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Quantification of Shear-Induced Platelet Activation: High Shear Stresses for Short Exposure Time

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Abstract: Thrombosis and thromboembolism are the life-threatening clinical complications for patients supported or treated with prosthetic cardiovascular devices. The high mechanical shear stress within these devices is believed to be the major contributing factor to cause platelet activation (PA) and function alteration, leading to thrombotic events. There have been limited quantitative data on how the high mechanical shear stress causes platelet activation. In this study, shear-induced PA in the ranges of well-defined shear stress and exposure time relevant to cardiovascular devices was quantitatively characterized for human blood using two novel flow-through Couette-type blood shearing devices. Four markers of platelet activation—surface P-selectin (CD62p), platelet-derived microparticles (PMPs), platelet-monocyte aggregation (PMA), and soluble P-selectin—were measured by flow cytometry and enzyme-linked immunosorbent assay (ELISA), respectively. The results indicated that PA induced by high shear stresses with short exposure time could be reliably detected with surface P-selectin, and, to a lesser extent, PMPs rather than soluble P-selectin. It was also verified that PMA can be a highly sensitive indirect marker of platelet activation. The quantitative relationship between percentage of activated platelets indicated by surface P-selectin expression and shear stress/exposure time follows well the power law functional form. The coefficients of the power law models of PA based on surface P-selectin expression were derived. **Key Words:** Blood-shearing device—Shear-induced platelet activation—Shear stress—Exposure time—Power law model.

Prosthetic cardiovascular devices are commonly used in surgical treatment or replacement of diseased cardiovascular organs (1–4). These prosthetic devices include heart valves, vascular grafts, stents, circulatory assist devices, blood oxygenators, and respiratory assist devices. Treatment with these devices increases survival for many patients with otherwise hopeless medical conditions. Unfortunately, these devices may often cause adverse pathological complications, particularly thrombosis and thromboembolism, which are directly related to nonphysiological conditions (flow and blood contacting surface) in these devices (5,6). Elevated and nonphysiological shear stresses commonly exist in these devices, either global or localized regions (7,8). It is well known that elevated shear stresses can cause platelet activation (PA) (8–10).

Platelets have been long regarded as the prominent cells involved in physiologic hemostasis and pathological thrombosis. When stimulated with agonists, such as adenosine diphosphate (ADP), collagen, or thrombin, a sequence of intracellular and molecular events occur in platelets, leading to structural and functional changes. Inside-out signaling leads to conformational changes of GPIIb/IIIa complexes (9), exposing conformation-dependent activation epitopes with high affinity for their ligands (fibrinogen, von Willebrand factor). The release reaction of platelets is associated with the neo-expression of α-granule glycoproteins such as P-selectin (CD62p) expressed on platelet surface (10) or in plasma of blood (soluble P-selectin) (11). In addition, platelet-derived microparticles (PMPs), small membrane vesicles, are released from the

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platelets after activation. PMPs are enriched with the membrane receptors for the coagulation factor, thus they can activate the coagulation cascade (12) and contribute to an acceleration of the thrombin generation (13). Additionally, activated platelets and monocytes can form aggregates via the binding of surface P-selectin on platelets to P-selectin glycoprotein ligand 1 (PSGL-1) on monocytes (14,15). All of these events can be used to represent or indicate PA.

Several investigative teams have been attempting to quantify the effect of shear stress on PA (16–19). In these studies, platelets were often exposed to the shear stress level under 50 Pa for extended duration (from a few seconds to minutes) using cone-plate viscometers or parallel flow chambers. The comparative study by Holme et al. (18). showed that high shear stress at 315 dynes/cm^2 (shear rate 10 500 s^{-1}), as encountered in severe atherosclerotic arteries, activated platelets and triggered platelet microparticle formation. In contrast, no significant PA or formation of platelet microparticles was observed at physiological shear rate (420 s^{-1}) or at the condition in arteries with a less severe stenosis (2600 s^{-1}). The study by Shankaran et al. (20) suggested that a threshold shear stress of 80 dyne/cm^2 is necessary to induce significant activation. The results of these studies have been used semi-quantitatively to illustrate relationships between fluid dynamics shear stress and potential clinical complications or cardiovascular diseases. However, in many cardiovascular blood-contacting devices, such as heart valves and ventricular assist devices (VADs), blood is often exposed to extremely high shear stresses (>100 Pa) for very short exposure times (<1 s) (21,22). Very few studies have been dedicated to understand how platelets respond to those high shear stresses for short exposure times and to quantify the relationships between shear stress and markers of PA, partially due to the limitation of the shear stress level which a viscometer or flow chamber could generate.

In this study, we characterized the shear-induced PA of human blood under nonphysiological shear stresses (from 25 to 350 Pa) with short exposure times (from 0.039 to 1.5 s) using two novel blood-shearing devices. Based on previous studies by our group (8,23) and others (19,24), shear stress that ranges from 25 to 350 Pa and the exposure times that range from 0.039 to 1.5 s should cover the shearing conditions existing in clinical VADs such as axial and centrifugal pumps. The most commonly used marker of PA, surface P-selectin, after exposure to high shear stresses for a short time, was quantified. Soluble P-selectin, PMPs, and platelet-monocyte aggregation (PMA) in blood were also examined. The functional relationship between shear-induced PA and shear stress/exposure time was derived based on surface P-selectin expression.

MATERIALS AND METHODS

Blood-shearing devices

Two novel blood-shearing devices were used in this study. The details of the design features and operational principles of the two devices can be found in reference (25). The first device (named as Hemolyzer-H) is an axial flow-through Couette device whose rotating spindle is supported with a pair of tiny pin-bearings, adapted from the adult Jarvik VAD. There is a small gap (0.1 mm) between the middle spindle surface and outer housing wall. The second device (named as Hemolyzer-L) is a centrifugal flow-through Couette device whose rotor is magnetically suspended with bearingless motor technology, adapted from the CentriMag VAD. The corresponding shear stresses range from 117 to 338 Pa for the Hemolyzer-H and from 21 to 212 Pa for the Hemolyzer-L, respectively, for a typical blood viscosity of 0.0036 Pa s.

When pressure is driven to flow through the narrow annular gap between the rotating inner spindle (or the magnetically suspended rotor) and the stationary outer housing in the axial direction, blood is exposed to a uniform high shear stress throughout the narrow gap. Computational fluid dynamics (CFD) simulations confirmed that a uniform high shear stress region can be generated inside the small gap while the shear stresses elsewhere are relatively small (25). The magnitude of the shear stress and exposure time can be controlled by adjusting the rotational speed of the spindle (or the rotor) and the axial flow rate, respectively. The equations for calculating the shear stress and exposure time were described previously (25). Since regions outside the gap have much larger dimension, shear stresses in those regions within these two Hemolyzers could be neglected, and the associated PA is also negligible.

Experimental procedure

Fresh human blood was drawn from 21 healthy donors, who did not take any medication for at least 10 days before the blood donation and mixed with anticoagulant ACD-A (9:1). The blood collection was carried out in accordance with the protocol approved by the Institutional Review Board for human subject research. All donors signed a consent form before the blood donation and were informed

of the purpose of the study. All experiments were conducted at room temperature.

The viscosity of blood was first measured with semi-micro viscometers (CANNON Instrument Company, State College, PA, USA). Blood-contacting surfaces, including tubing, connectors, and the blood-shearing device, were rinsed with saline before each experiment. Blood was pumped by a syringe pump (PHD2000, Harvard Apparatus, Holliston, MA, USA) through the blood-shearing devices in a single pass. Flow rates were chosen according to desired exposure time and rotating speeds of the spindle, or the rotor was selected according to desired shear stress levels. The baseline blood sample was collected at the inlet of the shearing devices. For each condition (combination of shear stress level and exposure time), blood samples were collected from the outlet of the shearing devices. The level of shear-induced PA was indicated by the difference in measured quantity of PA markers between the sheared blood samples and the baseline sample.

Initially, the four markers of shear-induced PA were quantified at three levels of shear stresses (150, 225, and 300 Pa) for two exposure times (0.05 and 0.5 s) using the Hemolyzer-H. For each combination of shear stress and exposure time, the blood-shearing experiments were repeated six times. Blood preparations from six different donors were used. Because the viscosity of blood from different donors was slightly different, rotational speeds were adjusted accordingly to achieve the same shear stress levels. For each blood preparation, the data at the six experimental conditions were collected. Table 1 lists the shear stresses, rotating speeds, exposure times, flow rates of the six testing conditions (three shear stress levels and two exposure time), and dynamic viscosity based on the blood of one typical donor. To collect the additional data for deriving the model of shear-induced PA, the uniform experimental design was used to select the experimental conditions (26). The shear stress range was set to be $25 \le \tau \le 350$ Pa, and the exposure time was set to be $0.039 \le t \le 1.5$ s. Blood was exposed to high shear stresses ranging from 150 to 350 Pa in Hemolyzer-H and low shear stresses ranging from 25 to 125 Pa in Hemolyzer-L, respectively. The data of shear-induced PA under additional 31 experimental combinations of shear stresses and exposure times were collected. Under each of these additional experimental conditions, the blood-shearing experiment was repeated at least two times.

Flow cytometry assays

Whole blood flow cytometric assay was used to determine PA in collected blood samples. Fluorescein isothiocyanate (FITC) conjugated anti-CD61 antibody (catalog number 555753, clone VI-PL2, BD Biosciences, San Jose, CA, USA) and phycoerythrin (PE) conjugated anti-CD62P antibody (product code MCA2420PE, clone Psel.KO.2.12, AbD Serotec, Raleigh, NC, USA) were used for identifying platelet population and determining activated platelets, respectively. Briefly, 5 μL of whole blood was mixed with 25 μL of 10 mM HEPES buffer and incubated with 10 μL anti-CD61 antibody and 10 μL anti-CD62p antibody for 20 min. ADP (200 μM)-stimulated blood sample was used as the positive control. Blood samples incubated with PE-conjugated IgG1 antibody (product code MCA928PE, AbD Serotec), which is the isotype of PE-conjugated anti-CD62P, were used as the negative control. The samples were fixed with 1% paraformaldehyde (PFA) in phosphate buffered saline (PBS) and stored at 4°C before flow cytometric analysis. PA was expressed as the percentage of CD62p positive platelets out of total platelets.

The PMPs detection was carried out according to the method described in the reference (27). The PMP count was determined according to forward scatter (FSC) characteristics of all CD61 positive events. Briefly, CD61 positive events were divided into subpopulations according to FSC characteristics corresponding to their size. The 2.5% smallest particles in the baseline blood sample (un-sheared) were defined as PMPs. The corresponding gate for the 2.5% smallest particles in the baseline sample was used to determine PMPs in all other sheared blood

TABLE 1. *Experimental parameters for three shear stress levels and two exposure times based on one donor's blood*

Experimental condition	Shear stress τ (Pa)	Rotational speed (rpm)	Exposure time t (s)	Flow rate (mL/min)	Dynamic viscosity of blood (Pa s)
1	150	4613	0.05	13.7386	0.0045
2	225	6920	0.05	13.7386	
3	300	9227	0.05	13.7386	
4	150	4613	0.5	1.3739	
5	225	6920	0.05	1.3739	
6	300	9227	0.05	1.3739	

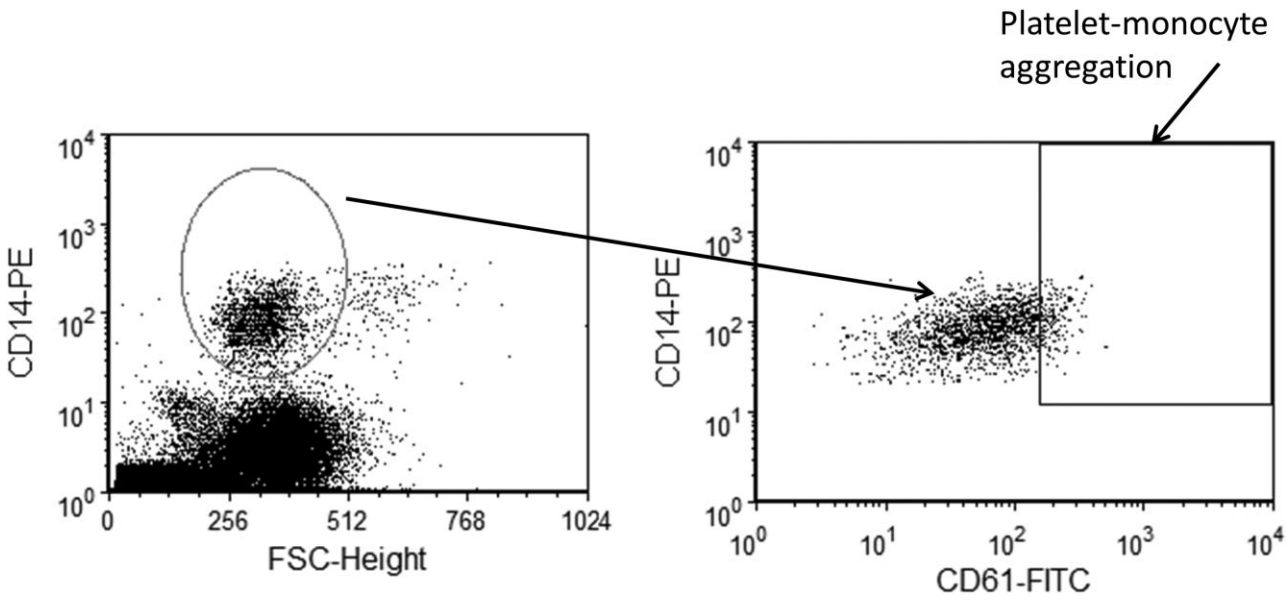

FIG. 1. Identifying PMA from lysed blood sample labeled with anti-CD61-FITC and anti-CD14-PE by flow cytometry. (A) Monocytes were distinguished from other blood cells by setting a CD14 positive gate in CD14-PE/FSC dot plot. (B) In CD14 positive gated events, platelet-monocyte aggregation was distinguished from other cells by setting CD61 positive gate. Platelet-monocyte aggregation was both CD14 and CD61 positive events.

samples. The shear-induced PMP generation was expressed as the percentage of PMP events in total CD61 positive events.

For detecting the PMA, whole blood was labeled with FITC conjugated anti-CD61 antibody (BD Biosciences) and PE conjugated anti-CD14 antibody (product code MCA1568PE, clone TÜK4, AbD Serotec). Thereafter, samples were fixed and red blood cells were lysed using ACK lysing buffer (Lonza, Walkersville, MD, USA). The PMA analysis was performed with the flow cytometer initially triggering on FSC (size) and then on the fluorescence channel 2 to identify CD14-PE positive events (monocytes). Platelet-monocyte aggregates were defined as the events positive for both CD61 and CD14. The gate of CD61-FITC positive was defined by using its isotype (IgG1k-FITC, catalog number 555748, clone MOPC-21, BD Biosciences) in negative control. Figure 1 shows the dot plots for PMA detection procedure. The PMA was expressed in percentage of both CD14 and CD61 positive events out of total CD 14 positive events. All flow cytometric data were recorded with a four color flow cytometer (FACS Calibur, BD Biosciences) and analyzed offline using the software FCS Express 3.0 (De Novo Software, Los Angeles, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Aliquots of blood samples were centrifuged at $160 \times g$ for 15 min at room temperature to obtain supernate. Cell-free plasma (CFP) was obtained by further centrifugation of the supernate at 14 000 rpm ($18\,800 \times g$) for 15 min at 4°C and was stored at –80°C for analysis. A commercial ELISA kit for human P-selectin (R&D Systems, Minneapolis, MN, USA) was utilized to quantify the concentration of soluble P-selectin in CFP as described previously (28).

Data analysis

Data are presented as mean ± standard error of mean (SEM). Statistical analysis was performed with the Student's *t*-test. Significance level was set at $P < 0.05$. A multivariate linear regression analysis was performed to fit the data of the measured markers of shear-induced PA with shear stress and exposure time to derive the model of shear-induced PA. *F*-test (26) was implemented to test the hypothesis of the power law model. Coefficient of determination was applied to indicate the goodness of fit for model coefficients.

RESULTS

Figure 2 shows the percentage of activated platelets induced by three levels of high mechanical shear stresses (150, 225, and 300 Pa) for durations of 0.05 and 0.5 s, respectively. In general, the level of PA indicated by P-selectin expression on the platelet surface increased with applied shear stress and exposure time. For the exposure time of 0.05 s, the percentage of activated platelets induced by the shear

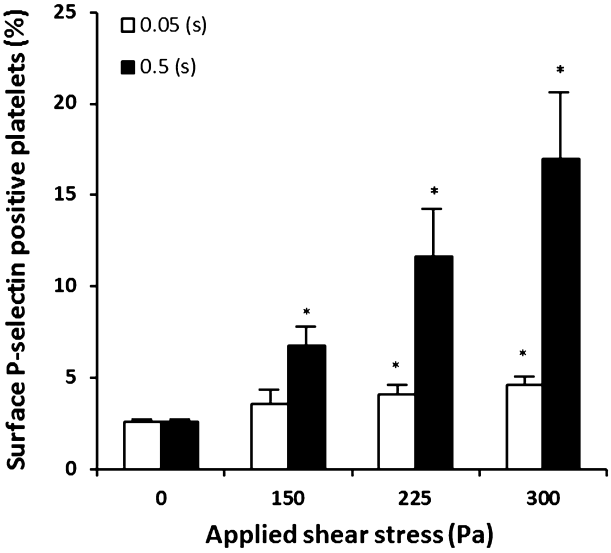

FIG. 2. The percentage of surface P-selectin (CD62p) positive platelets of total platelets in different conditions of shear stresses with exposure time ($n = 6$).

stresses of 150, 225, and 300 Pa increased from $2.60 \pm 0.12\%$ for the baseline blood sample (0 Pa), to 3.59 ± 0.78, 4.10 ± 0.49, and $4.59 \pm 0.50\%$. The increase in the PA became significant when the shear stress was at 225 Pa. When the exposure time was increased to 0.5 s, the percentage of activated platelets induced by the shear stresses of 150, 225, and 300 Pa increased from $2.60 \pm 0.12\%$ for the baseline to 6.74 ± 1.08, 11.66 ± 2.61, and $16.99 \pm 3.65\%$, respectively. These increases were significant compared with the level of activated platelets in the baseline. When comparing the percentages of activated platelets induced by the same level of shear stress for the two exposure times, the differences were all significant (not shown in Fig. 2).

Figure 3 shows the changes in the other three indirect markers of PA in the blood samples after being exposed to the three levels of shear stresses for 0.05 and 0.5 s, respectively. There was a slight increase in the concentration of soluble P-selectin in the blood samples for the three levels of shear stresses for the exposure time of 0.05 s (Fig. 3A). However, the

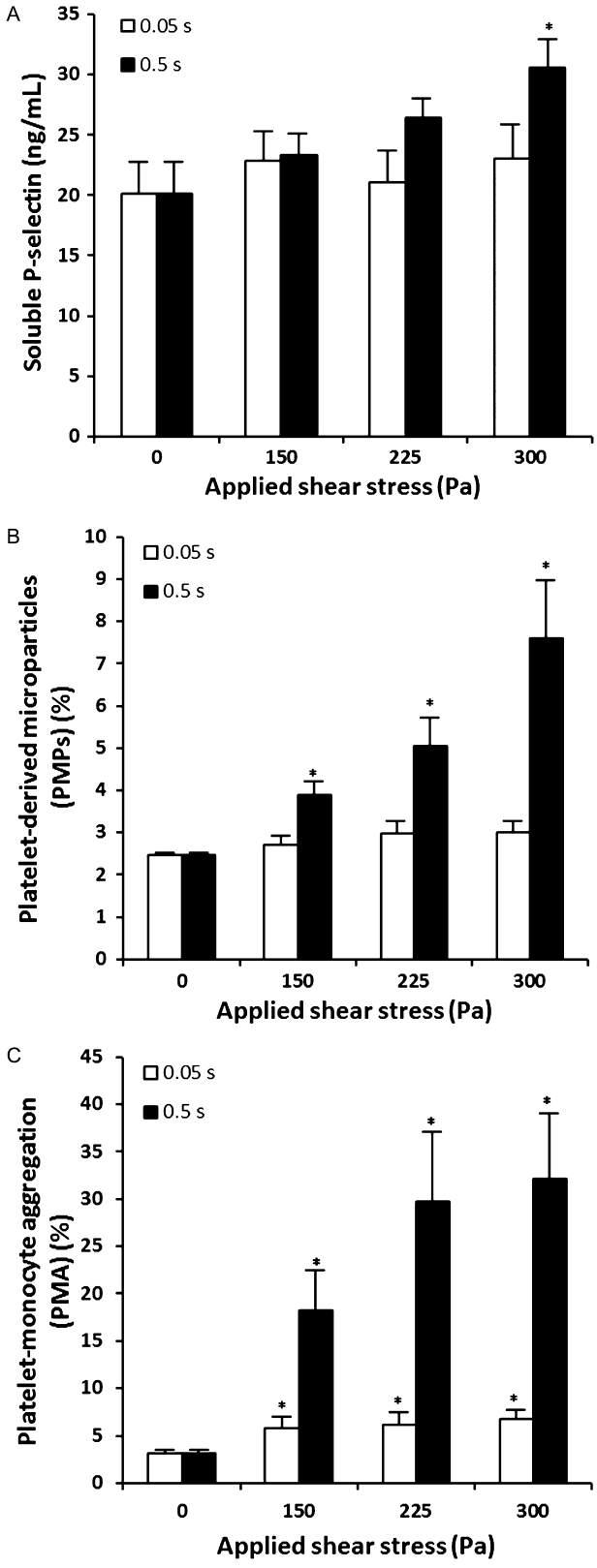

FIG. 3. (A) The concentration of soluble P-selectin (CD62p) in the CFP of blood in different conditions of shear stresses and exposure time ($n = 6$). (B) The percentage of PMPs out of total CD61-positive events in different conditions of shear stresses and exposure time ($n = 7$). (C) The percentage of PMA (both CD61 and CD14 positive events) out of total CD14-positive events in different conditions of shear stresses and exposure time ($n = 6$). Shear stress equal to zero is baseline. The asterisk (*) represents significant difference ($P < 0.05$) between the sheared condition and baseline. Solid bars and error bars represent mean ± SEM.

increase of soluble P-selectin became apparent as the exposure time increased to 0.5 s. The concentration of soluble P-selectin only became significantly different at a shear stress of 300 Pa for the exposure time of 0.5 s from that in the baseline. Overall, the relative change of soluble P-selectin in the sheared blood samples was smaller compared with surface P-selectin expression of activated platelets.

Both the PMPs and PMA in the sheared blood samples exhibited a similar increasing trend as surface P-selectin of activated platelets when the applied shear stress and exposure time increased (Fig. 3B,C). The generation of PMPs became significant at the shear stress of 150 Pa for the exposure time of 0.5 s compared with the PMPs in the baseline. The PMA became significant at the shear stress of 150 Pa for the exposure time of 0.05 s. It is interesting to note that about 5 to 6% of monocytes formed aggregates with platelets for three levels of shear stresses for exposure time of 0.05 s. When the exposure time increased to 0.5 s, 18 to 32% of monocytes aggregated to platelets.

To derive a quantitative relationship between PA and applied shear stress/exposure time, the power law model was used by multivariate regression to fit the data of shear-induced PA under 37 experimental conditions. Figure 4 shows the measured data points (green dots) of PA based on surface P-selectin expression induced by the 37 combinations of shear stress and exposure time collected from the two Hemolyzers and the surface of the fitted power law relationship. When the human blood was exposed to the shear stress of 350 Pa for 1.5 s, almost half of the platelets became activated. As expected, the shear-induced PA increased nonlinearly with both shear stress and exposure time. The *F*-test was performed by transforming the experimental data and the fitted power model into linear form in log scale. Based on the *F*-test, the linear model for shear-induced PA in log scale ($F_{35,\ 60} = 1.6475$, $P = 0.0440$) could not be rejected. The power law model of shear-induced PA was obtained with a coefficient of determination of 0.7963 (R^2):

$$\mathrm{PA} = 4.08 \times 10^{-5} \times \tau^{1.56} \times t^{0.80}$$

where τ is shear stress applied on blood and t is exposure time. The results of PA are obtained in decimals from the equation above given shear stress levels and exposure time, and can be converted to percentages by multiplying them by 100. This model can be used to estimate the percentage of activated platelets induced by the local high shear stress at a particular high shear region of blood contact cardiovascular devices. The shear stress field and exposure time can be obtained through CFD analysis.

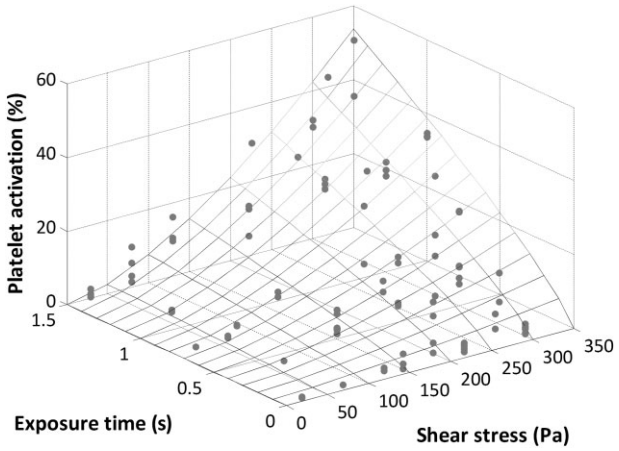

FIG. 4. The power law representation of shear-induced platelet activation based on surface P-selectin expression with the experimental data points.

DISCUSSION

Direct or indirect markers of PA (surface P-selectin, soluble P-selectin, PMPs, and PMA) of human blood were quantified after being exposed to a matrix of high shear stresses (150 to 300 Pa) for a short time (0.05 and 0.5 s). These levels of shear stresses represent typical nonphysiological conditions in most cardiovascular devices. It was shown that surface P-selectin and PMA increased significantly as the shear stress increased for the exposure time of both 0.05 and 0.5 s, whereas PMPs only rose significantly as the exposure time increased to 0.5 s. The concentration of soluble P-selectin did not change significantly for most of the conditions. Based on this observation, an expanded experiment was performed to derive the functional relationship between shear-induced PA and shear stress/exposure time. The power law relationship was found to fit well the experimental data based on the surface P-selectin expression with the multivariate regression. The derived power law model of shear-induced PA had the coefficient of determination around 0.8 and should have a fairly well predictive power.

PA is a complex process, involving a sequence of intracellular and molecular events in platelets, including physical changes of receptors, secretion of intracellular contents, development of procoagulant surface, generation of PMPs, and formation of PMA. Markers representing these changes have been proposed to indicate PA in the literature. Several studies have been performed to relate shear stress and exposure time to PA. These models include linear model

(29), power law model (21), cumulative power law model (30), and modified power law model combined with dynamic loading (31). Among those models, the power law model is most commonly used. A power law relation of the relative concentration of lactate dehydrogenase (LDH) from platelet lysis to shear stress (Pa) and exposure time (s) ($A = 3.31 \times 10^{-6}$; $\alpha = 3.075$; $\beta = 0.77$) was initially proposed by Giersiepen et al. (21). Although agonists such as ADP activating other platelets is believed to be released from platelet lysis (32,33), production of LDH is not directly relative to PA, and therefore may not be used as a direct indicator of PA. The study by Sheriff et al. used prothrombinase-based platelet activation state (PAS) assay to derive a power law model ($A = 1.47 \times 10^{-6}$; $\alpha = 1.04$; $\beta = 1.30$) (31) (shear stress: dyne/cm^2, time: sec). In the present study, we based on surface P-selectin expression to derive the power law relationship of PA ($A = 4.08 \times 10^{-5}$; $\alpha = 1.56$; $\beta = 0.80$) (shear stress: Pa, time: s). The coefficient A of this study is one order higher in magnitude than that in Sheriff et al.'s study. However, the coefficients α and β are in the same order. Sheriff et al.'s PAS assay measured the thrombin generation of certain condition normalized to that of totally activated platelets. However, the P-selectin assay in the present study presented the percentage of activated platelets of total platelets using surface P-selectin expression. Therefore, the two assays, which measured different aspects of PA and expressed results in distinct ways, might contribute to these differences. Further, Sheriff et al. used a hemodynamic shearing device similar to the cone plate viscometer to apply low shear stresses (1–7 Pa) for long exposure time (1–4 min), whereas this study utilized hemolyzers to apply high shear stresses (25–350 Pa) for short exposure time (0.039–1.5 s). Platelets may react diversely to distinguished shear stresses and exposure time ranges, leading to the difference of the coefficients.

Linear model of PA (assuming $\alpha = \beta = 1$) was also proposed to evaluate thrombogenic potential of implantable cardiovascular devises (29). The advantage of the linear model is easier to be incorporated into CFD simulations because of its linearity. However, compared with the linear model, the power law model (30,34) is preferred in predicting PA by CFD because it fits the results of in vitro studies better (31,35).

Soluble P-selectin, PMPs, and PMA were also investigated as markers of shear-induced PA in this study. The soluble P-selection was relatively insensitive compared with the surface P-selectin expression. The measured quantities of PMPs and PMA induced by the three levels of shear stresses for the two different exposure times exhibited the similar trend as that for surface P-selectin expression. By comparison, PMA was more sensitive than PMPs. As PMA is a subsequent step of P-selectin expression on platelets (14,15), it is not surprising that PMA had a similar sensitivity as the surface P-selectin. The rising trend of PMA induced by shear stress in our study is consistent with previous studies (36,37). However, because PMA is not directly related to PA, it can only be used as an indirect marker of PA, with high sensitivity for shear stress from 150 to 300 Pa with the exposure time of 0.05 and 0.5 s. Overall, surface P-selectin expression was proved to be a sensitive direct marker of shear-induced PA under high shear stress (150–300 Pa) for short exposure time (0.05–0.5 s) in the present in vitro study. The data from the present study may be used for estimating device-induced PA by linking fluid mechanical shear stresses in a device to the relationship of shear stress/exposure time and PA. As the data were obtained from the single pass in vitro experimental condition, extrapolation of the data to in vivo settings should be carried with caution. It has been reported that CD62p (P-selectin) expressed on platelet surface could be cleaved in vivo and result in the increase in soluble P-selectin (38). Thus, soluble P-selectin may be a more sensitive marker for initial PA than the expression of P-selectin on the surface of platelets in clinical setting (39,40). Additionally, PMA was also proved to be a more sensitive marker of in vivo PA than surface P-selectin (41).

CONCLUSION

Platelet activation induced by high shear stresses with short exposure time could be reliably detected with surface P-selectin, and, to a lesser extent, platelet-derived microparticles rather than soluble P-selectin. platelet-monocyte aggregation can also be a highly sensitive indirect marker of PA. The shear-induced PA generally elevated with increasing shear stress magnitude and exposure time. The quantitative relationship between percentage of activated platelets indicated by surface P-selectin expression and shear stress/exposure time closely follows a power law functional form. The coefficients of the power law model of shear-induced PA based on surface P-selectin were derived with the coefficient of determination of above 0.79.

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