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Platelet activation in a circulating flow loop: combined effects of shear stress and exposure time[1]

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Measurement of small changes in platelet activation state (PAS) in circulating stenotic systems *in vitro* has been problematic because of a paucity of real-time assay methods and circulation systems of low platelet-activating potential. PAS was measured by a modified prothrombinase assay in which activated platelets provide the essential cofactors in the activation of prothrombin by factor Xa. Chemical modification of the prothrombin ensures that the thrombin produced, while assayable, does not activate platelets. Human platelets were circulated in loops in which exposure to shear stress was adjusted by independently varying flow rate, viscosity, and the time of exposure to shear. Although with some differences in platelet response to different conditions of stress, the PAS directly increased with time of circulation, shear stress, and time of exposure to shear. The results show that low-level platelet activation caused by shear stress in a circulation loop can be quantitatively assessed in near-real time in a system of tube geometry. They confirm previous results obtained in non-circulating systems that exposure of platelets to shear conditions on the same order as found in the vasculature causes significant platelet activation, and that this activation is dependent on both shear stress and time of exposure.

Introduction

Coronary heart disease is the most common form of heart disease, manifested by a narrowing, or stenosis, of the coronary arteries by atherosclerotic plaque. Severe stenoses limit blood flow and can cause local ischemia and arrythmia. Moreover, if the atherosclerotic plaque ruptures, coagulation is initiated and a thrombus rapidly forms, blocking the coronary artery and leading to a heart attack and myocardial infarction.[1] Platelet activation is involved in both stages: (i) upon exposure to abnormal flow patterns and high shear stresses during passage through a stenosis, and (ii) in thrombus formation, which involves both platelet aggregation and fibrin formation. Thrombosis is fundamentally linked to hemodynamics, not only inducing shear stresses that can cause platelet activation and aggregation but also transporting cells and proteins to the thrombus.[2] Folts *et al.* described a canine model in which coronary artery constriction initiated platelet-dependent thrombus formation,[3–5] and, using the same model, Strony *et al.*[6] demonstrated that the acceleration as platelets approach the converging stenotic region, and the deceleration downstream, can cause deformation of 400–800%. Such data demonstrate the need for platelet activation criteria to be established under flow conditions, in systems that are reasonable facsimiles of vascular geometry.[7,8]

The effects of hemodynamic forces on platelets has been demonstrated by several investigators using non-stenotic models. Ramstack *et al.* showed that platelet stimulation is a nonlinear phenomenon, where the extent of platelet trauma increases exponentially as a function of shear rate and time.[9] These and other data led Hellums *et al.* to depict a locus of platelet activation on a shear stress-exposure time

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[1] JJ and DB have an interest in a pending US patent application concerning the modified prothrombinase method used in this study to measure platelet activation.

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plane, which is commonly used as a standard for platelet activation threshold.[10]

The criteria for platelet activation in such studies have been established under conditions of constant bulk stress, e.g., in cone-and-plate devices. However, as platelets flow in arteries, and especially through a stenosis, they are exposed to varying durations and extents of flow stress. For instance, Sutera *et al.* showed that pulsed exposure to shear stress resulted in more platelet aggregation than continuous exposure,[11] and Purvis and Giorgio reported that platelets exposed to elongational stresses are activated at lower shear levels and tend to form larger aggregates.[12] Boreda *et al.* estimated the combined effect of shear stress and duration in a moderately stenosed canine coronary model.[13] Applying Hellums' criterion for platelet activation, they estimated their maximal shear stress/exposure time combinations to reach approximately half the activation threshold. In this study, we describe a simple circulating stenotic model and an assay of platelet activation state (PAS) that enables the measurement of platelet activation in real time under varying conditions of flow and shear stress.

Materials and methods

Platelets

Informed consent, approved by the Stony Brook University IRB, was obtained from healthy adult volunteers of both sexes who had taken no aspirin or ibuprofen for 2 weeks. Blood, 30 ml, was drawn by venipuncture and collected into 0.3 ml 40% trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at $400\times g$ for 2.5 min. PRP, 12 ml, was gel filtered at 3 ml/min through a 220-ml column of coarse BioGel A50M (2.7% agarose; Bio-Rad, Hercules, CA) equilibrated in platelet buffer, which is a Hepes-modified Ca^{2+}-free Tyrodes buffer containing 0.1% bovine serum albumin.[14] (To minimize the risk of platelet activation during gel filtration, freshly washed gel filtration medium must be used: after each use the agarose beads were stirred in 50 mM NaOH in 0.5% sodium dodecyl sulfate, rinsed exhaustively with water, repacked in the column, and then equilibrated with platelet buffer.) The gel-filtered platelet pool was counted (Z1 particle counter; Coulter, Hialeah, FL) and adjusted with platelet buffer to a count of 10^5 per μl. Platelets were maintained with gentle agitation at room temperature and used within 6 h of gel filtration. Platelet samples of high viscosity were prepared by the addition of 420-kDa dextran (Sigma–Aldrich, St Louis, MO) to the gel-filtered platelet pool. At the 9% dextran concentration used, the contribution to the osmolarity is 0.16 mM, which is negligible. Control experiments on uncirculated platelets showed no detectable effect on either the base PAS activity of platelets or the activity obtained after maximal stimulation with Ca^{2+} ionophore.

Platelet activation state (PAS)

PAS was measured by a modification of the chemically modified prothrombinase method of Jesty and Bluestein.[15] Differences between current practice and the original report are as follows. (i) Acetylation of prothrombin was done by treatment of 10 μM prothrombin, in 100 mM $NaHCO_3$ + 5 mM $CaCl_2$, with 3 mM sulfo-*N*-succinimidyl acetate for 20 min at 22°C. (ii) The PAS assay—the generation of Ac-FIIa in response to platelet prothrombinase—is done for exactly 10 min at 37°C in a total volume of 100 μl containing (final concentrations) 20 000 platelets per μl, 200 nM Ac-FII, 5 mM Ca^{2+}, and 100 pM factor Xa. (iii) Duplicate 20-μl samples from each PAS incubation were assayed for generated thrombin using 0.3 mM Chromozym-TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide, Roche Diagnostics, Indianapolis, IN) in a microplate reader (VMax; Molecular Devices, Sunnyvale, CA) at 25°C. (iv) Activation of platelets with Ca^{2+} ionophore A23187, which provides a positive control and reference value for the PAS activity of maximally activated platelets, was done by the addition of 1/40 volume of a solution of 0.2 mM A23187 in dimethylsulfoxide (DMSO) followed by a 3-min incubation immediately before the addition of factor Xa to the incubation. The low level of DMSO (2.5% final concentration) has no effect on the PAS assay. The present methodology routinely reports base PAS activities of freshly prepared platelets of 0.5–2% of the maximum attainable. This is shown, for a typical preparation of acetylated prothrombin, in Figure 1, which demonstrates substantial improvements in linearity, assay range, and sensitivity compared with the initial report.[15] Whereas normal thrombin generation is highly nonlinear because of feedback platelet activation, acetylated thrombin generation is near-linear and, with unactivated platelets, remains very low.

It may be noted that PAS activity is an intrinsic property of a platelet preparation or an experimental sample. Although the rate of thrombin generation in the assay depends on the platelet count, relative (normalized) activities, which are referenced to maximally activated samples, are independent of platelet count. The count used here, 20 000/μl (nonphysiological, as are the other conditions of this assay), was adjusted to maximize the sensitivity range of the assay.

Flow cytometry

Flow cytometry was done on a FACScan instrument (Becton-Dickinson, Franklin Lakes, NJ), using fluorescein (FITC)-labeled annexin V (Molecular Probes, Eugene, OR). Platelets, 4×10^6 in 40 μl platelet buffer

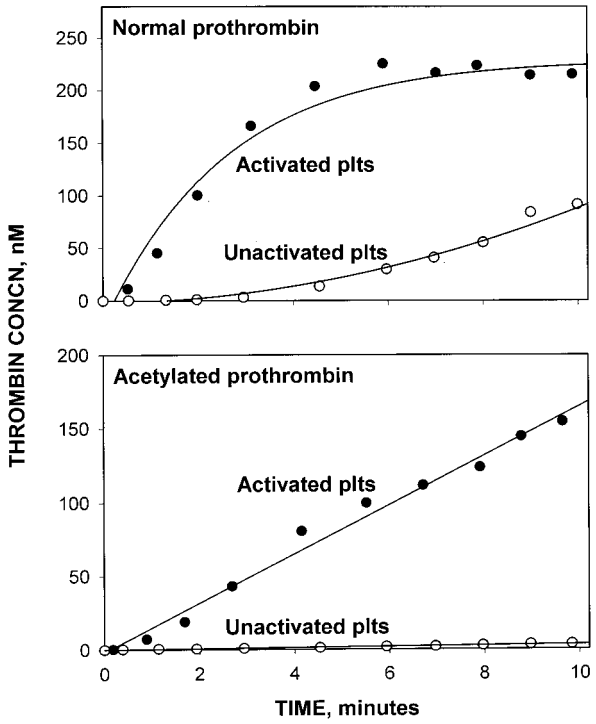

Figure 1. The modified prothrombinase assay: time course of thrombin generation. The time course of activation of normal (top) and acetylated (bottom) prothrombin by factor Xa in the presence of unactivated (○) and Ca^{2+} ionophore-activated (●) platelets (5 μM ionophore A23187). In the routine PAS assay, unlike here, only a single 10-min sample is taken to determine platelet activation state.

+ 2.5 mM Ca^{2+}, were treated with 5 μl FITC–annexin V (used as supplied) at room temperature for 10 min and then diluted into 1 ml Ca^{2+}-containing buffer before cytometry. The analysis gating includes all normal platelets and larger particles, but not the smaller microparticles (roughly, < 1 μm).

Circulation experiments

Circulation experiments were done in a flow loop consisting of an approximately 35-cm length of 2.8-mm (ID) PVC manifold-pump tubing (Fisherbrand, Fisher Scientific), two 3.2-mm polypropylene T-connectors fitted with Luer side ports (Cole-Parmer, Vernon Hills, IL) and a variable length of 0.97 (tolerance + 0.1−0.05) mm PTFE tubing (Small Parts Inc, Miami Lakes, FL) to mimic stenotic flow conditions. All-new tubing was used for each day's set of experiments. Between individual experiments the loop was rinsed with 10% bleach (Clorox), followed by water, 10 mM HCl, and finally platelet buffer containing bovine serum albumin. The loop was mounted on a variable-speed peristaltic pump (Rainin Instrument, Boston, MA).

The total loop volume, less the volume in the stenotic tube, was 2.3 ml. For this study, variables affecting shear stress exposure were (i) the length of stenotic tubing section, (ii) the flow rate, and (iii) the viscosity. All circulation experiments were run at $37 \pm 2^{\circ}$C for 30 min. The ratio of the stenotic volume to the total loop volume determines the fractional period of high shear stress to which the platelets are exposed (see Discussion). At stenotic lengths of 0, 0.5, 1, and 1.5 m, the fractional high-shear exposure times are approximately 0, 14, 24 and 32% respectively. Applying the Poiseuille relation for laminar pipe flow, the wall shear rate, $\gamma = 4Q/\pi R^3$, where Q is the flow rate and R the tube radius. Shear stress, τ, is given by the relation $\tau = \mu\gamma$, where μ is the kinematic viscosity. The shear rate in the non-stenotic region of the loop is $< 4\%$ of that in the stenotic section. However, this number does not account for non-hydrodynamic stresses on the platelets that are likely caused by the peristaltic pump action.

Platelet variability; statistics

Individual gel-filtered platelet preparations, usable for only 6 h, vary in both their base activation state and their sensitivity to activation. No standard platelet preparation is available. Each day's experimental study involved the comparison of just two conditions, each being repeated twice. Two such sets of data obtained with different platelet preparations are included in each data set shown, for a total of four paired comparisons. In any one experimental set, the two conditions were performed in palindromic order to minimize artifacts caused by time-dependent changes in platelets, i.e., for conditions A and B (low viscosity versus high viscosity, for example), the order was A–B–B–A. (Most gel-filtered platelet preparations activate slowly over the course of several hours at room temperature, typically from ca. 0.8 to 2% of maximal PAS activity over 6 h.) PAS determinations were normalized to the maximum activity of the platelet preparation in use, obtained by treatment of the platelets for 3 min with 5 μM Ca^{2+} ionophore A23187. The normalized PAS activities presented are thus ratios.

The differences in platelet activation rates (slopes of PAS activity versus time) were analyzed for statistical significance as follows. The normalized data were fitted by linear regression, and the regression lines then tested for difference in slopes according to a generalization of Student's paired t-test.[16] With a particular set of experimental conditions (the four experimental paired sets of Figures 2–5), the results yielded slopes that follow Student's t distribution. To test the null hypothesis that two samples were drawn from populations with the same slope the collected mean slopes were therefore analyzed by Student's paired t-test in the normal way.[16]

Results

To demonstrate the validity of the PAS assay in real time, and to examine the properties of platelets in the circulation loop, a control loop without stenosis, and a loop with a 1-m stenosis tube, were compared at a

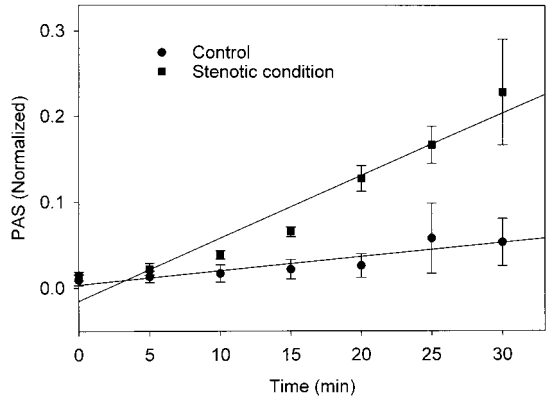

Figure 2. The effect of a stenotic region on the activation of platelets in a circulation loop. Gel-filtered platelets were circulated in a flow loop in platelet buffer, as described in Materials and methods, at a flow rate of 6.25 ml/min. Samples were removed immediately after injection into the loop (zero time), and then at 5-min intervals, for assay of platelet activation state (PAS) (●). In a parallel experiment a 1-m section of 0.97-mm PTFE tubing was inserted in the circulation loop to provide a region of high shear rate (■). Other details are described in Materials and methods. Each data point is the mean of 4 PAS values (± SEM), obtained from duplicate experiments performed twice, on different preparations of platelets. Lines were fitted by unweighted linear regression.

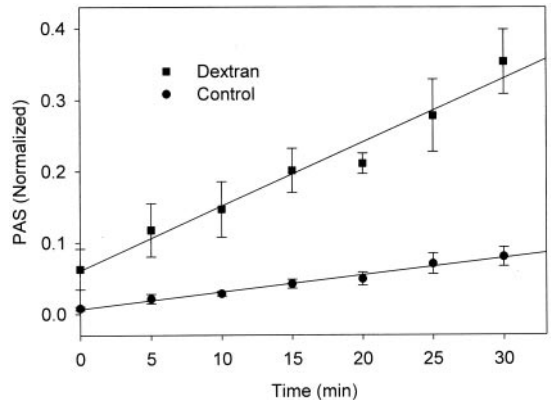

Figure 3. The effect of increasing viscosity on the activation of platelets. Gel-filtered platelets were circulated at 1.67 ml/min in a flow loop containing a 1-m section of 0.97-mm PTFE tubing, in normal platelet buffer (●) and in buffer containing 9% (w/v) 450-kDa dextran, which triples the viscosity and the shear stress (■). Other details are as described for Figure 2.

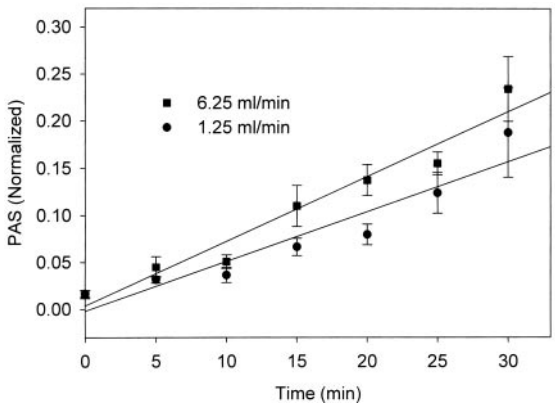

Figure 4. The effect of flow rate on the activation of platelets. Gel-filtered platelets were circulated in platelet buffer at 1.25 (●) or 6.25 ml/min (■) in a flow loop containing a 1-m section of 0.97-mm PTFE tubing. Other details are as described for Figure 2.

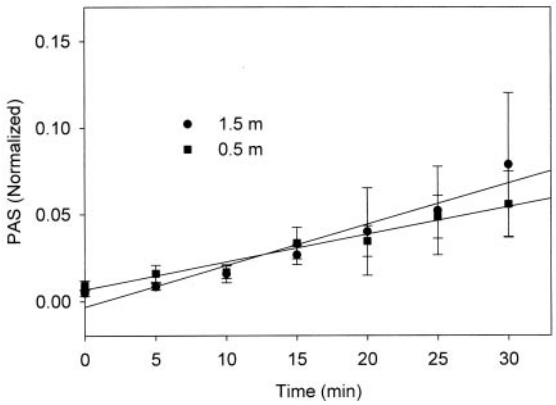

Figure 5. The effect of high-shear exposure time on the activation of platelets. Gel-filtered platelets were circulated in platelet buffer at 1.67 ml/h in flow loops containing sections of 0.97-mm PTFE tubing of 0.5 (■) and 1.5 m (●). Other details are as described for Figure 2.

flow rate, 6.25 ml/min, that produced a wall shear stress of 41 dyne/cm^2. The shear stress in the nonstenotic section of the loop (chiefly the 35-cm section of pump tubing) was 1.4 dyne/cm^2. The combined results of four comparison pairs are shown in Figure 2. The rate of activation in the loop containing the stenosis was almost 5-fold higher (slope = 0.0073 min^{-1}) than in the control loop without stenosis (slope = 0.00164 min^{-1}), the difference being highly significant ($P < 0.001$). The low rate of activation in the control flow loop without stenosis was at least 10-fold greater than that in platelets not subjected to circulation. The combined data demonstrate convincingly that the stenosis tube causes an increased rate of platelet activation, but they do not preclude mechanisms other than shear stress as the cause; e.g., interaction of the platelets with the loop materials. Relevant here is the fact that the junctions at the beginning and end of the stenotic capillary are sharp discontinuities, and do not correspond with the smoother geometry of vascular stenoses. It is therefore possible that a small part of the baseline rate of platelet activation is caused by this very brief exposure to turbulent conditions at the junctions. We note, however, that even at the highest flow rate platelets circulate through the loop less than 3× per minute.

To address the question of whether shear stress directly caused platelet activation in the circulation loop, we compared activation in a 1-m stenotic loop at different viscosities. In this case the tubing materials and other mechanical aspects of the loop are identical, so that differences in shear stress may be isolated. Figure 3 shows the collected results of four comparison pairs, the viscosity differing by 3-fold. The difference between the conditions is clear ($P < 0.001$), the rates of activation differing by 3.8-fold (0.0090 min^{-1} at 33 dyne/cm^2, compared with 0.0024 min^{-1} at 11 dyne/cm^2). However, even here it is difficult to prove that the difference in shear stress is the direct cause, since it is feasible that

dextran present in the high-viscosity experiments causes activation under conditions of shear. High molecular weight (HMW, 450 kDa) dextran was chosen as a viscogen because of its lack of charge, high specific viscosity, and low osmolarity. Moreover, suspension of platelets in 9% 450-kDa dextran in the absence of flow does not cause platelet activation beyond the minimal storage-dependent rate previously mentioned. While low molecular weight (40- and 70-kDa) dextrans are known to interfere with platelet deposition on surfaces (such as vascular grafts), the effect is on adhesion and not activation.[17,18] In this regard it is pertinent that the buffer in which the platelets are suspended in the present study contains no von Willebrand factor (vWf), which is the major bridging molecule in platelet adhesion to surfaces. It is, however, possible that small amounts of vWf may be supplied by the platelets themselves on activation. Overall, we judge the possibility of an effect of HMW dextran on platelet activation to be very small, and may therefore conclude that the increased rate of activation at high viscosity is caused by the increased shear stress.

A third approach to confirming that platelet activation in the circulation loop is caused by shear stress involved altering the shear rate by changing the flow rate. Although the viscosity and the surface to which the platelets are exposed remain the same in this series of experiments, increases in flow rate increase the number of passages through the pump, giving a higher probability of mechanical damage. The data in Figure 4 show that at a flow rate of 6.25 ml/min (wall shear stress of 41 dyne/cm^2 in the stenotic section) the rate of platelet activation was 0.0069 min^{-1}, whereas the rate of activation at a flow rate of 1.25 ml/min (shear stress of 8.2 dyne/cm^2) was 0.0053 min^{-1}. Although not large, the difference is statistically significant ($P = 0.019$).

We mentioned in the Introduction the concept of the integral of shear-stress with respect to time[10,13] and its relevance to platelet activation (see Discussion). We thus compared loops with different lengths of stenosis tube, circulated at the same flow rate. Although the 30-min time course of the experiment does not change, the time of exposure to stenotic shear stress increases in proportion to tube length. Figure 5 compares platelet activation with tube lengths of 0.5 and 1.5 m at a flow rate of 1.7 ml/min (wall shear stress = 11 dyne/cm^2). Although the mean rate of activation is 1.5-fold greater (0.00237 vs. 0.00158 min^{-1}) when the stenosis length is increased 3-fold, statistical significance was not achieved.

While the PAS method provides a quantitative measure of the ability of platelets to support prothrombin activation, the cellular events involved are complex and involve at a minimum two processes: the scrambling of the membrane bilayer to provide anionic phospholipid on the outside, and the release of FV(a) from the α granules to the membrane. To

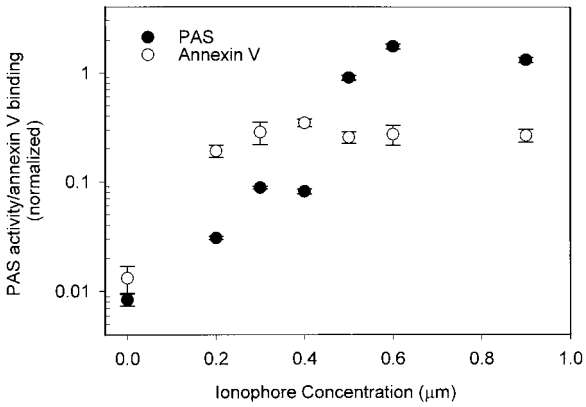

Figure 6. Comparison of PAS activities and annexin V binding as measures of platelet activation. Platelets, $10^5/\mu$l in platelet buffer + 5 mM Ca^{2+}, were treated at 37° with 1/50 volume of varying concentrations of calcium ionophore A23187 in DMSO, to give the final concentrations shown. After 5 min unstirred incubation at 37°, separate samples were assayed for PAS activity and for annexin V binding. Cytometry data were obtained as the percentage of gated particles that were labeled. Data (± SEM) are the mean of three treatments at each ionophore concentration, normalized to the PAS or cytometry response obtained by treatment with 5 μM ionophore.

demonstrate correlation between the PAS assay and an alternative measure of platelet activation, we compared the extents of platelet activation over a range of concentrations of calcium ionophore, measuring PAS and annexin V binding on each sample. Annexin V binds to anionic phospholipid, but does not involve FV. It may thus be expected to be in part correlated with PAS activity. In addition to the fact that the two methods are not measuring identical properties, it should be noted that because of the time delay in the flow cytometry measurement of annexin V binding, the two data sets are not properly comparable. Fixed platelets cannot be used for annexin V binding measurements. Thus, in contrast to the PAS assay, which allows immediate measurement of the activity state of a sample, it is possible that some minor level of continuing platelet activation continues during treatment of the platelets with FITC-annexin V, and during the brief storage of these pre-labeled platelets before flow cytometry. This caveat in fact points up the advantage of the PAS assay in being able to handle multiple samples in near-real time. Additionally, we note—over the course of six series of experiments—the very high variability among platelet samples in their annexin V-binding response to calcium ionophore. Figure 6 shows a typical set of comparison data, demonstrating—given the caveats mentioned—a reasonable quantitative correlation between the two methods.

Discussion

A platelet circulation loop

A major goal was to demonstrate a circulation system and an assay method that permit real-time measurments and enable the measurement of

flow-dependent activation of platelets in a tube geometry. While much informative work has been done with platelets exposed to well-controlled shear fields in cone-and-plate instruments,[19] the conditions are less applicable to flow conditions in the vasculature or through prosthetic devices. Circulating systems incorporating a parallel-plate chamber have also been used to examine the adhesion and aggregation of platelets on a number of materials, including cultured cells, but the overall platelet activation state in these systems must reflect the combined stress history of both the pumped circulation system and the experimental chamber. Apart from the fundamental interest in understanding the role of platelet activation in vascular disease, the development of reliable circulating systems for studies of platelet activation also bears on the development of such devices as artificial hearts, ventricular assist devices, prosthetic heart valves, blood pumps, etc.

Given the extreme sensitivity of platelets to shear stress and mechanical damage, the design of circulation pump systems is problematic. Reciprocating pumps are unusable because they commonly use ball and similar valves and small passageways that produce very high local shear stress as a valve opens, and shear stress and mechanical shock when the valve closes. Peristaltic pumps, while better, nonetheless also cause platelet activation, as we see here in the control loop (Figure 2, ●), probably mainly through the trapping and rolling of platelets at the rotating points of tube closure. Nonetheless, we demonstrate that with careful attention to materials and geometry, such loops can be designed with acceptably low platelet-activation potential. We emphasize especially the frequent need for new tubing, and the avoidance of bore changes in the loop.

Measurement of platelet activation state

This report validates well the modified prothrombinase assay that we initially described and have since improved.[15] It depends on two corollaries of activation that are central in platelet function in hemostasis: the provision of anionic phospholipid and factor Va. Anionic phospholipid is required for the platelet-dependent activation of factor X by factors IXa+VIII, and for the platelet-dependent activation of prothrombin; while factor Va is an additional requirement for the latter. Although flow cytometry methods provide valid assessment of platelet activation—for instance, the measurement of P-selectin expression or annexin V binding[20] (Figure 6)—they are not well suited to the analysis of multiple timed samples. Additionally, they report the percentage of cells and larger particles with activation-dependent ligand-binding properties, rather than the total amount of such binding in the population. The question of the extent of microparticle formation under high- and low-shear conditions as a function of circulation time and other conditions is currently under investigation. The PAS assay allows a relatively simple determination of this parameter, since it enables measurement of the activity of both the total platelet sample, and the activity of the supernatant after removal of whole platelets—but not the smaller microparticles—by appropriate centrifugation.

Platelet stimulation function (PSF)

In shear-induced platelet activation, the platelet activation threshold depends on the shear-stress history of the platelets,[10] and is related to the integral of shear-stress exposure with respect to time. Boreda *et al.* derived a platelet stimulation function that is defined by $\mathrm{PSF} = \tau \times t^{0.452}$, where t is the time of exposure at a constant shear stress τ.[13] In this study the PSF is determined from the product of the 30-min experimental time course and the fractional high-shear exposure times (see Materials and methods). The present data allowed us to test the PSF hypothesis, its key feature being the prediction that platelet activation should vary linearly with shear stress (i.e., proportional to viscosity and flow rate), but nonlinearly (as a fractional power) with time of exposure.

With regard to changes in shear stress effected by changing the viscosity, the data are in fair agreement with prediction, the platelet activation rate increasing 3.8-fold with a 3-fold increase in viscosity (Figure 3). Changes in flow rate, however, did not agree with the PSF prediction, a 3-fold increase in flow rate leading to only a 30% increase in platelet activation rate (Figure 4). The reason for this discrepancy is unknown and is currently under study over a wide range of flow rates.

With regard to time of shear-stress exposure, the PSF hypothesis would predict that a 3-fold increase in stenosis tube length, and hence t , should increase the platelet activation rate by $3^{0.452} = 1.64$-fold. This assumes that the shear-stress contribution of the non-stenosis section of the loop (1.4 dyne/cm^2) is insignificant compared with the stenosis tubing section (41 dyne/cm^2). Experimentally, although the statistical significance of the results is questionable, we observed a 1.5-fold increase in the rate of activation, in good agreement with prediction.

During the circulation experiments, the PAS activity of a sample represents the average bulk properties of all the platelets. Even though the wall shear stress is known, the distribution of shear stress in the population, and hence the shear stress history, depends on the distribution of trajectories and cannot be predicted. Accordingly, one should not necessarily expect a linear correspondence between τ (the value of the shear stress at the wall), which is the parameter of choice for characterizing the flow conditions, and the platelet activity actually measured.

In summary, the PAS assay was used to quantify the time-resolved activation of platelets by flow-induced stresses in a circulating system, under shear stresses on the order of those found in the vasculature. Our results confirm that, as is found in other, noncirculating, geometries, elevated shear stress in a circulating system causes significant platelet activation. The results in some aspects also support the proposals of Boreda *et al*.[13] that the time of exposure to shear stress is less significant than the magnitude of the shear stress experienced by the platelets. Additionally, they signal significant possibilities for the measurement of the platelet-activation potential of various vascular devices in circulation models *in vitro*.

Acknowledgements

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References

1. Delouphery T. Atherosclerosis: Overview. *New Engl J Med* 1992; **326**: 1406.
2. Wootton D M, Ku D N. Fluid mechanics of vascular systems, diseases, and thrombosis. *Annu Rev Biomed Eng* 1999; **1**: 299–329.
3. Folts J D, Crowell E B, Rowe G G. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. *Circulation* 1976; **54**: 365–70.
4. Folts J D, Gallhager K P, Rowe G G. Hemodynamic effects of controlled degrees of coronary artery stenosis in short-term and long-term studies in dogs. *J Thorac Cardiovasc Surg* 1977; **73**: 722–7.
5. Folts J D, Gallhager K P, Rowe G G. Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation? *Circulation* 1982; **65**: 249–55.
6. Strony J, Beaudoin A, Brands D, *et al*. Analysis of shear stress and hemodynamic factors in a model of artery stenosis and thrombosis. *Am J Physiol* 1993; **265**: 1787–96.
7. Bluestein D, Niu L, Schoephoerster R T, *et al*. Fluid mechanics of flow through a stenosis: relationship to the development of mural thrombus. *Ann Biomed Eng* 1997; **25**: 344–56.
8. Bluestein D, Gutierrez C, Londono M, *et al*. Vortex shedding in arterial stenosis and its relevance to mural platelet deposition. *Ann Biomed Eng* 1999; **27**: 763–73.
9. Ramstack J M, Zuckerman L, Mockros L F. Shear induced activation of platelets. *J Biomechanics* 1979; **12**: 113–25.
10. Hellums J D, Peterson D M, Stathopoulos N A, *et al*. Studies on the mechanisms of shear-induced platelet activation. In: Hartman A, Kushinsky W, eds. *Cerebral Ischemia and Hemorheology,* New York, NY: Springer, 1987; 80.
11. Sutera S P, Nowak M D, Joist J H, *et al*. A programmable, computer-controlled, cone-plate viscometer for the application of pulsatile shear stress to platelet suspensions. *Biorheology* 1988; **25**: 449–59.
12. Purvis N B, Giorgio T D. The effect of elongational stress exposure on the activation and aggregation of blood platelets. *Biorheology* 1991; **28**: 355–67.
13. Boreda R, Tatemi R S, Rittgers S E. Potential for platelet stimulation in critically stenosed carotid and coronary arteries. *J Vasc Invest* 1995; **1**: 26–37.
14. Neuenschwander P, Jesty J. A comparison of phospholipid and platelets in the activation of human factor VIII by thrombin and factor Xa, and in the activation of factor X. *Blood* 1988; **76**: 1761–70.
15. Jesty J, Bluestein D. Acetylated prothrombin as a substrate in the measurement of the procoagulant activity of platelets: elimination of the feedback activation of platelets by thrombin. *Anal Biochem.* 1999; **272**: 64–70.
16. Glantz S A. *A Primer of Biostatistics*, 4th edition. New York: McGraw-Hill, 1997.
17. Shoenfeld N A, Eldrup-Jorgensen J, Connolly R, *et al*. The effect of low molecular weight dextran on platelet deposition onto prosthetic materials. *J Vasc Surg* 1987; **5**: 76–82.
18. Garrett K O, Pautler S V, Johnson P C. The inability of low-molecular-weight dextran, aspirin, and prostaglandin E1 to inhibit monolayer platelet adhesion to polyethylene. *J Lab Clin Med* 1993; **122**: 141–8.
19. Kroll M H, Hellums J D, McIntire L V, *et al*. Platelets and shear stress. *Blood* 1996; **88**: 1525–41.
20. Merten M, Chow T, Hellums J D, *et al*. A new role for P-selectin in shear-induced platelet aggregation. *Circulation* 2000; **102**: 2045–50.