. Data of 7 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated vs Intact platelets by one-way ANOVA: ** - $p < 0.01$.

Figure 8. Uniform shear stress does not induce caspase 3 activation, while sonication and biochemical agonists potentiate an increase of caspase 3 activity.

Recalcified GFP (20,000 cells/ μ L, 2.5 mM CaCl_2) were exposed to shear stress in the HSD or incubated with biochemical agonists for 10 min at room temperature. Platelet caspase 3 activity was detected using FITC-conjugated caspase 3 inhibitor DEVD-FMK (BioVision, Milpitas, CA). **A**: representative diagrams of FITC-fluorescence intensity distribution in platelets: blue – intact platelets, red – 70 dynes/cm² shear stress, grey – sonication, green – ADP, maroon – thrombin. **B**: fluorescence intensity (shown as times increase of median fluorescence intensity). Data of 4 independent experiments with different donors are summarized, $M \pm SD$, ANOVA: * - $p < 0.05$, ** - $p < 0.01$.

Figure 9. Chelation of intracellular calcium with BAPTA-AM has no effect on platelet phosphatidylserine externalization mediated by shear stress or sonication (A). Chelation of extracellular calcium with EDTA inhibits shear-mediated phosphatidylserine externalization though has no effect on PSE mediated via sonication (B).

BAPTA-AM- or EDTA-treated GFP were exposed to shear stress in the HSD for 10 min or sonication. Platelet phosphatidylserine externalization was measured via annexin V binding. Data of 4–6 independent experiments with different donors are summarized, $M \pm SD$ as error bars are plotted. P values were calculated by one-way ANOVA: $p < 0.01$ as compared with Intact platelets with (##) or without (**) calcium chelators added; ns – no significant difference, $p > 0.05$.