Activation status of platelet aggregates and platelet microparticles shed in sheared whole blood

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The role of temperature and shear rate in the activation status of aggregating platelets and platelet microparticles (MPs) was investigated in a modified concentric-cylinder rotational viscometer. Whole blood anticoagulated with citrate was exposed to a range of shear rates typical of cardiopulmonary bypass circuits (0, 1000, 2000 and 4000 s^{-1}) over four temperatures spanning hypothermic to mildly hyperthermic conditions (24, 30, 37 and 42 °C) for short durations (100 s). Aliquots of blood were double-stained for CD41 (platelet GPIIb/IIIa) and CD62 (P-selectin). Platelets, platelet aggregates, MPs and red blood cell-platelet and -MP aggregates were identified by flow cytometry by acquiring only CD41-positive particles and differentiating on a plot of CD41 versus forward light scatter. The activation status of each particle was quantified by measuring CD62 expression (α-granule release). A degree of correlation between the shedding of MPs and the formation of platelet–platelet aggregates was observed for the data as a whole ($r = 0.85$ for $p < 0.01$), although this trend was not observed for a shear rate of 4000 s^{-1}. The mean expression of CD62 on both platelets and MPs was maintained at a very low level for all temperature and shear rate combinations. There was, however, a number of very highly activated MPs associated with red blood cells at high shear rates.

1. Introduction

Cardiopulmonary bypass (CPB) is used to replace circulation and ventilation in patients undergoing open heart surgery, more commonly coronary artery bypass grafting. Despite there being 250 000 CPB procedures each year a decade ago in the United States alone [1], the risk of morbidity and mortality is still relatively high [2, 3], although the nature of the surgery and critical status of the patients influence these figures. Nevertheless, large numbers of patients are affected neurologically [2] whilst many others suffer bleeding disorders [4, 5]. The picture is complicated by the fact that CPB is usually performed under hypothermic conditions. Many clinical studies have looked at different parameters of the cellular and non-cellular components of coagulation and have arrived at differing conclusions [6]. Many researchers blame some form of transitory acquired platelet dysfunction for the bleeding disorders [5, 7], whilst others suggest that induced hypothermia alone can significantly affect the functioning of components of the coagulation cascade [8]. Few have studied isolated parts of the process *in vitro* in depth, however.

There are a number of possible alternative explanations for the complications associated with CPB. There is undoubtedly an influence on the blood from contact with the oxygenation unit. An additional trauma comes from the unnatural shear forces placed on the blood components. In a membrane oxygenator, blood passes through a series of narrow channels or hollow fibres of 200 μm or less at high velocities for prolonged periods of time. Platelets are relatively labile cells and have been shown to activate and form aggregates in response to high levels of sustained shear forces. Recently, the presence of platelet-derived microparticles (MPs) has been observed *in vitro* in sheared whole blood samples in which the platelets have undergone aggregation [9] without chemical agonist involvement. There have been many reports of MP generation in response to platelet-stimulating species [10], mostly activated complement fragments or complexes [11–13]. Fragments of platelets have been observed in blood from patients undergoing CPB [14], although the causative factor of this shedding was not identified. MPs contain, and express on their surface, procoagulant species [15] which are involved in the activation and potentiation of thrombosis and platelet and leukocyte reactions. It is possible that MPs are important enhancers of the aggregation and activation states of single platelets,

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amplifying the effect a device has on flowing blood. Indeed, they have been implicated in the potentiation and modulation of atherosclerotic plaques [9], fibrin growth [16] and the activation of granulocytes in injury and inflammation [17]. This study investigated the hypothesis that MP production is correlated with platelet activation and aggregate formation over a range of temperatures and shear rates, and that hypothermic surgical procedures may potentiate such vesiculation.

2. Materials and methods

2.1. Collection of blood

Blood was taken from healthy, male volunteers who had been free from medication for at least 14 days and anticoagulated with tri-sodium citrate (3.8% wt/vol) at a ratio of 1 part citrate to 9 parts blood, by venepuncture of a median cubital vein with a 19-gauge needle. Before any experiments were performed, the platelets were allowed to recover from the shock of phlebotomy by incubating the blood for 30 min at 37 °C.

2.2. Shear-force exposure to blood samples

A series of experiments was performed in which blood was exposed to shear rates of varying magnitude (0, 1000, 2000 and 4000 s^{-1}) at a range of temperatures (42, 37, 30 and 24 °C). In each experiment, 310 µl whole blood was exposed to the desired temperature/shear rate combination in a modified concentric cylinder viscometer (Contraves LS-2, Zurich, Switzerland) which was housed in a thermostatically controlled environmental chamber. The torque-measuring apparatus of the LS-2 was isolated and its motor replaced with a faster unit, the speed of which was under computer control, thus allowing precise, linear acceleration and deceleration of the cylinder to the desired level of shear. The environmental chamber was heated by a 200 W solid-state heating element with integral fan (RS Components, Corby, Northamptonshire, UK) coupled to a proportional temperature controller. The aliquots were sheared for 100 s plus 15 s acceleration and deceleration phases at the beginning and end of each cycle. The blood for each experiment was taken from a 2 ml sample which had been held at the appropriate temperature for at least 7 min. For each temperature, the sequence of shear rates to which the blood was exposed was randomized to prevent a systematic error.

2.3. Analysis of platelet reactions

At the end of each shearing period, a 40 µl aliquot was removed from the sample chamber and placed into fixative (1% (wt/vol) final concentration paraformaldehyde in PBS) and incubated at room temperature for 10 min. Fixed blood was then double-stained with anti-CD62P directly conjugated with fluorescein isothiocyanate (FITC) (specific for P-selectin, from Serotec, Oxford, UK) and anti-CD41 directly conjugated with phycoerythrin (PE) (specific for platelet glycoprotein receptor GPIIb/IIIa, from Pharmingen, San Diego, CA, USA) and incubated for 20 min at 4 °C. The samples were analysed for platelet-positive particles using a Becton Dickinson FACSort (San Jose, CA, USA) by thresholding on FL2 (PE fluorescence); 30 000 such particles were acquired per sample. MPs were identified by reference to a non-exposed control sample (one for each temperature) in terms of its forward scatter (FSC) and PE fluorescence, both being less than for platelets. The PE fluorescence was cross-correlated with FL1 (FITC fluorescence), being a measure of platelet α-granule release.

2.4. Data analysis

Particles were quantified in terms of their FSC and platelet-marker fluorescent intensity (FL2). For each subject, a two-dimensional dot-plot (FSC versus FL2) of the 37 °C control sample was constructed. The dot-plot was separated into five polygonal regions to identify (I) normal platelets, (II) platelet–platelet aggregates, (III) platelet–red blood cell (RBC) aggregates, (IV) microparticle (MP)–RBC aggregates, and (V) MPs. These regions were then applied to dot-plots generated from the treatment samples and platelet aggregation was quantified (Fig. 1). The mean number of platelets per aggregate (MPA) was estimated by dividing the mean value of FL2 for the platelet aggregate group (II) by the mean value of FL2 for the normal platelet group (I). A platelet aggregation index (PAI) could then be calculated by the following formula

$$\mathrm{PAI}(\%) = \frac{N(\mathrm{II}) \cdot \mathrm{MPA}}{N(\mathrm{I}) + [N(\mathrm{II}) \cdot \mathrm{MPA}]} 100 \qquad (1)$$

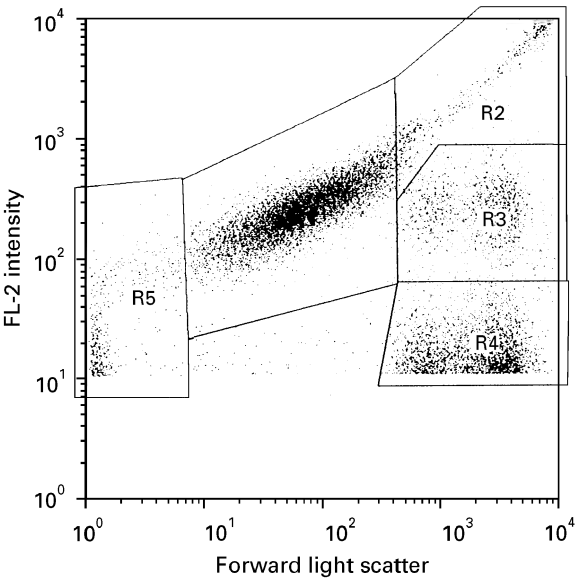

Figure 1 Dot-plot of fluorescence intensity against forward light scatter for 30 000 platelet-positive particles in whole blood exposed to 4000 s^{-1} at 24 °C for 100 s. Regions consist of (I) normal platelets; (II) platelet–platelet aggregates; (III) platelet–red blood cell aggregates; (IV) microparticle–red blood cell aggregates; (V) microparticles.

TABLE I Values of MPSI (%), as defined in Section 2.4. Values compared to non-exposed control samples held at 37 °C

Temp (°C)	MPSI (%)			
	$0\,s^{-1}$	$1000\,s^{-1}$	$2000\,s^{-1}$	$4000\,s^{-1}$
24	1.40[a]	1.61[a]	1.87[b]	3.94[b]
30	1.07	1.32[c]	1.38[a]	2.46[c]
37	1.76	1.91	1.09[a]	1.62[b]
42	0.946	0.979	0.973	1.23[a]

[a] $p < 0.05$ compared with control value.
[b] $p < 0.01$ compared with control value.
[c] $p < 0.005$ compared with control value.

where $N(\mathrm{I})$ and $N(\mathrm{II})$ are the number of particles within regions I and II.

An index of microparticle shedding (MPSI) was defined as the number of particles observed in polygonal region V divided by the total number of platelets involved in both platelet–platelet aggregates and single cells, as follows

$$\mathrm{MPSI}(\%) = \frac{N(\mathrm{V})}{N(\mathrm{I}) + [N(\mathrm{II}) \cdot \mathrm{MPA}]}\,100 \qquad (2)$$

3. Results

A two way analysis of variance with replicants was performed on the MPSI data. Significant variation in the data was found with both temperature and shear-rate ($p < 0.05$). It was additionally noted that significant differences between treatment samples and control samples at 37 °C existed due to both temperature and shear rates as analysed by a paired, one-tailed t-test (Table I). The greatest increase over the control was observed at $4000\,s^{-1}$ and 24 °C ($p < 0.005$).

There appeared to be similarities in the shape of the three-dimensional distributions of MPSI and PAI and over all the combinations of conditions a correlation coefficient of 0.85 was estimated. A Fisher's z test indicated that there was a relationship between PAI and MPSI ($p < 0.01$). Visual comparison of the two graphs in Fig. 2 suggests that as functions of temperature there may be differences between platelet aggregation and microparticle shedding at a shear-rate of $4000\,s^{-1}$. A paired one-tailed t-test indicated that MPSI was lower at 42 °C than at 37 °C ($p < 0.01$) at this shear-rate, while a similar test for PAI did not demonstrate this difference. Indeed, there appears to be a minimum at 37 °C in platelet aggregation though this cannot be shown to any level of significance.

Surprisingly, mean P-selectin expression was maintained at a low level, insignificantly different from the control sample, for all treatments, although there were subpopulations of platelets and MPs that had undergone a high degree of α-granule release (Fig. 3). These highly activated particles were, *in every case*, associated with an RBC.

4. Discussion

Previous studies have shown that MP generation occurs in response to similar stimuli that cause platelets to aggregate [9]. This study provides evidence that the two processes may not, however, be absolutely correlated. Increasing temperature appears to have a diminishing effect on MP shedding, whilst the presence of platelet aggregates is significantly higher than control values at the highest temperature at $4000\,s^{-1}$ (Table I). Platelet aggregation can be initiated by a variety of routes: shear-induced aggregation (SIPA) via binding of von Willebrand factor (vWF) to platelet GPIb [18] or agonist-induced aggregation by ADP via a P_{2y} class receptor [19], collagen via GPIa/IIa

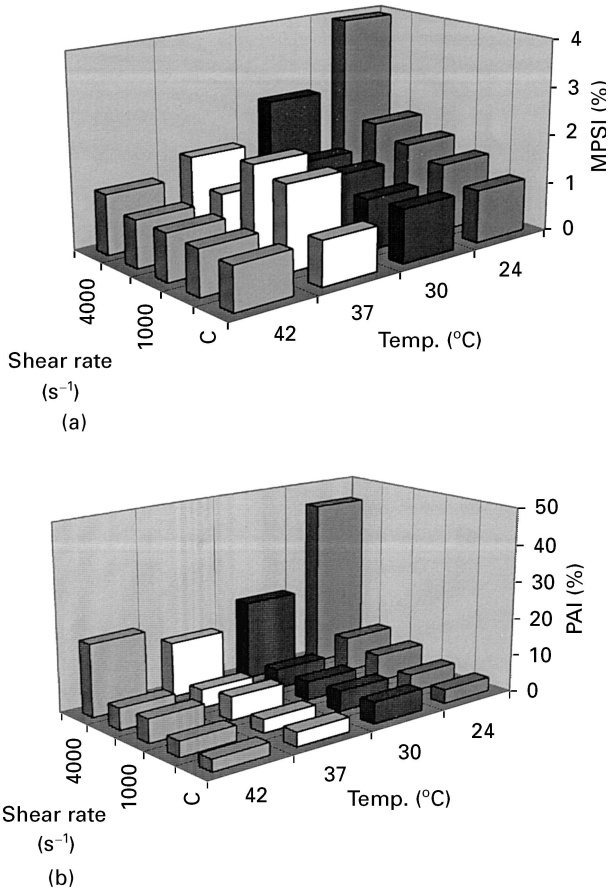

Figure 2 Indices of (a) microparticle shedding, and (b) platelet aggregation, as defined in Section 2.4., for 100 s exposure of whole blood to different shear rates at a range of temperatures.

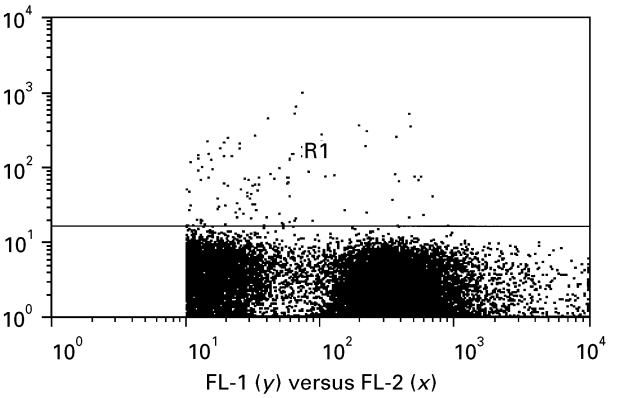

Figure 3 Dot-plot of P-selectin expression against CD41 for whole blood exposed to $4000\,s^{-1}$ at 24 °C for 100 s. R1 indicates the population of greater than background CD62 expression.

($\alpha_2\,\beta_1$) [20] or thrombin via an undesignated receptor [21] or synthetic molecules which ultimately involve the induction of GPIIb/IIIa ($\alpha_{IIb}\beta_3$) to bind fibrinogen. MP shedding has been studied to a smaller degree, probably because, until recently, their size has fallen below the detection limit of many laboratory instruments. MP generation can also occur along a number of routes, although shear-induced shedding has also been demonstrated to be dependent upon vWF-GPIb interaction [9]. Our findings suggest, however, that temperature alone may cause differential modulation of MP shedding compared to platelet aggregation, given that the non-exposed 42 °C control contained fewer MPs than did the 37 °C control ($p < 0.05$). It is possible that this effect is due to additive contributions to aggregate formation, for example from ADP, for which there is an inhibitor in plasma which is temperature sensitive. Alternatively, it is possible that there are contributions to MP shedding from additional biochemical interactions. Clearly, further studies are needed to elucidate the exact nature of the differential response.

Despite minimal differences in mean CD62 expression between control and sheared samples, the shape of fluorescence histograms was more complex in the sheared samples (Fig. 4). CD62 expression in the control samples was typical of a weak or non-expressive sample, whereas a bimodal response of varying magnitude was observed in the sheared samples. This distribution could indicate a population of platelets with a low level of P-selectin expression. Data modelling techniques would be required to enumerate this population and this is beyond the scope of this paper.

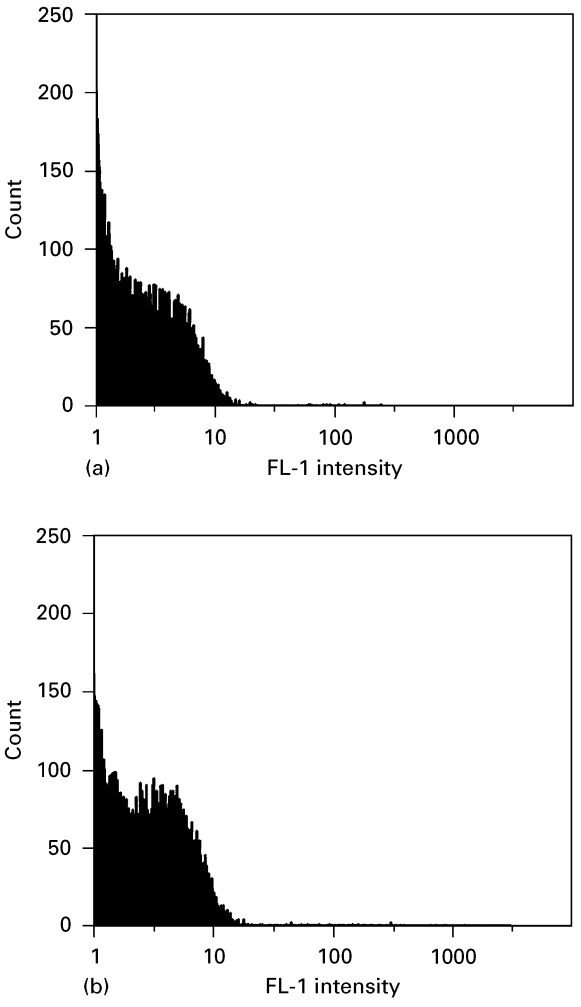

Figure 4 Histograms of CD62 expression on platelet-positive particles in blood exposed in a viscometer at 37 °C for 100 s at: (a) 0 s^{-1} and (b) 4000 s^{-1}.

It is known that high shear environments have the dual manifestations of MP shedding and high MP procoagulant content [9], as demonstrated by the expression of activated phospholipids on the MP surfaces. It would not, therefore, be unreasonable to expect that they would also have a higher-than-baseline expression of CD62. This was not the case here, however, given that the activation status of single platelets and platelet–platelet aggregates was minimal. This apparent lack of stimulation is similar to findings by Konstantopoulos *et al.* [22], and demonstrates the ability of platelets to undergo differential activation. There were, however, a very small number of microparticles that were highly activated (in terms of α-granule release), but these were, in every case, associated with a red blood cell. This demonstrates a possible mechanism by which some highly activated procoagulant particles may be (i) prevented from adhering to a local surface, and (ii) transported to distal sites. It is likely, however, that a large number of highly activated, fluid-phase MPs and platelets adhere to the viscometer walls under such shear forces. The adhesion of platelet material to the chamber walls has not been quantified in this study.

The degree of MP shedding in all the control samples appears to be rather high. This is not inconsistent with results from other studies. It has been demonstrated that citrated blood consistently contains a higher number of MPs than does heparin or K_2EDTA blood [23]. The use of citrate can be justified by the non-specific expression of P-selectin and aggregate formation observed in heparin and K_2EDTA regimens.

It has been suggested that the interaction of platelets with RBCs may be due to coincidence of these cell types as they pass through the cytometer's laser beam [22] as many cells pass through this beam in a short period of time contained within a narrow column of fluid. Because further dilution of the samples with isotonic saline did not appear to reduce the apparent association, we conclude that the observed aggregates of platelets and MPs with RBCs is a genuine phenomenon.

5. Conclusion

MP shedding does not mirror platelet aggregation in every combination of shear rate and temperature and there are probably contributions to aggregate formation which do not affect MP generation. Our results suggest that most platelets and MPs are not stimulated to secrete α-granules by virtue of shear alone, even at the highest rates studied, but this does not preclude the possibility that some platelets have been highly activated and have adhered to the test system.

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