Granule secretion markers on fluid-phase platelets in whole blood perfused through capillary tubing

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The effect of material composition and shear rate on fluid-phase platelet activation was investigated using a capillary perfusion model. Citrated whole blood was perfused along the lumens of tubes constructed from silicone, PVC, Pellethane, W124 (an experimental polyetherurethane), and glass. Platelet activation was determined by measuring the increase in α-granule membrane protein P-selectin (GMP-140, CD62) and the lysosomal granule membrane protein GP-53 (CD63) on fluid-phase platelets by flow cytometry. All tubes caused an increase over the negative control in the number of P-selectin and GP-53 molecules detectable on the surface of these platelets. The activation response of platelets to changes in shear rate was also investigated. It was found that lysosomal release paralleled α-granule release in glass, but not in Pellethane, over a range of wall shear rates (100–1,000 s⁻¹). © 1994 John Wiley & Sons, Inc.

INTRODUCTION

When a material is placed within flowing blood, a number of well-defined events occur. After a small degree of inorganic ion and water adsorption,1 the adsorption of proteins commences, typically within the first few milliseconds.2 This layer then forms the interface at which certain reactions between blood and the surface will take place. Depending upon the chemical and physical nature of the surface, and consequently on the nature of this protein layer, blood cells, including erythrocytes, leukocytes, and platelets may become altered, either within the fluid-phase or at the material surface itself. Reactions involving platelets are particularly important. It has been demonstrated that in humans a relatively small percentage of circulating platelets is at any time adherent to the surface.3 The presence of activated fluid-phase platelets has importance not only to thrombosis but also in the propagation of reactions of other blood cells to artificial surfaces. Many constituents of the platelet granules (e.g., platelet derived growth factor (PDGF), platelet factor 4 (PF4), and other chemotactic factors) propagate reactions by activating neutrophils,4,5 which, in addition, have the ability to adhere to exposed platelet P-selectin.6

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Various assays have been utilized for studying platelet activation, but they have generally been concerned with adherent platelets through either the study of their morphology by SEM,7,8 or quantification of the presence of platelet activation markers within the blood or plasma, such as 5-HT,9 PF4,10 TxB2,11 or β-TG.12 The status of fluid-phase cells is usually not discussed. Therefore, it was thought desirable to investigate the fluid-phase platelet activation after perfusion of whole blood through tubes of differing materials. This was performed by measuring perfusion-induced increases in both α-granule and lysosomal granule secretion markers and by studying the manner in which shear rate affects these parameters.

MATERIALS AND METHODS

Polymers

The experimental materials used were medical grade tubing (1.0 mm internal diameter). Silicone (Shore hardness 50) was purchased from Altec (Alton, Hampshire, U.K.). PVC (Shore hardness 80) was from Portex (Hythe, Kent, U.K.), Pellethane (Shore hardness 90) was from Dow (Midland, MN), and
glass was from Chance Brothers (Malvern Links, Worcestershire, U.K.). W124 was an experimental material, based on polyetherurethane with a Shore hardness of 90, containing PEO in its soft segment to achieve a greater hydrophilicity.

**Blood material contact**

Fresh blood was withdrawn from an antecubital vein of healthy male volunteers who had refrained from any medication for at least 14 days and had fasted for at least 4 h. Collection was achieved using a 19-gauge needle placed such that vascular damage and platelet trauma were minimized. Withdrawn blood was immediately placed in trisodium citrate (3.8% w/v) at a volume ratio of 1 part citrate to 9 parts blood.

Anticoagulated blood was used for material contact after an incubation period of at least 20 min at 37°C to prewarm the blood and allow platelet recovery after blood withdrawal. Whole blood was perfused through 120 cm lengths of tubing in a thermostatically controlled hot room at 37°C at wall shear rates of up to 1,000 s⁻¹ for periods of 10 min. After material contact, the platelets were stabilized by the addition of 1 µg PGI₂ (Sigma, St. Louis, MO) per 1 ml whole blood.

Perfusion was performed by way of a Harvard model 11 microprocessor-controlled syringe pump (Harvard Apparatus Ltd., South Natick, MA). Five milliliters of whole blood was perfused along the length of the tube, which was connected at each end to 50-ml polypropylene syringes. After the blood had been completely exhausted from one syringe the pump was changed to the other end to allow blood to be perfused along the same length of tubing almost indefinitely. The capillary tubing was placed inside a 1-m length of glass tubing to ensure that the tube remained straight. The syringes were connected to the capillary tubing by 4.5-cm length polypropylene ramped connectors. From the physical characteristics of the tubing and the rate of perfusion it was estimated that the development length of the flow was less than 1 mm at the highest wall shear rate performed (1000 s⁻¹) and considerably smaller for the other shear rates as defined by Caro et al.¹³ for Reynolds numbers in the range 10–2,500. This ensured that a fully developed, laminar flow profile was experienced in excess of 98% of the capillary tubing length after taking start and end effects into account.

**Platelet staining for flow cytometry**

One hundred microliters of citrated whole blood containing stabilized platelets was incubated with 20 µl of primary antibody, specific either for P-selectin (Mo.ab 2.17), an α-granule membrane protein (CD-62), or GP-53 (Mo.ab 2.28), a 53 kDa lysosomal membrane protein (CD63). Both antibodies were donated by Dr. H.K. Niewenhuis (University Hospital of Utrecht, The Netherlands). Fifty microliters of goat anti-mouse antibody conjugated with fluorescein isothiocyanate (FITC) (Becton Dickinson, San Jose, CA) was added to form the fluorescing complex and incubated for 30 min. The cells were then washed, first by centrifugation at 1,500 g for 10 min, then by resuspending them in 100 µl of a modified tyrosides buffer (138 mM NaCl, 2.9 mM KCl, 1 mM MgCl₂, 3 mM Na H₂ PO₄, 5 mM dextrose, 20 mM Hepes, and 0.3% w/v BSA [Fraction V; Sigma, Poole, Dorset, U.K.]), and again by centrifugation as before. Fixation was then achieved by resuspension and incubation of the cells with 60 µl of 1% w/v paraformaldehyde for 10 min. Fixative was removed by centrifugation at 1,500 × g for 10 min, with the cells resuspended in 100 µl of modified tyrosides buffer.

Fluorescein isothiocyanate-containing samples were kept in the dark, and all incubations were performed on ice to reduce nonspecific binding of the antibodies. All solutions and buffers required for flow cytometry were filtered to 0.22 µm prior to use to remove any contaminating platelet and sub-platelet–sized particles.

**Flow cytometry**

Platelets were analyzed by passing fixed, whole blood, single-labeled for platelet granule membrane proteins, through the flow cytometer (Epics Profile II; Coulter Electronics, Hialeah, FL). Platelets were identified on a log–log plot of forward scatter against side scatter. This plot revealed three distinct populations: red and white blood cells, platelets, and debris and sub-platelet–size cellular particles. The identity of the platelet population was confirmed by the ability of the cells to stain for GP Iib/IIa using a mouse anti-human GP Iib/IIa antibody conjugated to phycoerythrin (Serotech, Oxford, U.K.). A two-dimensional gate was set around this platelet population for each different blood sample used, and platelet-staining data were collected by reference to this gate. Data for 50,000 platelets were collected for each sample.

**Treatment of fluorescence data**

Conventional scoring of cells into negative and positive populations could not be applied to the P-selectin and GP-53 measurements because these proteins are to a variable degree expressed on all platelets in a population. Changes in the expression
after perfusion of blood through narrow bore tubing were relatively small. Only with stimulation by strong agonists such as collagen, epinephrine, or thrombin could a histogram of clearly fully activated cells be observed. However, by making use of the accurate fluorescence detectors within the flow cytometer that allow reproducibility of readings typically better than 1% of background fluorescence levels, the small, perfusion-induced changes in P-selectin and GP-53 expression could still be measured. The background fluorescence was defined as that obtained by class-specific but epitope nonspecific control antibody binding. This nonspecific binding was reduced to a minimum by performing all incubations on ice. Differences in the expression of either P-selectin or GP-53 between populations of platelets were determined from an increase in detected fluorescence of the whole platelet population expressed in fluorescence units as given by the instrument. These changes were then analyzed against positive and negative controls. The positive control was platelets stimulated with thrombin; the negative control was platelets stabilized with PGJ₂ immediately after withdrawal from the body.

RESULTS

The ability of various materials to cause α-granule and lysosomal granule secretion was determined for perfusion at 1,000 s⁻¹ at 37°C for 10 min (Figs. 1 and 2). This showed that the perfusion of whole blood through a material clearly has an effect on the expression of granule membrane proteins on platelets. There were few marked differences between any of the polymeric materials for α-granule release. The experimental polyurethane W124 was slightly less activating than silicone or PVC. Although perfusion always enhanced the expression of platelet activation markers above the negative control, glass was, of course, far more activating than the other surfaces for both α-granule release and lysosomal release. W124 did not cisplcy the same high degree of platelet compatibility when comparing lysosomal granule release, whereas Pellethane and silicone were apparently both better in this respect than PVC.

In addition, the platelet granule secretion associated with differing shear rates was investigated for Pellethane and glass, and the result of every perfusion was confirmed in another, separate experiment. In each case perfusion was for 10 min at 37°C. In glass, lysosomal release paralleled α-granule release for differing wall shear rates; 100 s⁻¹ caused the greatest activation (Fig. 3). The peak lysosomal granule release was greatest at 333 s⁻¹ in Pellethane, whereas α-granule release was still increasing at 1,000 s⁻¹ (Fig. 4).

DISCUSSION

Platelet granule secretion studies

Because of the sensitivity of fluorescence detectors, differences in the response of platelets to small, differing stimuli, such as those arising from perfusion along catheter tubing, may be readily detected. Under these experimental conditions the changes were not great, even after perfusion along glass; to put the figures in perspective, thrombin stimulated cells registered approximately 20 fluorescence units.

The observed platelet release reactions are probably due to the stimulation of different membrane glycoprotein receptors, of which platelets have a large number. The main receptor involved in adhesion to biomaterials has often been considered to be
GPIIb/IIIa, the fibrinogen receptor. However, the stimulation of platelets would appear from our data to be more complex, as the α-granule and lysosomal release seem to be differentially induced by different surfaces and flow regimes. This could have been because selective protein adsorption to different materials caused different platelet receptors to be activated. In this respect it is interesting that the release of substances from different granules is selectively sensitive to inhibition by different pharmacologic agents.

Because of the sensitivity of the method and the fact that small differences may be detected following perfusion of whole blood through catheters of different materials, we propose that the method may be appropriate for the evaluation of blood–material interactions in vitro.

**Fluorescence measurement**

In conventional flow cytometry, it is usually considered that a cell population is positive or negative for a particular antigen. A gate is placed at a particular fluorescence limit, indicating a baseline for positivity, and the cell count above and below is used to determine the percentage of positive and negative cells in the population. However, when a cell population displays a continuum of antigenic expression, positive or negative identity is meaningless, in which case a gate is set at a value of fluorescence below which cells stained with an isotypic nonspecific control have 100% expression. The fluorescence observed below this level cannot be distinguished from nonspecific binding of the antibodies. The degree of antigenic expression is calculated from a change in displayed mean fluorescence of cells above this threshold compared with a control whose antigenic properties are known. It is possible to find cells that have weak antigenic expression sufficient that the fluorescence due to antibody staining falls below this nonspecific baseline. Indeed, resting platelets fall into this category with regard to P-selectin.

Therefore, there are several choices for analysis of the data. First, one could simply set a gate, using the nonspecific control, and count the number of cells that have positivity above this level. However, this would be misleading in several ways. Simply quantifying the number of cells above this point would not take into account the degree of shift in fluorescence of these cells. A population of cells with many weakly activated cells, all of which would be counted as positive, and a population of many highly activated cells would deceptively register the same. In addition, a small increase in antigenic expression could increase fluorescence, but not beyond the nonspecific control; therefore, this shift would be missed.

An alternative method would be to quantify and compare means of fluorescence of cells above the nonspecific baseline, but this, too, would be misleading for the same reasons. The only way in which it is possible to quantify the data without recourse to misleading alternatives is to compare the raw fluorescence data to establish small differences in population expression. This is made feasible by the advanced computerized statistical analysis on the more advanced flow cytometers in which the fluorescence data are split into 1,024 channels. Any errors in analysis are minimized by basing the mean fluorescence on at least 50,000 cells, resulting in a maximum difference of approximately 1% between analyses of the same cells (in the order of 0.01 fluorescence units) and a maximum of 3% between analyses of the same population of cells stained separately. This degree of accuracy allows any difference in fluorescence to be directly attributable to real differences in antigenic expression.
CONCLUSIONS

Tubes constructed from medical grade polymers and control materials were assessed for their ability to cause platelet α-granule and lysosomal granule release. No correlation was found between these two parameters for identical experimental conditions using different polymeric materials. The α-granule and lysosomal granule release did follow the same pattern in response to glass over a range of shear rates, but not for Pellethane over the same range. It is concluded that under some circumstances, the secretion of the two granules can be differentially induced, possibly by different platelet activation pathways. In addition, fluid-phase platelet granule status is considered a useful marker of blood compatibility.

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References


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