Duration of exposure to high fluid shear stress is critical in shear-induced platelet activation-aggregation

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Summary
Platelet functions are increasingly measured under flow conditions to account for blood hydrodynamic effects. Typically, these studies involve exposing platelets to high shear stress for periods significantly longer than would occur in vivo. In the current study, we demonstrate that the platelet response to high shear depends on the duration of shear exposure. In response to a 100 dyn/cm² shear stress for periods less than 10-20 sec, platelets in PRP or washed platelets were aggregated, but minimally activated as demonstrated by P-selectin expression and binding of the activation-dependent αIIbβ3 antibody PAC-1 to sheared platelets. Furthermore, platelet aggregation under such short pulses of high shear was subjected to rapid disaggregation. The disaggregated platelets could be re-aggregated by ADP in a pattern similar to unsheared platelets. In comparison, platelets that are exposed to high shear for longer than 20 sec are activated and aggregated irreversibly. In contrast, platelet activation and aggregation were significantly greater in whole blood with significantly less disaggregation. The enhancement is likely via increased collision frequency of platelet-platelet interaction and duration of platelet-platelet association due to high cell density. It may also be attributed to the ADP release from other cells such as red blood cells because increased platelet aggregation in whole blood was partially inhibited by ADP blockage. These studies demonstrate that platelets have a higher threshold for shear stress than previously believed. In a pathologically relevant timeframe, high shear alone is likely to be insufficient in inducing platelet activation and aggregation, but acts synergistically with other stimuli.

Keywords
Shear stress, stenosis, platelet activation, aggregation

Introduction
Until recently, platelet functions were traditionally measured under static conditions or with low speed stirring, for example, as found in a lumiaggregometer. This approach fails to consider the hydrodynamic effects that platelets experience in flowing blood in vivo. Two types of instruments are now commonly used to assess platelet behaviour under shear stress: a parallel-plate flow chamber system and a cone-and-plate viscometer (1). The former is best used to evaluate adhesion interactions between platelets and immobilized substrates (endothelial cells and subendothelial matrix), whereas the latter is commonly used to test platelet activation and aggregation in fluid phase, a process representing what may occur in a stenotic vessel (1, 2). These technologies have provided new insights into how fluid shear stress affects platelet interactions with other platelets, and
with leukocytes, erythrocytes, and endothelial cells (1-3). Using a cone-and-plate viscometer, it has been demonstrated that high flow shear stress can activate and aggregate platelets, a process that is initiated by a GP Ib-VWF interaction and requires platelet integrin αIIbβ3 (4-9). Recent studies showed that shear-induced platelet aggregation was enhanced in patients with acute myocardial infarction compared to control subjects (6-13). An obvious concern regarding this experimental approach is that the time of high shear exposure is significantly longer than would be expected to occur in the area of localized stenosis in vivo. For example, it takes approximately 60 sec for blood to make a complete circuit through the vasculature (14). Flow rates through the internal carotid arteries and coronary arteries are 200 to 300 cm/sec (15-18) and 10 to 25 cm/sec (19-22), respectively, such that platelets are exposed to potential areas of high shear for less than a few seconds. It is, however, largely unknown how or whether platelets respond to such short high shear pulses that last no more than a few seconds. Recently, we found that platelets in plasma aggregated in response to a high shear pulse (100 dyn/cm²) lasting as short as 2.5 seconds, but platelet aggregation under this condition involves minimal activation as determined by P-selectin expression. This distinction is critically important in understanding the effects of high fluid shear stress on platelets and other blood cells. Here, we present results from experiments designed to further explore how platelets respond to short high shear pulses.

Materials and methods

Blood sample collection and platelet preparation

These studies were approved by the Institutional Review Board for Human Subject Research for Baylor College of Medicine. Blood from 26 healthy donors, who were medication-free for at least two weeks prior to phlebotomy, was collected in a syringe containing 0.38% sodium citrate (final concentration). Blood was also collected in 75 µM of PPACK (final concentration) to compare the effects of calcium on platelet activation and aggregation. Our donor pool consisted of 10 males and 16 females (24-52 years old). Samples were used as either whole blood or platelet-rich plasma (PRP). To obtain PRP, blood was centrifuged at 150 × g for 15 min at 24°C to remove red blood cells and leukocytes. Platelet counts in PRP were determined by a Coulter Counter (model Z2, Beckman-Coulter, Miami, FL) and normalized to 2-3 × 10⁸ platelets/ml using platelet-poor plasma (PPP), which was obtained by centrifuging PRP at 1500 × g for 10 min at 24°C.

Shear-induced platelet aggregation

Whole blood or PRP (500 µl) was loaded onto a cone-and-plate viscometer (RS1, HAAKE Instrument Inc., Paramus, NJ) and exposed to a pathological level of shear stress of 100 dyn/cm² for various time intervals (23). This level of shear stress can be found in the area of arterial stenosis. The time from zero to a desired shear stress takes 0.1 sec.

Shear-induced platelet aggregation was determined as the percentage reduction of single platelets as compared to the unsheared controls from the same donors as described previously (4, 23). Briefly, ten microliters of sample was fixed in 0.5% glutaraldehyde and the platelet count was determined by a Z2 Coulter Counter (for sheared PRP) or a Coulter Epics XL-MCL flow cytometer (Beckman-Coulter) (for sheared whole blood). Preliminary testing showed that aggregation obtained from both methods was closely correlated and the fixative glutaraldehyde had no effect on platelet activation and aggregation. As a positive control, we also measured the extent of platelet aggregation in response to 20 µM ADP.

We also determined shear-induced reversible platelet aggregation of paraformaldehyde fixed platelets. In this case, PRP was prepared from whole blood collected into acid-citrate dextrose as anticoagulant (85 mM sodium citrate, 111 mM glucose, and 71 mM citric acid, 10% v/v). To isolate platelets, PRP was centrifuged at 900 × g for 10 min at 24°C, the platelet pellets were washed once with a CGS buffer (13 mM sodium citrate, 30 mM glucose and 120 mM sodium chloride, pH 7.0), and resuspended in phosphate buffered saline (PBS) containing 1% paraformaldehyde. After a 20 min incubation at room temperature, platelets were washed with PBS and resuspended in autologous plasma to a final platelet concentration of 200,000/µl. Disaggregation was also determined using washed platelets in the presence of 10 µg/ml of purified human VWF and 2 mg/ml of purified human fibrinogen.

Measurement of platelet activation

Expression of CD62P (P-selectin) on the platelet surface indicates α-granule release and is a commonly used measure of secretion and platelet activation. To measure CD62P expression, 10 µl of PRP that was exposed to shear stress was mixed with 90 µl of Ca²⁺ and Mg²⁺ free Tyrode’s buffer (137.0 mM NaCl, 2.0 mM MgCl₂, 0.4 mM NaH₂PO₄, 11.9 mM NaHCO₃, 2.9 mM KCl, 5.5 mM Glucose, pH 7.35) containing 1% bovine serum albumin and 5 µg/ml of a R-Phycocerythrin-conjugated monoclonal anti–CD62P antibody (BD-PharMingen, San Diego, CA) for 20 min at 24°C. After incubation, 1 ml of PBS containing 1% paraformaldehyde was added and the samples were analyzed for CD62P expression by flow cytometry. Unsheared PRP was used as a negative control. For comparison, P-selectin expression was also determined in platelets stimulated with 20 µM of ADP. Previous studies have shown that P-selectin expression is temperature-dependent with the highest level of expression at 37°C (7, 24). However, we did not find significant difference in P-selectin expression between 24 and 37°C in a pilot study of 12 donors, therefore, all measurements were made at 24°C.
Although P-selectin expression, which indicates the platelet granule release, is commonly used as an indication of platelet activation, it may not be sensitive enough to measure shear-induced platelet activation. To address this concern, platelet activation in response to high shear was also determined in washed platelets by measuring the binding of the monoclonal antibody PAC-1, which recognizes only the activated form of αIIbβ3 (25).

To confirm the role in ADP in shear-induced platelet aggregation, we sheared whole blood in the presence of 500 µg/ml of creatine phosphate (Sigma Chemical, St. Louis, MO, final concentration) and 100 µg/ml of creatine phosphate kinase (Sigma Chemicals, final concentration). The ADP converting enzyme system of CP/CPK has previously shown to block ADP-induced platelet aggregation (26, 27).

**Statistical analysis**

All experimental data were presented as mean ± SEM. The unpaired 2-tailed Student’s $t$ test was used for all the data analysis and a $p$ value less than 0.05 was considered to be statistically significant.

**Results**

**Platelet aggregation and activation were proportional to the shear duration**

To determine how shear-induced platelet aggregation is affected by the duration of high shear exposure, we measured aggregation and P-selectin expression of platelets that had been exposed to a constant shear stress of 100 dyn/cm$^2$ for periods from 2.5 to 120 sec. As shown in Figure 1A, platelets in whole blood aggregated after they were sheared for as short as 5 sec and reached maximal aggregation after 10 sec. This extent of aggregation was comparable to non-sheared platelets stimulated with 20 µM of ADP (Fig. 1A, grey bar). Shear induced platelet aggregation using PRP lacking red and white blood cells was significantly delayed, showing significant differences in the time required for both initiation and maximal aggregation (Fig. 1A).

To correlate aggregation to secretion, we determined the expression of P-selectin on these same samples of sheared platelets. P-selectin expression was not detectable until platelets were exposed to the 100 dyn/cm$^2$ shear stress for 40 and 20 sec in PRP and in whole blood, respectively (Fig. 1B). In both PRP and whole blood, P-selectin expression reached maximum only after 80 sec of shearing. Even then, the maximal level of P-selectin expression in sheared platelets was 32.4% of that of ADP treated platelets (9.56±0.87 vs. 29.5±1.02, Student’s $t$ test, $n = 26$, $p < 0.001$), despite the fact that the amounts of platelet aggregation in PRP and whole blood after 80 sec shear exposure was comparable to that of ADP treated platelets (Fig. 1A).

To address the possibility that platelet aggregates might not permit exposure of CD62P binding site for anti-CD62P antibody, we performed the same type of experiment in the absence of platelet aggregation. We found no significant difference in P-selectin expression in the presence or absence of the αIIbβ3 blocking antibody 7E3 (Fig. 2), suggesting that P-selectin expression was similar in aggregated or non-aggregated platelets exposed to shear.

To further evaluate activation of sheared platelets, we measured binding of the monoclonal antibody to the platelet integrin αIIbβ3, PAC-1. As shown in Figure 3, PAC-1 binding was also minimal on platelets that were exposed to a 100 dyn/cm$^2$ of shear stress for either 10 or 60 sec as compared to that of ADP stimulated platelets.

Figure 1: Effects of shear duration on platelet activation and aggregation in PRP and whole blood. PRP or citrated whole blood was exposed to a constant high shear stress of 100 dyn/cm$^2$ for 2.5 to 120 sec, after which platelet aggregation (A) and P-selectin expression (B) were then determined by particle counting and flow cytometry, respectively. The results were compared to that induced by 20 µM of ADP. The values are mean±SEM, unpaired Student’s $t$ test, $n = 14$ experiments for both PRP and whole blood (* $p<0.001$, ** $p < 0.01$).
Free calcium concentration did not affect these data, since similar results were observed for both shear-induced platelet aggregation and secretion when using sodium citrate or PPACK as anticoagulants (data not shown).

**Shear aggregated platelets rapidly disaggregated in PRP, but not in whole blood samples**

Since shear-induced P-selectin expression and PAC-1 binding were less than what was seen with agonist stimulation, we hypothesize that “shear-induced platelet aggregation” may actually be a process of shear-induced reversible platelet crosslinking involving minimal platelet activation. To test this hypothesis, we exposed platelets (PRP and whole blood) to a 100 dyn/cm² shear stress for 10, 20 or 60 sec. Platelet aggregation was determined immediately after shear and also after the samples were allowed to stand at room temperature for 1, 5, 10, or 30 min. For PRP samples, disaggregation occurred rapidly after shear exposure and the extent of disaggregation was inversely proportional to the duration of shear with rapid disaggregation after 10 sec shear and substantially less disaggregation after exposure to 60 sec of high shear (Fig. 4). For a 10 sec shear, more than 50% of the platelet aggregates disaggregated within one minute of incubation and 90% after 10 min.

Similarly, washed platelets also aggregated in response to a 10 sec exposure of 100 dyn/cm² shear stress in the presence of exogenous VWF and fibrinogen. The aggregates were again rapidly disaggregated within 10 min (data not shown).

In contrast to PRP and washed platelets, disaggregation in whole blood was not detectable after 60 sec of high shear, and significantly less than in PRP for 10 sec of high shear (the Student’s t test, n = 4 experiments, p<0.01). As a control, disaggregation was not detectable in ADP-induced platelet aggregation (20 µM), even after 30 min for both PRP and whole blood (Fig. 4). This enhanced platelet aggregation in whole blood was partially blocked by pretreating blood with ADP-converting enzyme system CP/CPK (data not shown).

**Sheared platelets in PRP retained ability to be aggregated by ADP**

Since platelet activation is generally considered to be an irreversible process, one way to determine the functional status of these disaggregated platelets is to determine if they retain their aggregability to other agonists. We therefore tested whether the disaggregated platelets can be re-aggregated by ADP. Platelets were first exposed to a 100 dyn/cm² shear stress for 10 sec and then incubated at room temperature for 5 min to allow disaggregation to occur. The disaggregated platelets were then treated with 4 µM ADP and monitored for aggregation in a lumaggregometer. As shown in Figure 5, the sheared platelets aggregated in a pattern similar to that of unsheared platelets, although to a lesser extent.
Paraformaldehyde-fixed platelets were shear-agglutinated

These data suggested that shear-induced platelet aggregation is reversible and activation-independent. We explored this possibility by shearing the reconstituted PRP containing paraformaldehyde-fixed washed platelets and fresh plasma from the same donor. This reconstituted PRP was unresponsive to ADP, but could be agglutinated by ristocetin (data not shown). The reconstituted PRP was exposed to a 100 dyn/cm² of shear stress for 10, 60 or 120 sec and reversible platelet aggregation determined by particle counts. As shown in Figure 6, the fixed platelets were agglutinated under high shear stress for the durations of 60 and 120 sec, but was minimal for a 10 sec exposure.

Discussion

We have studied the activation and aggregation responses of platelets after exposure to pathological shear stress for various periods of time. The major findings in this study include: 1)
short periods of high shear stress induce reversible aggregation (<20 sec for PRP, <5 sec for whole blood). 2) shear has a minimal effect on platelet activation. 3) platelets aggregated by short pulses of high shear undergo rapid disaggregation and the shear-aggregated-disaggregated platelets remain responsive to agonist stimulation.

When exposed to high shear pulse for a short period that is physiologically relevant (2.5-20 sec), platelets either fail to aggregate or form unstable aggregates that undergo rapid disaggregation when shear ceases. The rate of disaggregation is inversely proportional to the time period that platelets were exposed to high shear (Fig. 4). For example, nearly half of the platelet aggregates induced by a 10 sec exposure of high shear disappeared within 1 min of post-shear incubation, whereas in the same time period, less than 10% disaggregation occurred for a 60 sec exposure of high shear (Fig. 4). The disaggregated platelets appear to be functionally normal because they can be re-aggregated by ADP in a pattern similar to that of unsheared platelets (Fig. 5). Furthermore, platelets that were exposed to a shorter high shear pulse express minimal levels of P-selectin on their surfaces (Fig. 1) and binds minimally the activation-dependent antibody PAC-1 (Fig. 3), indicating that the sheared platelets may not be activated by short pulses of high shear.

Taken together, these results suggest that, contrary to popular belief, platelets may have intrinsically a higher threshold for shear stress and cannot be activated and aggregated by the short exposure of high shear that may be encountered in vivo in the area of arterial stenosis. Instead, they undergo reversible clustering that involves minimal activation.

Our results are consistent with previous studies, showing that high shear stress alone had no effect on the release reaction of washed platelets (28, 29). The crosslinking nature of shear-induced platelet clustering is also supported by our recent observation that platelets can be induced to aggregate in response to a short exposure of high shear (2.5 sec), but the aggregation only occurs when high shear exposure (100 dyn/cm²) is followed by a low shear segment (5 dyn/cm²), a shear profile similar to that in the area of arterial stenosis in vivo. Interestingly, platelets under this condition also show minimal activation as determined by the surface expression of P-selectin (23). The high tolerance of platelets for shear stress may play an important physiological role in protecting platelets from temporary exposure to very high shear stress, which occurs frequently in response to constant variations of blood flow caused by such changes as body posture, muscle tone, and exercise (14).

Compared to PRP, a greater extent of platelet activation and aggregation occur in whole blood during a short exposure of high shear (Fig. 1A, 1B). Platelet aggregates in whole blood are also much more stable (Fig. 4) and are associated with a higher level of P-selectin expression (Fig. 1), suggesting that other blood cells, especially erythrocytes, may play a critical role in shear-induced platelet aggregation. This observation is supported by previous reports showing that platelet aggregation in whole blood is significantly greater than that in PRP (30, 31). The requirement for other blood cells can be two fold. First, heterogeneous populations of cells at high density promote cell-cell collision. Erythrocytes, which constitute 97% of cellular contents of blood (32), promote cell-cell collision and prolong the lifetime of platelet-platelet interaction (29, 33). The increased lifetime of platelet-platelet interaction will in turn increase the probability of crosslinking platelet αIIbβ3 by VWF, which possess a slow on-rate in the ligand-receptor interaction (34), as well as form a fibrinogen cross bridge between adjacent platelets. Second, other blood cells, especially erythrocytes, may release platelet agonists such as ADP. Previous studies have demonstrated that shear-induced platelet aggregation is significantly enhanced when platelets are pretreated with ADP (35) and is inhibited by ADP scavengers (33). Our results using the ADP converting enzyme system of CP/CPK is consistent with these studies. High fluid shear stress induces ADP release from erythrocytes through hemolysis (33, 36), which results from reducing the deformability of erythrocytes (37). This may explain why fresh erythrocytes are twice as effective as glutaraldehyde-fixed and ADP depleted erythrocytes in promoting shear-induced platelet aggregation (38).

In summary, we found that shear-induced platelet aggregation induced by short pulses of high shear is reversible and requires minimal platelet activation, suggesting a high threshold for shear-induced platelet activation and aggregation. Instead of activation and aggregation, platelets may be temporarily agglutinated by high fluid shear stress. This process is more efficient in a complex shear profile containing both high and low shear as commonly found at the site of arterial stenosis. Our results...
therefore suggest caution in interpreting data generated by prolonged shear exposure to platelets because it may overestimate the effect of shear, which may only be encountered in vivo when platelets first adhere to the vessel wall (wall shear stress). We further demonstrated that shear-induced platelet aggregation is greatly enhanced by the presence of other blood cells, primarily erythrocytes. The enhancement is likely to be mediated through increased platelet-platelet interaction and agonists released by erythrocytes such as ADP, indicating that shear-induced platelet aggregation may be resultant from combined mechanical and biochemical forces.

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References
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