Platelet activation: assessment and quantification

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Introduction

The platelet is one of the key elements of human blood. Platelets play a central role in the process of thrombus formation (thrombogenesis)\(^1\), as well as an important role in atherogenesis\(^2\) and the progression of atherosclerotic lesions.

The interaction of the platelet with the vessel wall and its subsequent contribution to atheroma formation and thrombosis is of pivotal importance in the aetiology and pathogenesis of peripheral, coronary, cerebrovascular and other vascular diseases\(^3,4\). Acknowledgement of the fact that platelets have a central role to play in these disease states has led to a considerable amount of research into its pathophysiology. Indeed, inappropriate platelet activation is common in atherosclerosis\(^5,6\) and many of its risk factors, such as smoking and diabetes\(^7,8\). The role of antiplatelet therapy (such as aspirin) in reducing the risk of many cardiovascular and cerebrovascular disorders is also well established\(^9\).

Whilst there is increasing realization that inappropriate platelet activation plays a prime role in the increasing heart disease burden of society, there is still no generally accepted ideal measure of platelet activation that would indicate a state of ‘high risk’. There is also a need for the objective assessment of the relative effectiveness and safety of antiplatelet agents. Furthermore, despite the great success of aspirin, it is clearly far from perfect\(^10\). Greater attention directed to the understanding and appreciation of platelet pathophysiology and its quantification should yield valuable information that may translate into improved means to counter inappropriate platelet activation. The objective of this review is to provide a comprehensive overview of the literature that would provide a guide to a better understanding of the pathophysiology and quantification of platelet activation.

Platelet production, structure and life span

The normal platelet count is in the range 150–350 x 10\(^9\) /\(l\)\(^1\) and the platelet has a life span of approximately 8–10 days\(^12\). Resting platelets are discoid and have a smooth, rippled surface\(^13\) with a diameter which averages 1–2 \(\mu\)m and a mean cell volume of around 5–6 fl.

Platelets are derived from the megakaryocytes in the bone marrow. These megakaryocytes arise by a process of differentiation from the haemopoietic stem cell and undergo fragmentation of their cytoplasm to produce platelets\(^14\). Platelet production is under the control of humoral agents such as thrombopoietin\(^15\).

The glycocalyx and plasma membrane

The outermost layer of the platelet (the glycocalyx) is a surface coat made up of glycoproteins. This glycoprotein (GP) layer of the platelet plays a very important role in platelet function, including adhesion and aggregation, and thus, contributes to haemostasis\(^16,17\).

Indeed, there are various receptors on this glycoprotein layer, which bind to various adhesive agents, aggregating agents, inhibitors and procoagulant factors. These include the selectins (such as GMP 140, or P-selectin), the integrins (such as GP I, GP II, GP III, GP IV, GP V), the immunoglobulins and other receptors, such as those for adenosine diphosphate (ADP), collagen, epinephrine, thrombin, etc., which assist in platelet adhesion, aggregation and coagulation\(^17–20\).

For instance, glycoprotein (GP) Ia/IIa facilitates adhesion to collagen\(^21\), whilst GP Ib is important in the attachment of platelets to von Willebrand factor (vWF)\(^22\) and the vascular subendothelium. In particular, GP IIb/IIIa contributes towards adhesion to fibrin and the binding of soluble ligands (for example, fibrinogen) that facilitate platelet–platelet interaction\(^23\).

Key Words: Platelets, thrombosis, thromboembolism, antiplatelet therapy.


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Platelet granules

The platelet is an enucleate cell and the intracellular organelles in the cytoplasm include the mitochondria, alpha (α) granules, electron dense granules, dense tubular system, peroxisomes and the lysosomes\(^{[36]}\) (Table 1). Alpha granules store a variety of proteins such as platelet factor 4 (a heparin antagonist), platelet-derived growth factor (PDGF), beta thromboglobulin, fibrinogen, vWF, fibronectin and other clotting factors, although these molecules are all not entirely specific to platelets\(^{[37]}\). Some of these molecules are also secreted by the endothelial cells (for example, vWF), some are already present in the plasma (for example, fibrinogen), and a few are specific to the platelets (for example, beta thromboglobulin).

The beta thromboglobulin molecule is a tetramer and has identical subunits of 81 amino acids, each with a molecular weight of 8851\(^{[38]}\). Another alpha granule component, Platelet Factor 4 has identical subunits containing 70 amino acids, each with a molecular weight of 7756\(^{[39]}\). Both exist in similar quantities in the alpha granules, and are released during platelet activation\(^{[40]}\). The exact physiological function of these two factors is not clear and what constitutes ‘normal’ plasma levels of both molecules is also not entirely known. In addition, Platelet Factor 4 has antithrombin activity\(^{[39]}\) and its binding to the specific sites on the surface of platelets may modulate platelet aggregation and secretion induced by low level of platelet agonists\(^{[41]}\).

Thrombospondin, a 450 kDa adhesive glycoprotein present in high concentration in platelet alpha granules, is readily secreted following platelet activation\(^{[42]}\). Its main function appears to be the mediation of cell adhesion and also platelet–platelet recognition during irreversible aggregation\(^{[43]}\). However, thrombospondin is also synthesized by a variety of other cells and thus, plasma thrombospondin levels are not specific for platelet release\(^{[44]}\).

Finally, dense platelet granules contain calcium, serotonin and nucleotides, such as adenosine diphosphate (ADP)\(^{[45]}\). Other structures include the lysosomes, which contain hydrolytic enzymes, and peroxisomes, which contain the enzyme catalase\(^{[45]}\).

Another example is P-selectin, which is a member of selectin family of cell surface receptors\(^{[24]}\). It is also known as PADGEM (platelet activation dependent granule external membrane protein) or GMP 140 (granule membrane protein). This molecule, which is cysteine rich and heavily glycosylated, with mass of 140 kDa, is located in the membrane of the secretory granules (alpha granules) and in the membrane of the Weibel–Palade bodies of the vascular endothelial cells\(^{[25,26]}\). P selectin redistributes from the membrane of the granules to the plasma membrane when platelets and endothelial cells are activated and thus degranulated\(^{[27]}\).

Thus, activated platelets and not resting platelets express P-selectin on their plasma membrane, acting as receptors for neutrophils and monocytes\(^{[28]}\). Increased membrane expression of P-selectin as well as increased levels in plasma are therefore indicative of platelet activation\(^{[29,30]}\). By acting as an adhesion receptor and promoting cell-to-cell contact, P-selectin recruits both platelets and neutrophils to the sites of tissue injury\(^{[31]}\), and acute inflammation\(^{[32]}\). Platelet–leukocyte interaction(s) brought about by P-selectin in areas of tissue injury and inflammation results in the deposition of fibrin by the leukocytes within the thrombus\(^{[43]}\). It is also possible that P selectin may modulate the function of one cell type by the other.

Beneath the glycocalyx lies the platelet plasma membrane, which has phospholipid as its most important component. There is also a system of platelet contractile proteins, which form the sub-membranous filaments which help to maintain the discoid shape of the platelet\(^{[34]}\). The membrane of phospholipids promotes the cascade of coagulation by converting coagulation factor X to Xa and prothrombin to thrombin\(^{[35]}\).

Table 1  Contents of platelet granules

| 1. Electron dense granules                  | ADP (metabolically inactive storage form) |
| 2. Alpha granules                        | Factor V                                  |
| 3. Lysosome                              | Catalase                                  |
| 4. Peroxisomes                           | Thrombospondin                            |


Platelet function

The main function of platelets is the formation of mechanical plugs during the normal haemostatic response to the vessel wall injury, following platelet activation. As mentioned previously, the platelet surface has various receptors and there are diverse stimuli, which activate platelets, with equally diverse platelet responses to these stimuli, mediated by the binding of various stimulants to specific platelet receptors. For example, the occupation of ADP receptors by ADP, collagen receptors by collagen, or thrombin receptors by thrombin all produce changes within the platelet,
leading to the transformation of specific proteins (GP IIb/IIIa complexes) into a form that binds fibrinogen\textsuperscript{46,47}.

It is possible to categorize platelet responses into the ‘reversible’ platelet responses, which include adhesion, shape change and reversible aggregation and, the ‘irreversible’ platelet responses that comprise release reaction and secondary irreversible aggregation\textsuperscript{47}. Platelet adhesion and a change in shape are the initial physiological responses towards the development of haemostatic plug or thrombus\textsuperscript{48}.

Platelet shape change

When platelets adhere to the subendothelial matrix, they change their shape from discoid to spherical with the extrusion of the pseudopods\textsuperscript{59}. There is a suggestion that the procoagulant activity and subsequent production of thrombin increases on the platelet surface due to this platelet shape change\textsuperscript{60}.

Platelet aggregation

The linking of the platelets via fibrinogen brings about platelet aggregation. A great number of agents (including ADP, epinephrine, collagen and thrombin) can induce platelet aggregation\textsuperscript{61}. Simplistically, vWF and fibrinogen bind to receptors on one platelet and cross-links to the other platelet by binding on to receptors on the latter\textsuperscript{62}.

‘Reversible’ platelet aggregation is induced by low concentrations of platelet stimuli in the presence of extracellular Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+}, whereas high concentrations of the agonists can cause ‘irreversible’ reaction. The latter is the result of the platelet release reaction, which relates to the release of arachidonic acid metabolites\textsuperscript{63,64}, especially endoperoxides and thromboxanes, and the secretion of platelet constituents from the dense granules (ADP, ATP, serotonin, Ca\textsuperscript{2+}), alpha granules (beta-thromboglobulin, Platelet Factor 4, platelet derived growth factor etc.) and from the lysosomes\textsuperscript{65-68}.

Arachidonic acid is derived from platelet membrane phospholipids by the action of phospholipases, which is further acted upon by the cyclo-oxygenase to form the prostaaglandin endoperoxide intermediates, PGG\textsubscript{2} and PGG\textsubscript{2}. These endoperoxides can either be converted to prostaglandins, thromboxane B\textsubscript{2} via thromboxane A\textsubscript{2} or, non-prostone structures. These endoperoxide intermediates of the pathway themselves are potent platelet aggregators.

The release reaction, which augments the platelet aggregation, is regulated by two positive feedback loops. Firstly, the endoperoxides, thromboxane A\textsubscript{2} and ADP, which are released during the reaction cause further expression of the fibrinogen receptors on the platelet surface by intracellular mechanisms, thus inducing further platelet aggregation\textsuperscript{70}. Secondly, the synergism between the different platelet agonists augments platelet aggregation\textsuperscript{71-73}. Full platelet aggregation can also be induced by the simultaneous addition of subthreshold levels of platelet stimuli, which fail to induce platelet aggregation on their own merit.

Thus, the synergistic action of the primary platelet stimulus, other subthreshold agonist stimuli and the products of platelet release reaction build up an efficient ‘multi-stimulus’ for platelet aggregation. Increased intra-platelet levels of cyclic nucleotides also inhibit platelet aggregation\textsuperscript{74}. These activated platelets contribute to haemostasis and it is widely believed that these activities are relevant to thrombosis, especially to...
arterial thrombosis, where the bulk of the occlusion often seems to be due to platelet mass.

Procoagulant activity

Once platelet aggregates are formed, there is a tendency for the fibrin threads to be laid on them to form a clot. This process is facilitated by the platelets, possibly via more than one mechanism. Platelet membrane phospholipids potentiate the intrinsic pathway of coagulation, which eventually forms thrombin from prothrombin by activated factor X\cite{75}. The platelet surface also protects active coagulation factors from inactivation by their natural inhibitors\cite{76}. The constituents of the platelet release reactions play an important role in the formation of platelet thrombus. For example, Platelet Factor 4 seems to possess antiheparin activity, and fibrinogen that is released could potentially contribute further to the formation of the thrombus\cite{77}. As mentioned previously, P-selectin expression could result in platelet–leukocyte interaction leading to fibrin deposition by the leukocytes to form thrombus\cite{33}.

Recently, there has been more evidence on the role of procoagulant activity of platelets. For example, coagulation Factor V located in the alpha granules of the platelets becomes membrane bound when activated simultaneously with two agonists, thrombin and convulxin, an activator of the collagen receptor glycoprotein VI\cite{79}. These platelets are referred to as convulxin and thrombin induced Factor V platelets. These convulxin and thrombin platelets were capable of generating more prothrombinase activity than any other physiological agent and thus, are effectively more procoagulant. Factor V could also be expressed on the platelet membrane by simultaneous activation with thrombin and most of the subtypes of collagen, as would be expected at sites of endothelial damage. There is at least one description of a patient in the literature who developed a bleeding tendency due to the presence of an inhibitor directed against plasma and platelet Factor V\cite{79}.

Platelet receptor occupancy

Occupancy of the receptor on the platelet membrane by an agonist or antagonist results in intracellular mechanisms, which in turn modulate further platelet function and platelet–platelet interaction\cite{80}. Platelet receptor occupancy could also have an important role to play in platelet procoagulant activity\cite{81}.

The duration of occupancy of the receptor by the agonist appears to be critical in bringing about some of the changes in the platelets. For example, occupancy of the thrombin receptor by thrombin (0·025–0·12 U. ml⁻¹) for a duration of 45–60 s is mandatory for stimulation, whilst the addition of a thrombin inhibitor during this time period results in inhibition\cite{82}. The duration of receptor occupancy required to modulate a specific platelet action also depends on the concentration of the receptor effector substance.

Mechanisms of action of antiplatelet therapy

Drugs can modify platelet aggregation by interfering with one or more of the various pathways implied in the process of aggregation. For example, aspirin irreversibly inhibits the cyclo-oxygenase in the platelet by acetylating the latter and thus decreases the amplification of the aggregation produced by the intermediates (especially thromboxane A₂) of the arachidonic acid pathway\cite{82}. The ADP receptor antagonists, such as ticlodipine or clopidogrel, interfere with the binding of ADP to its receptors and prevent changes within the platelet that lead to aggregation, including the amplification processes that result from the release of stored ADP\cite{83}. The GP IIb/IIIa antagonists, such as abciximab, tirofiban and integrin, inhibit platelet aggregation by interfering with ligand binding to activated GP IIb/IIIa complexes\cite{83}.

The precise mechanism of platelet inhibition by dipyridamole is not known, although several have been proposed. For example, dipyridamole is thought to act by inhibiting the uptake of adenosine into the red blood cells, thus increasing the level of the former in the plasma, which then acts on the platelets to increase its cyclic AMP level. Dipyridamole also acts by inhibiting cyclic nucleotide phosphodiesterases and thus increasing intra-platelet levels of cyclic nucleotides\cite{84}. The third mechanism of action for dipyridamole is probably by the potentiation of the antiaggregating effects of endothelium derived relaxing factor\cite{84}.

Even though there appears to be significant progress in the field of antiplatelet therapy, many clinical trials have exposed the limitations of these drugs. Aspirin, for example, though widely used in vascular disorders, is a relatively weak antiplatelet agent, producing only partial inhibition of platelet aggregation, even in large doses\cite{85}. Incomplete inhibition of platelet aggregation or thromboxane A₂ synthesis, and the bypass of aspirin inhibition by non-thromboxane A₂ dependent activators, all probably contribute to the relative inefficacy of aspirin\cite{86}.

One important limiting factor in the usage of these drugs seems to be their potential action on the other systems of the body and the difficulties of their action being restricted to single, specific platelet receptors or metabolic pathways. However, interruption of the final step of platelet aggregation is now possible with the new GP IIb-IIIa inhibitors and clinical trials have already provided significant evidence on this view, at least with the intravenous agents (such as abciximab). Nevertheless, the areas where there is further potential for platelet inhibition include the inhibition of the initial step of platelet adhesion, platelet collagen receptor inhibition, antithrombins and thromboxane A₂ receptor inhibition.

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Table 2  Methods of assessing platelet function

<table>
<thead>
<tr>
<th>Functional indices</th>
<th>Flow cytometry</th>
<th>Soluble markers (plasma and/or urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Spontaneous aggregation</td>
<td>• Expression of glycoproteins such as P selectin</td>
<td>• Beta thromboglobulin</td>
</tr>
<tr>
<td>• Aggregation response to ADP, collagen, epinephrine, thrombin, etc.</td>
<td>• Platelet Factor 4</td>
<td>• Platelet Factor 4</td>
</tr>
<tr>
<td>• Adhesion to a substratum such as collagen or endothelial cells</td>
<td>• Soluble P selectin</td>
<td>• Soluble glycoprotein V</td>
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**Detection and quantification of platelet activation**

Platelet activation has been implicated in the pathogenesis of a number of diseases, which include atherosclerosis\textsuperscript{[5,6]}, coronary vascular disease\textsuperscript{[87,88]}, and cerebrovascular disease\textsuperscript{[89]}. Abnormal platelet activation has also been associated with atrial fibrillation\textsuperscript{[90]}, cancer, peripheral vascular disease, Alzheimer disease\textsuperscript{[91]}, inflammatory bowel disorders\textsuperscript{[92]} and recently deep vein thrombosis\textsuperscript{[93]}. Platelet activation seems to be influenced by diabetes mellitus\textsuperscript{[7]}, smoking\textsuperscript{[8]}, hypertension and also the use of oral contraceptives\textsuperscript{[94]}

Platelet activation comprises a change in platelet shape, platelet aggregation and the release of platelet constituents. Platelet activation may therefore be quantified by factors such as a change in shape and a tendency to aggregate, and also by measuring the blood and urine levels of relevant platelet metabolic products. For example, the alpha granule components, beta thromboglobulin and platelet factor 4\textsuperscript{[85,96]}, and the soluble form of the adhesion molecule P-selectin\textsuperscript{[50]} can be measured as markers of platelet activation in the plasma. Thromboxane B\textsubscript{2}, a product of platelet cyclo-oxygenase activity can also be measured in the urine\textsuperscript{[97]}. Undoubtedly blood tests, which reflect platelet activation, are potentially very helpful in evaluating and possibly managing patients with various thrombotic disorders. A battery of tests are often used for the detection of platelet activation in various disease states, as these tend to measure different aspects or markers of platelet activation and may differ from each other in terms of their sensitivity and potential to be influenced by various laboratory procedures of storage and analysis (Table 2).

**Platelet aggregation**

Assessment of platelet aggregation is performed in a platelet aggregometer in response to the addition of different agonists of different strengths such as ADP, collagen, epinephrine, and thrombin. The extent of aggregation of the platelets is detected and quantified by the amount of light that passes through the platelet-rich plasma in comparison with platelet free plasma. This is assessed in response to the addition of the agonists, which gives an indication of the degree of platelet aggregation\textsuperscript{[102]}. However, aggregometry techniques do not seem to correlate well with other tests of platelet activation and may have serious limitations in measuring in vivo platelet activation\textsuperscript{[103]}

Even though the reasons for discrepancy between different methods of quantification of platelet activation are not entirely clear, it should be borne in mind that different principles are involved in different methods of quantifying platelet activation. For example, methods such as platelet aggregation quantify in vitro platelet activation in response to agonists, whereas the measurement of platelet release products in the plasma quantifies in vivo platelet activation. Platelet aggregation requires the presence of whole platelets, whereas factors such as beta thromboglobulin in the plasma could be increased with platelet lysis\textsuperscript{[104,105]}. Furthermore, venepuncture itself could potentially affect platelet activation. It should be highlighted that platelet aggregation is a moderately time-consuming technique, excluding its application to large epidemiological studies, and there is the uncertainty of whether the ex vivo conditions in the platelet aggregometer truly reflect in vivo aggregation.
Platelet metabolic products — alpha granule proteins (beta thromboglobulin and Platelet Factor 4)

Detection of platelet alpha granule contents in the plasma such as beta thromboglobulin and Platelet Factor 4 can easily be performed using an ELISA or radioimmunoassay. These two proteins are specific to platelets and are detected both in the megakaryocyte and the platelet with the use of immunofluorescence and immunoperoxide methods. Measurement of plasma levels of beta thromboglobulin and Platelet Factor 4 are specific to platelet release and have been suggested as a means for detecting increased platelet activation in vivo. However some problems are evident with respect to measurement of these proteins. For example, Kutti et al. failed to show any such correlation. Beta thromboglobulin levels are also raised in renal failure as they are normally metabolized in the kidney, and thus, in patients with renal failure, the plasma levels of beta thromboglobulin may be abnormally elevated and Platelet Factor 4 may be a better marker of platelet activation in patients with renal failure. Platelet Factor 4 has a short plasma half-life as it seems to be rapidly bound to the endothelial cells, and in addition, levels are raised during heparin therapy, as heparin releases Platelet Factor 4 from its binding sites (for example, on endothelial cells). As mentioned earlier, ‘normal values’ of these molecules are not exactly known. Beta thromboglobulin and Platelet Factor 4 are theoretically present in platelets in similar amounts and are released in similar quantities, but plasma levels of beta thromboglobulin greatly exceed plasma levels of Platelet Factor 4. This is possibly due to more rapid binding of Platelet Factor 4 to the endothelial cells and thus, its removal from plasma. Therefore a higher ratio of beta thromboglobulin to Platelet Factor 4 is always maintained in vivo. The plasma levels of these two proteins are considerably different and vary independent of each other under different conditions. A comparable increase in these two markers may indicate in vitro release as the endothelial cells do not take up Platelet Factor 4 and the concurrent measurement of both proteins in each blood sample may allow distinction between in vivo release and artefactual in vitro release.

Platelet surface receptors (selectins, integrins and immunoglobulins)

As mentioned before, the platelet–endothelial interaction is mediated through adhesion molecules on the surface of platelets, which belong in the families of selectins (P-selectin), integrins (GP Ia, GP Ic, GP IIa, GP IIb-IIIa) and immunoglobulins.

Raised levels of soluble P-selectin are found in a variety of thrombotic disorders, including ischaemic heart disease, cerebrovascular disease, congestive cardiac failure, and hypertension. Levels of soluble P-selectin do not seem to be influenced by the various anticoagulants and different methods of preparation of plasma, unlike the levels of beta thromboglobulin and Platelet Factor 4. Chong et al. also demonstrated that soluble P-selectin levels are not influenced by the presence of renal failure and that it could be a useful marker for thrombotic disorders.

Detection of activated platelets by measuring the surface expression of P-selectin can be performed using fluorescence-activated flow cytometric techniques, in combination with a monoclonal antibody. Even though the various markers are considered as markers of platelet activation and are useful in assessing the participation of platelets in disease processes, they do not necessarily correlate with the severity of the underlying disorder. Though this remains partly unexplained, it is not too surprising, as an increasing severity of the underlying disorder does not necessarily result in a corresponding increase in platelet activation.

Flow cytometry

This technique can be used to characterize the alteration in the structure of the platelets, which could be due to platelet activation, haemostatic function or due to the maturation process. Flow cytometric analysis of platelets is also useful for the characterization of primary or secondary platelet abnormalities. This test can even be performed independent of the platelet count (if performed under appropriate conditions such as antibody saturation) and allows follow-up monitoring during antiplatelet therapy.

Flow cytometry can be used with multiple fluorescent staining of platelets or in combination with monoclonal antibodies to measure circulating activated platelets, leukocyte platelet aggregates and the procoagulant platelet-derived microparticles. Furthermore, flow cytometry detects activated platelets by determining the change in shape of the activated platelets, the detection of specific antigens on the membrane of activated platelets (P-selectin, GP IIb-IIIa), or platelet surface bound proteins (such as fibrinogen), or the detection of the expressed procoagulant surface. Alterations in the density of these surface glycoproteins, their ligands, or the expression of new epitopes on these structures provides a possible means of detecting and quantifying platelet activation. For example, when platelets are activated the platelet surface GP IIb-IIIa complex undergoes conformational changes, which generate new epitopes detectable by using monoclonal antibodies that could complement or even replace conventional platelet assays. This technique involves incubation of whole blood with monoclonal or polyclonal antibodies directed against platelet glycoproteins (such as PAC1, a monoclonal antibody specific for fibrinogen receptor or S12, a monoclonal antibody specific for P-selectin) or their receptor–ligands (such as fibrinogen). The targets of detection can be measured in whole blood, washed platelets or platelet-rich plasma.
for these monoclonal antibodies are found only on activated platelets and not on resting or inactive platelets. These platelet-bound antibodies are then detected with streptavidin conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). With the increasing number of FITC or PE labelled antibodies, the same thing can be achieved during one incubation step.

Platelets need to be differentiated from other blood cells and this could be done by using antibodies to detect membrane proteins specific to platelets, such as GP Ib or by their light-scatter profile. For example, flow cytometry has a very high sensitivity and could detect activation in as low as 0.8% of platelets. However, flow cytometry is performed on unseparated whole blood obtained by using a wide bore needle in a highly standardized manner to avoid stasis and subsequent activation in vitro platelet activation. Phosphate buffered saline is a recommended buffer for incubation and washing, whereas paraformaldehyde can be used to fix whole blood samples. A combination of sodium citrate, theophylline, adenosine and dipryridamole appears to be an ideal anticoagulant for blood collection, though sodium citrate is also commonly used before the platelets are fixed with paraformaldehyde.

In summary, flow cytometry is used to detect platelet activation with a very high sensitivity. Flow cytometry may also be a helpful tool in assessing the efficacy of antiplatelet therapy in clinical disorders, the study of platelet immunology, assessment of the quality control of stored platelet concentrate before transfusion, and as a marker of platelet activation. However, prostacyclin is not specific to platelets, also being released by endothelial cells. Urinary prostacyclin measurement would perhaps be more appropriate for patients who are difficult to bleed, as difficult venuupuncture may lead to artefactual platelet activation.

**Is there an ideal way to quantify platelet pathophysiology?**

Plasma samples for ELISA can be stored for a number of months at −70 °C and the results are comparable to those obtained by flow cytometry. Nevertheless there are few large, comprehensive studies that directly compare different methods of quantifying platelet pathophysiology, although one may argue that different methods may actually reflect different aspects of platelet physiology.

It appears that more than one test could be applied to assess the state of platelet activation in disease states, although interpretation would be confined to the relationship between the method used and disease state studied. Furthermore, the use of plasma markers of platelet function may also make it easier to undertake large-scale epidemiological investigations, compared to more tedious methods that require expensive equipment, such as flow cytometry. The ability to assess platelet pathophysiology would enable us to identify suitable candidates for antiplatelet therapy as well as to evaluate the efficacy of such drugs.

**Urinary metabolites**

**thromboxane B₂, prostacyclin**

Thromboxane A₂ is the predominant cycloxygenase product of human platelets and is a potent platelet activator and a vasconstrictor. Furthermore, 11-dehydro-TxB₂ and 2,3-dinor-TxB₂ are the stable metabolic products of two major pathways of thromboxane metabolism in man and are excreted in the urine. As platelet activation in vitro may confound the plasma concentration of thromboxane B₂, the measurement of the these metabolites in the urine could arguably be regarded as more reliable markers of in vivo platelet activation, unless cyclooxygenase inhibitors are administered.

Measurement of urinary metabolites of thromboxane B₂ is simple, non-invasive and could readily be applied to clinical studies. Urinary thromboxane B₂ is also a useful adjunct to diagnosing acute thromboembolic disorders, especially when used with other non-invasive tests. For example, increased urinary thromboxane B₂ may correlate with acute cardiac ischaemia or infarction, as well as cerebral ischaemia.

Prostacyclin (PGI₂) is a metabolite of the endoperoxides of the cyclooxygenase pathway, and urinary excretion of prostacyclin (or its metabolites) has been used as a marker of platelet activation. However, prostacyclin measurement would perhaps be more appropriate for patients who are difficult to bleed, as difficult venuupuncture may lead to artefactual platelet activation.

**Risk stratification in thrombotic disorders — other clinical applications**

What does platelet activation mean in terms of patient management? At present, platelet activation is mainly employed as a valuable research tool, but it appears to have a number of potential clinical implications.

Measurement of these markers of platelet activation could be useful in identifying patients with a variety of cardiovascular disorders at high risk of thromboembolism. For example, Markowitz et al. demonstrated that markers of platelet activation could probably be employed to identify patients with high risk of restenosis following coronary angioplasty. Nevertheless, not all the studies have provided convincing evidence of its usefulness in assessing thromboembolic risk. For example, Hollander et al. demonstrated that although platelet activation markers were useful in risk stratification of patients presenting with chest pain, they were no better than existing blood markers of ‘high risk’, such as creatinine kinase-MB. However, platelet activation markers could perhaps be a useful guide to distinguish different subgroups of patients, following the observation that stroke patients with carotid artery disease exhibit significantly more platelet activation than those strokes with a cardioembolic aetiology.
is also the potential of confounding effects from concomitant drug therapy, such as the non-steroidal anti-inflammatory agents or steroids. Indeed, Klinkhardt et al. reported that a combination of aspirin and clopidogrel is more beneficial than aspirin alone for the prevention of cardiovascular events, which act by different mechanisms. Thus, it would be possible, for example, to demonstrate whether a combination of aspirin and clopidogrel is more effective than either of the medications alone in terms of inhibition of the platelets. Indeed, Klinkhardt et al. recently reported that there was augmentation of the antiaggregatory effects of IIb/IIIa inhibitors by aspirin and clopidogrel, and were additive to changes observed with aspirin or clopidogrel alone.

By assessing the effect of an antiplatelet medication on platelet activation, it may be possible to choose the appropriate antiplatelet therapy for an individual and subsequently assess the impact of introduction of the medication or to estimate the appropriate treatment dose(s). For example, Sturzenegger et al. found that the lack of reduction in the rate of cerebral emboli measured with transcranial Doppler in the group of high-risk patients due to introduction of additional antiplatelet therapy correlated with the lack of beneficial effect on the platelet activation. Furthermore, patients with primary antiphospholipid syndrome have increased platelet activation, which has been correlated with the higher thromboembolic risk in such patients.

Conclusion

Whether or not reliable detection of platelet activation is practically feasible in the day-to-day acute clinical management of patients still remains to be seen. Assessment of platelet function would be important in providing a greater understanding of platelet physiology as well as the development and validation of new anti-thrombotic agents. Tests that detect activation are potentially useful in the diagnosis, risk stratification and management of thromboembolic disorders. Nevertheless, no single test for the quantification of platelet activation is perfect and each has its own merits and disadvantages that would need to be carefully considered.

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