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Assays for quality control of platelets for transfusion

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Quality control of platelets relies on the determination of the extent by which platelets are still able to react with known agonists, and here knowledge of the biochemistry of platelet activation may guide to decide which tests are useful. *In vivo*, platelets will adhere to collagen exposed upon vessel wall damage, and especially under high shear conditions through von Willebrand factor (VWF) that forms a bridge between collagen and platelet glycoprotein (GP) Ib. Binding of GPVI to collagen next results in the activation of a tyrosine kinase cascade, finally resulting in phosphorylation and activation of phospholipase C (PLC) γ2, which leads to an increase in cytosolic Ca^{2+} levels. High Ca^{2+} levels trigger the production of thromboxane A2 (TXA2) and release of platelet granules containing among others, adenosine diphosphate (ADP). TXA2 and ADP now will activate their receptors on platelets which are, among others, linked to a G-protein that activates PLCβ2 with more Ca^{2+} increase. This ultimately provokes activation of the integrin $\alpha_{IIb}\beta_3$ (or GPIIbIIIa), which now can bind the symmetrical fibrinogen, thus allowing cross-linking of platelets or platelet aggregation. From the above it is clear that testing whether platelets are fully reactive ideally should be looking at as many of the above parameters as possible, while at the same time being simple and fast. Systems in which blood is flown over collagen surfaces mimic best the physiological situation; however, these tests are not fit for stored platelet testing as haematocrit is a critical factor in these experiments. Thromboelastography at best provides a test for G-protein dependent activation (through thrombin) and integrin $\alpha_{IIb}\beta_3$ involvement. Platelet agglutination induced by ristocetin (surrogate for the adhesion phase) next to aggregation (involvement of integrin $\alpha_{IIb}\beta_3$) induced by collagen (Tyr kinase pathway) and ADP (G-protein mediated pathway) can give a comprehensive view on the platelet quality. However, flow cytometry is also an excellent technique to detect (i) bound VWF to platelets in the presence of ristocetin, (ii) collagen- or ADP-induced activation of integrin $\alpha_{IIb}\beta_3$ (by determining the binding of fibrinogen or of the activation-dependent PAC-1 antibody) and (iii) secretion (by detecting surface expression of P-selectin).

Key words: adhesion, aggregation, platelets

Introduction

The term ‘platelet quality’ is commonly used to unite the wealth of parameters that describe thrombocyte morphology, function, responsiveness to (ant)agonists, receptor abundance, et cetera. It is a dubious term because the vast number of possibilities to assess ‘platelet quality’ requires researchers or (para)medical staff to select and therefore limit the assessment to specific aspects of platelet function. For instance, a platelet suspension may react vigorously to potent agonists in turbidometric aggregometry, but remain fairly unresponsive to specific immobilized matrix proteins in shear flow, leading to quite different conclusions on the suspension’s quality. It is up to the critical investigator to restrict conclusions of platelet assays to the sole parameter it measures.

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Some platelet assays are simple and only require a trained eye ('swirl') or a pH probe. These assays generally discriminate poor quality platelet concentrates from non-poor ones. Furthermore, these allow swift examination of a product in typical blood bank or routine quality control settings. However, advanced platelet assays like those described below can unravel much more than this binary result.

First quality parameters in blood banking

Platelets carry active mitochondria which generate 85% of the adenosine triphosphate supply through oxidative phosphorylation [1]. The remaining energy demand is largely fulfilled by anaerobic respiration [2] leading to an accumulation of lactic acid in suspensions like concentrates for transfusion. Acidosis adversely affects platelet behaviour (in) directly causing the cell to swell and adopt a spherical instead of discoid shape. Therefore, pH-determination is a straightforward method commonly used to report product quality. Sometimes, pH and other indicators of quality correlate, but this is not always the case with precedents on both sides, that is, issued concentrates with acceptable pH but poor quality or *vice versa*. This especially holds for platelet products prepared in additive solutions, which often contain buffering salts that thus may hold pH, but not necessarily biological integrity.

The morphological transition from disc to sphere mentioned above is often exploited for platelet concentrate quality determination [3]. The method results in a 'swirl (ing)' score, referring to the level of anisotropic and nematic light scattering behaviour of discoid particles in suspension. The elegance is mainly of practical nature as it is easy and quick allowing transfusion services to discard products below threshold scores if required. However, the assay hardly has dynamic range, is prone to subjectivity and does not entirely or necessarily correlate with other platelet (quality) markers.

An automated operator-independent method for platelet morphology testing was developed by Maurer-Spurej *et al.* [4] and is commercially developed under the name of Thrombolux™ (Lightintegra, Vancouver, BC, Canada). The technique uses dynamic light scattering in a small sample of platelet concentrate while applying multiple cycles of warming and cooling. Platelet size, number of microparticles and the cellular response to the temperature cycles finally result in a single score for the sample [5]. The dynamic range is substantially higher than with 'swirling' and the technique allows for enumeration of microparticles [6], which might be considered a marker for platelet lesion. In a pilot study, dynamic light scattering by Thrombolux™ has been shown to correlate with transfusion outcome [7] corroborating with the correlation of 'swirling' scores and corrected platelet count increment.

Determination of platelet reactivity *in vitro*

A second approach is to determine to what extent the platelets are still able to react with known agonists, and here knowledge of the biochemistry [8] of platelet activation may guide to decide which tests are useful (Fig. 1). Platelets will adhere to collagen exposed upon vessel wall damage, and especially under high shear conditions through von Willebrand factor (VWF) that forms a bridge between collagen and platelet glycoprotein (GP) Ib. Binding of GPVI to collagen next results in the activation of a tyrosine kinase cascade, finally resulting in phosphorylation and activation of phospholipase C (PLC) γ2, which leads to an increase of cytosolic Ca^{2+} levels. High Ca^{2+} levels trigger the production of thromboxane A2 (TXA2) and release of platelet granules containing among others, adenosine diphosphate (ADP). TXA2 and ADP now will activate their receptors on platelets which are, among others, linked to a G-protein that activates PLCβ2 with more Ca^{2+} increase. This ultimately provokes activation of the integrin $\alpha_{IIb}\beta_3$ (or GPIIbIIIa), which now can bind the symmetrical fibrinogen, thus allowing cross-linking of platelets or platelet aggregation.

From the above it is clear that comprehensive testing of all platelet functions (in haemostasis) ideally should be looking at as many of the above parameters as possible, while at the same time being simple and fast.

Systems under flow

Tests that are most mimicking *in vivo* platelet-dependent thrombus formation are systems in which blood is flown over or through collagen coated surfaces such as parallel-plate flow chambers, cone-and-plate viscometers or commercial devices like PFA-100. However, as haematocrit is an important parameter in these settings (e.g. PFA-100 does not close with a haematocrit <10%), it is clear that they are not immediately fit for quality testing of platelets from platelet concentrates in a routine setting. However, for research purposes, platelets from concentrates can be reconstituted [9] with red blood cells and/or plasma to fulfil the requirement of haematocrit. There is considerable variability when performing hydrodynamic flow chambers [10] for platelet function. There are outstanding review articles on basic and applied issues of flow chambers to which the reader is referred [11,12].

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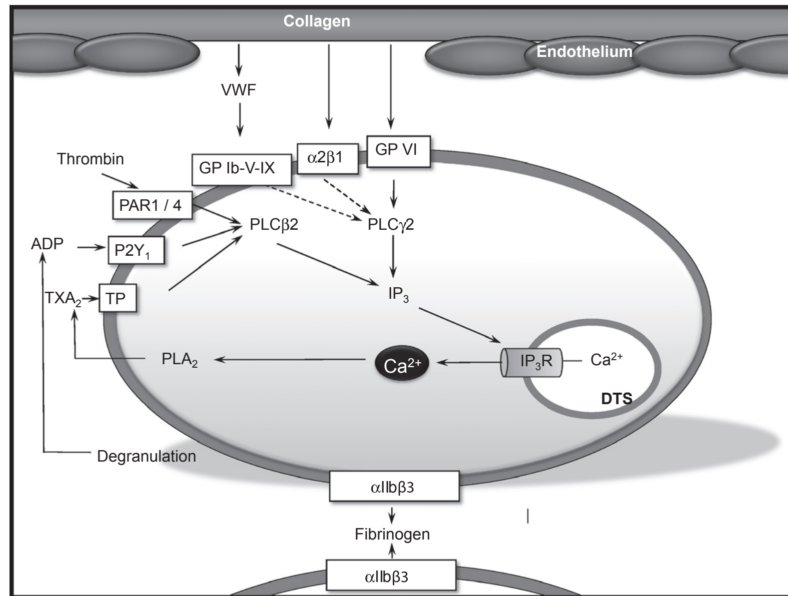

Fig. 1 Main platelet activators and activation pathways. Phospholipase C (PLC) γ2 is activated through tyrosine kinases, PLCβ2 by G-proteins. Both result in increase in cytosolic Ca^{2+}-levels, leading to $\alpha_{IIb}\beta_3$ (GPIIbIIIa) activation, secretion of granules with expression of P-selectin, and exposure of negatively charged phosphatidylserine.

Thromboelastography

Another quite popular test in the clinic is thromboelastography [13], in which the clotting (and fibrinolysis) of whole blood is followed by determining the torque exerted by the clotting blood on an oscillated device. The test is obviously a clotting test, but platelets do participate as they become activated by the thrombin formed, after which they accelerate further clot formation and cause clot retraction. Thrombin also activates platelets via G-proteins, whereas clot retraction is mediated by binding of integrin $\alpha_{IIb}\beta_3$to fibrin. In this system hence G-protein-mediated activation and aggregation is assessed, however, neither the adhesion step nor the tyrosine kinase-dependent platelet activation pathway, is involved. The test may therefore be of somewhat limited use in the quality control setting of platelet concentrates, despite its ease of use. Furthermore, it is clear that when platelets indeed are kept in diluted plasma this also will affect the outcome of this test.

Platelet aggregation

This brings us to platelet aggregation, which is rather cumbersome for routine use, requires specialized equipment and skilled personnel [14]. Nevertheless, with three experiments one can have a fairly good view of the overall platelet reactivity. Indeed, on the one hand (i) VWF-dependent platelet agglutination induced by ristocetin, will serve as a surrogate for platelet adhesion to the injured arterial vessel wall, whereas on the other hand activation (ii) by collagen is probing the tyrosine kinase pathway, or (iii) by ADP, G-protein dependent activation, both will lead to GPIIbIIIa-dependent aggregation, readily detectable with the aggregometer. In countries where platelet concentrates are stored in additive solutions, artefacts of lower plasma protein concentrations (VWF, fibrinogen) may arise. Minor fluctuations in ristocetin responses may also be seen in single donor concentrates (from apheresis) since plasma VWF concentrations and VWF biological properties are quite variable in the normal population. Also strong calcium-chelating agents often used as anticoagulants in acid-citric-dextrose or citric-phosphate-dextrose may attenuate calcium signalling and subsequent aggregation. Moreover, phosphate ions desensitize ADP receptors (P2Y12, P2Y1) making aggregation data from platelet concentrates difficult to compare with typical aggregation responses in freshly prepared platelet rich plasma.

Flow cytometry

Another technique that can be used, and that is more easily standardized is flow cytometry [15], where, however, everything essentially relies on surrogate endpoints, and hence becomes somewhat more remote from the actual *in vivo* aggregating function of the platelets. Nevertheless, activation of $\alpha_{IIb}\beta_3$ can be detected by antibodies such as

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PAC-1 [16], which specifically recognizes the activated form of $\alpha_{IIb}\beta_3$, or by detecting fibrinogen bound to platelets [17], which both are thus a surrogate for the aggregating capacity. Alternatively, granule secretion can be determined by detecting P-selectin (CD62P) that becomes surface exposed upon fusion of platelet α-granules with the outer membrane. Binding of PAC-1 or an anti-P-selectin antibody to platelets activated by collagen or by ADP, therefore, in addition is a measure of the tyrosine kinase or G-protein dependent platelet activation pathways respectively. Furthermore, flow cytometry can also give an indication of the platelet adhesive capacity by detecting platelet-bound VWF after addition of ristocetin, using anti-VWF detecting antibodies [18], although the latter is not yet one of the more commonly used tests.

Platelet viability can be tested by binding of labelled annexin V to phosphatidyl serine. This negatively charged phospholipid resides almost exclusively in the inner leaflet of the membrane of healthy viable and resting platelets. Upon cell death, the outer leaflet bears significantly more negative charge as a consequence of phosphatidyl serine diffusion and flip-flop. Interpretation is sometimes dubious, since cell activation also makes phosphatidyl serine flip sides, causing activated but not (yet) dead cells also to bind annexin V. Furthermore, the interaction with annexin V is calcium dependent requiring calcium in the final readout mix.

Flow cytometry is easily standardized within one lab, but absolute data are often very variable among groups [19] making it a tough method to compare directly and excluding it as a 'golden standard' method. Variation in flow cytometry results is caused mainly by differences in acquisition protocols, source and label of antibodies, choices of gatings as well as the hardware. For instance, when examining platelet concentrates in function of storage time (running over more than 1 day), one should take care of the day-to-day variation caused by, for example, photomultiplier voltage changes. Internal standards like synthetic beads coated with the same antibodies as those used in the assay may prove helpful to reduce this kind of variation.

Disclosure

The authors have no conflicts of interest to disclose.

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