Evaluation of Platelet Function by Flow Cytometric Measurement of Ligand Binding

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SUMMARY. Rapid and relevant evaluation of platelet function is often clinically important. By means of fluorescent labelled chicken antibodies (which do not bind to Fc-receptors) against fibrinogen and von Willebrand factor and flow cytometry, we have determined the time course of ligand association to platelets after stimulation with adenosine 5'-diphosphate and ristocetin respectively. The expression of guanosine 5'-phosphate (GMP)-140 was also measured. We have applied this technique to evaluate platelet function during platelet storage and cardiopulmonary bypass. There was a significant reduction of the binding of fibrinogen and von Willebrand factor and significantly increased expression of GMP-140 after 9 days of storage. Changes in metabolic variables such as lactate accumulation, glucose consumption and decrease in pH confirm that the functional impairment is due to a large extent to a deteriorated platelet metabolism. No significant differences were found between samples taken before and during cardiopulmonary bypass, but there was a tendency towards increased ligand binding as well as increased expression of GMP-140 at the end of cardiopulmonary bypass. The flow cytometric technique that is described may be useful for evaluation of platelet function and platelet activation in vivo.

Introduction

In many clinical situations an evaluation of platelet activation in vivo and of platelet function is wanted instantly.1,2,3 Many of the currently available assays for detection of platelet activation, such as measurement of platelet release products: β-thromboglobulin, platelet factor 4 and metabolites of thromboxane A2,4 require meticulous, time consuming techniques. Recent advances in flow cytometry have made it possible to rapidly provide information on platelet activation and platelet function, and sometimes give an indication on the etiology of platelet dysfunction.

However, since flow cytometric measurements are performed ex vivo they have in many cases been shown to be hampered by the same drawbacks as many other techniques, e.g. the major part of the activated platelets may not be found in the circulating blood but be trapped in vascular lesions. Consequently only a few platelets carrying activation markers might be expected to be found in peripheral blood. On the other hand, the platelets that are still circulating might be expected to react to 'sub-threshold' concentrations of agonists or respond 'supernormally' to low concentrations of the same agonists when stimulated ex vivo. Likewise the technique could reveal subnormal responses to external agonists.

In this study we compared platelets reactions over time ex vivo with and without external agonist stimulation. By means of flow cytometry and FITC-
conjugated chicken antibodies against human fibrinogen and von Willebrand factor we evaluated the capability of platelets to bind these ligand proteins with or without adenosine 5'-diphosphate (ADP) and ristocetin stimulation respectively. These studies could give information on the functional capacity of the ligand receptors and not only on their density. The membrane glycoproteins GPIb and GPIIIa and the expression of guanosine 5'-phosphate (GMP)-140 were also measured as representative markers of the external platelet membrane, the external platelet membrane and the α granule membrane, and α granule membrane, respectively.7

Studies were also performed on platelet concentrates (n=5) during 9 days of storage, and on platelets from patients (n=10) during cardiopulmonary bypass surgery. In the former study we were able to evaluate the sensitivity of the methods on a fixed population of platelets, and in the second study we could check our hypothesis that the circulating platelets are generally not carrying activation markers but might be 'primed' to respond supernormally. A comparison was made with standard methods for evaluation of platelet function.

Materials and Methods

Reagents

ADP, grade 1 and ristocetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chicken antibodies, IgG fractions purified from egg yolk, against human: fibrinogen, insulin, and von Willebrand factor were obtained from Immunosystem AB (Uppsala, Sweden). The procedure for FITC labelling of chicken antibodies was as previously described.8 FITC-conjugated mouse monoclonal antibodies against GPIb (AN51) and GPIIIa (CD 42 b and CD 61 respectively) were from Dako AS (Glostrup, Denmark). An additional FITC-labelled anti-GPIb (SZ2) antibody and an anti-CD 62 antibody specific for the platelet α granule membrane glycoprotein GMP-140 were purchased from Immunootech SA (Marseille, France). FITC-conjugated mouse monoclonal anti-human IgG1 and IgG2 antibody from Becton, Dickinson Immunocytometry Systems (Mountain View, CA, USA) were used for estimating non specific binding.

Patients and controls

Controls

Ten blood donors who had not ingested drugs known to affect platelet function for at least 10 d were used to determine the normal time course for platelet binding of fibrinogen and of von Willebrand factor, as well as for determination of the expression of GMP-140 (CD 62P) on the platelet surface. Six single-donor platelet concentrates were also investigated. Informed consent was obtained from all subjects.

Patients

Ten patients undergoing cardiopulmonary bypass (CPB) were investigated. Eight patients underwent coronary bypass operation and two patients had one heart valve replaced. Blood samples were obtained from each patient: before the start of surgery, after heparinization and at the end of cardiopulmonary bypass.

Platelet isolation

For controls and patients, blood was collected either in siliconized glass tubes containing 0.5 ml of 0.129 mol/l sodium citrate (Becton, Dickinson Maylan, Cedex, France) for analysis of fibrinogen, von Willebrand factor and GMP-140, or in tubes containing 0.5 ml of a solution containing trisodium citrate, adenosine, theophylline and dipyridamole (Diatube H, Diagnostica Stago, France) for analysis of GPIb and GPIIIa. Blood was centrifuged at room temperature (140 x g for 10 min) to prepare platelet-rich plasma. Platelet counts were performed with a Coulter S-Plus electronic particle counter (Luton, Beds England). The platelet counts were adjusted to 100 x 10⁹/l by addition of platelet poor plasma, which was prepared by subsequent centrifugation of the remaining blood at 2700 x g for 15 min.

Preparation and storage of platelet concentrates

Single-donor platelet concentrates were collected from six healthy blood donors by an apheresis procedure, using a blood cell separator (Cobe BCT Inc, Lakewood, CO, USA) and a closed-system apheresis kit (extended life platelet dual needle set, Cobe). The platelet concentrates were stored in a computerised environmental chamber (Melco Engineering, CA, USA) at 21.6-22.5°C with constant linear agitation, 60 rpm. Samples for analysis were drawn directly from the blood donor at the time of the donation and further samples were drawn under sterile conditions from each bag on days 0 (after filtration), 2, 5 and 9. Filtration was performed, routinely, using PALL LRP6 platelets filters (PALL, Newquay, Cornwall, UK).

Time course of binding of FITC conjugated anti-fibrinogen, FITC-conjugated anti-von Willebrand factor and expression of GMP-140

Five μl of platelet suspension (100 x 10⁹/l) were incubated in 50 μl Hepes buffer (137 mmol/l NaCl, 2.7 mmol/l KCl, 1 mmol/l MgCl₂, 5.6 mmol/l glucose, 1 g/l bovine serum albumin, and 20 mmol/l Hepes, pH 7.4) in the presence of ADP at 0.5 μmol/l, 5 μmol/l or ristocetin at 5 g/l. The incubations were carried out at room temperature without stirring for up to 60 min. One reaction mixture was prepared for each time point. At 0, 3, 5, 10, 20, 30, 40, 50 and 60
min 10 μl FITC-conjugated chicken anti-fibrinogen, anti-von Willebrand factor or anti GMP-140 antibodies were added. The reaction was stopped after 2 min by addition of 0.5 ml ice-cold phosphate-buffered saline (PBS) containing 1% paraformaldehyde (PFA). Control samples were performed substituting ADP or ristocetin with HEPES-buffer. A limited number of time points were used for patients. For fibrinogen binding and expression of GMP-140: 0, 5 and 60 min were used and for binding of vWF: 0, 10 and 60 min were used. The same time points were used for the investigation of stored platelet concentrates.

**Determination of expression GPIb and GPIIIa**

PRP diluted to 100 x 10^9/l, 10 μl further diluted in 60 μl PBS-buffer pH 7.4 was incubated with 10 μl FITC-conjugated anti-GP Ib or anti-GP IIIa for 30 min at 0°C followed by addition of 0.5 ml ice-cold PBS containing 1% PFA. Flow cytometry

Samples were analysed with an EPICS Profile II (Coulter Electronics, Hialeah, FL, USA) flow cytometer. The instrument was calibrated daily with Immuno-Check (Coulter Inc, Hialeah, FL, USA) to obtain optimal laser condition. The standardisation of the mean fluorescence intensity (MFI) was performed before each experiment with Standard-Brite (Coulter, Inc). A gate was set around the platelet population and 10,000 cells were analysed for green fluorescence. Control experiments were performed with an appropriate concentration of FITC-conjugated chicken anti-insulin or mouse IgG of corresponding subclass as a negative control antibody to determine the non-specific binding (limit set to less than 2% positive platelets). Results were expressed either as the MFI of the platelet population or as the percentage of antibody positive platelets.

**β-Thromboglobulin measurement**

β-Thromboglobulin was measured by the RIA technique using kits from Amersham International (Bucks, UK). The supernatant of the platelet concentrates was centrifuged at 5000 x g and stored at -70°C until the measurements were performed.

**pH, glucose and lactate**

pH was measured in samples from the platelet concentrates on a Radiometer ABL 520 (Copenhagen, Denmark). Glucose and lactate concentrations during the storage of platelets were assessed with the Kodak Ektachem 250 Analyser (Eastman Kodak Co., Rochester, NY).

**Statistics**

Results are presented as mean values ±SEM. Statistical significance was determined by paired t testing. A P value of 0.05 or less was considered significant.

**Results**

**Behaviour of normal platelets after stimulation ex vivo**

The time course of binding of FITC-conjugated anti-fibrinogen, anti-von Willebrand factor and anti-GMP-140 in normal subjects is illustrated in the Figure. The binding of anti-fibrinogen chicken antibody to ADP stimulated platelets reached a maximum within the first 5 min after addition of ADP. During this period of time 50% of platelets became positive for FITC-conjugated antibodies. Subsequently there was a marked decrease in the percentage of positive cells at a rate of 2%/min over the next 15 min and 0.25%/min during the last 40 min. Determinations of GPIIIa in ADP stimulated platelets showed an unchanged percentage of positive cells over time while there was a moderate decrease of the MFI values after 30 min.

Ninety percent of the ristocetin stimulated platelets became positive for anti-von Willebrand factor chicken antibodies in the first 30 min, with no subsequent decrease during the following 30 min. Similar results were obtained when an anti-GMP-140 was added to ADP-stimulated platelets, except that the percentage of positive cells was lower (30%) at 40 min, with a plateau thereafter.

**Stored platelet concentrates**

Platelet concentrates progressively lost their capacity to bind FITC-conjugated anti-fibrinogen antibody during storage. As can be seen in Table 1 the decrease was already evident directly after filtration of the platelets when only 18 ± 6.3% of platelets became positive.
Table 1 Changes in Fibrinogen, vWF and GMP-140 binding in platelet concentrates

<table>
<thead>
<tr>
<th>Platelet activation</th>
<th>Ligand</th>
<th>Percent positive platelets</th>
<th>Significance tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days of storage</td>
<td>BF vs AF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 2</td>
</tr>
<tr>
<td>ADP 0.5 µmol/l (5 min)</td>
<td>Fibrinogen</td>
<td>60.9±5.2</td>
<td>18.0±6.3</td>
</tr>
<tr>
<td>Ristocetin 5 g/l (10 min)</td>
<td>vWF</td>
<td>81.3±3.8</td>
<td>81.7±3.5</td>
</tr>
<tr>
<td>Buffer (60 min)</td>
<td>GMP-140</td>
<td>1.0±0.1</td>
<td>1.3±0.1</td>
</tr>
</tbody>
</table>

BF = before filtration; AF = after filtration. Values are expressed as mean ± SEM of five donors.

positive for the antibody after 5 min stimulation with ADP, compared with the value for unfiltered platelets, 60.9±5.2%. An extremely low fibrinogen binding was seen on Day 9, 0.8±0.2% positive cells.

The values for anti-von Willebrand factor binding to platelets 10 min after stimulation were contrary to fibrinogen binding, not affected by the filtration procedure. However, during storage the percentage of platelets binding antibody was continuously diminished and after 9 d of storage there were 35.2±4.4% positive cells compared to 81.3±3.8 Day 0.

Distribution of GMP-140 on unstimulated platelets demonstrated a significant increase in the surface expression of this marker. After 9 d of storage the FITC-conjugated anti GMP-140 bound to 31.1±6.8% of cells after 60 min of incubation compared to 1.0±0.1% on Day 0.

Changes in the expression of GPIb, GPIIIa and the concentration of β-thromboglobulin during storage are illustrated in Table 2. The filtration procedure did not change the expression of GPIb and GPIIIa determined as MFI. During storage there was a continuous decrease of the MFI for GPIb (SZ2). On Day 9 MFI was decreased to 116±5 compared to 175±20 (P=0.001) on Day 0. Similar results were obtained using an additional antibody, AN51. The MFI for GPIIb/IIIa showed an increase, though not significant, after 9 d of storage. There was a continuous and marked increase of the β-thromboglobulin concentration.

Changes in metabolic activity of stored platelets

There was a continuous increase of lactate, and a continuous decrease of the glucose concentrations during storage. pH was declined in the stored platelet concentrates (Table 3).

Effect of cardiopulmonary bypass on expression of Fibrinogen, von Willebrand factor and GMP-140

After injection of heparin the percentage of platelets binding anti-fibrinogen antibodies increased, though not significantly. However, during CPB there was no further change in the percentage of platelets exhibiting anti-fibrinogen antibody. The frequency of platelets binding anti-von Willebrand factor antibodies did not change after heparinization but an increased binding of anti-von Willebrand factor antibodies was seen at the end of CPB.

The frequency of platelets expressing GMP-140...

Table 2 Changes in GP Ib, GP IIb/IIIa, β-TG and Platelet count during storage of platelet concentrates

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>Mean fluorescence intensity</th>
<th>GP IIb/IIIa</th>
<th>β-TG (ng/mL)</th>
<th>Platelet count (x 10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GP Ib</td>
<td>AN 51</td>
<td>SZ 2</td>
<td>GP IIb/IIIa</td>
</tr>
<tr>
<td>Day 0 BF</td>
<td>86±17</td>
<td>174±20</td>
<td>147±7</td>
<td>ND</td>
</tr>
<tr>
<td>Day 0 AF</td>
<td>121±21</td>
<td>185±10</td>
<td>146±11</td>
<td>1942</td>
</tr>
<tr>
<td>Day 2</td>
<td>100±11</td>
<td>210±9</td>
<td>143±6</td>
<td>10554</td>
</tr>
<tr>
<td>Day 5</td>
<td>90±9</td>
<td>178±14</td>
<td>139±7</td>
<td>18688</td>
</tr>
<tr>
<td>Day 9</td>
<td>56±3</td>
<td>116±5</td>
<td>164±13</td>
<td>26880</td>
</tr>
</tbody>
</table>

BF = before filtration; AF = after filtration; ND = not done. Values are expressed as mean ± SEM of five donors.

Table 3 Changes in metabolic activity of stored platelets concentrates

<table>
<thead>
<tr>
<th>Days of storage</th>
<th>Lactate (mmol/L)</th>
<th>Glucose (mmol/L)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>1.5±0.1</td>
<td>19.0±0.2</td>
<td>7.0±0.03</td>
</tr>
<tr>
<td>Day 2</td>
<td>6.1±0.4</td>
<td>17.3±0.4</td>
<td>7.0±0.06</td>
</tr>
<tr>
<td>Day 5</td>
<td>12.8±0.5</td>
<td>13.5±0.5</td>
<td>7.0±0.05</td>
</tr>
<tr>
<td>Day 9</td>
<td>23.3±4.5</td>
<td>6.9±1.1</td>
<td>6.2±0.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM of five donors.
was only slightly increased at the end of CPB (Table 4).

Discussion

In this study we demonstrate that analysis of the ligand-binding capacity of functional receptors on platelets may be a useful tool in evaluating platelet function in different clinical settings.

Our studies in normals showed that ligand binding and the expression of CD 62P initially were parallel phenomena. After 5 min the GPIIb/IIIa receptors appear to be internalised or possibly they lose their capacity to bind ligand proteins. This finding shows the necessity to measure both ligand density of receptor and of ligand protein.

Under normal conditions platelets are circulating in a nonactivate state but on contact with damaged endothelium they become activated. Platelet activation is initiated by the binding of an agonist to their membrane receptors and is associated with changes in the platelet surface membrane. These changes include activation of surface membrane receptors, the fusion of granule membrane with plasma membrane and binding of substances released from granule to the platelet surface. These changes also give the possibility to identify activated platelets with various techniques. In our study, we investigated changes in glycoprotein GPIb and glycoprotein GPIIIa expression during storage of platelet concentrates. We found a progressive loss of surface GPIb with appearance of a surface GPIb negative population during storage, while GPIIIa showed a slight nonsignificant increase. A decrease of GPIb during storage is consistent with observations by other groups.13,14 This phenomenon could be due to proteolysis of this structure leading to an increase in the supernatant plasma concentration of glycocalcin (fragment of the extracellular domain of GPIb).15 In agreement with our findings other investigators have also demonstrated an increased surface concentration of GPIIIa during storage of platelets.16,17 It is possible the platelets translocate some of their internal GP IIIa structures to the platelet surface during storage/activation.18

Granule membrane structure GMP-140 (CD 62P) was increasingly exposed during storage. Similar findings have been reported by other groups.16,17 However, Goodall19 has reported decreased exposure of GMP-140 during storage of platelet concentrates and considers that this discrepancy could be due to different blood banking routines. Since we also find a dramatic increase of α-granule material, β-thromboglobulin, it seems reasonable to find an increase of the GMP-140. Recent evidence suggests that GMP-140, apart from being a marker for platelet activation, could also serve as a receptor for neutrophils and monocytes.20 The association of other cells to the platelet is correlated with an increased platelet clearance from the circulation.21

Thus expression of GMP-140 could give an indication on how the platelet concentrate survive in the circulation. This also implies that platelets by this mechanism might take part in an inflammatory reaction.

The capacity of platelets to bind fibrinogen after stimulation with ADP decreased continuously during storage. Goodall19 obtained similar results in studying platelet concentrates for 4 d. We interpret this finding as a loss of platelet function—the receptor has lost its capacity to bind fibrinogen either due to structural changes or to depleted energy resources of the platelets. Similarly the capacity of the stored platelets to bind von Willebrand factor upon stimulation with ristocetin decreased during storage. Garcia et al14 have reported similar findings but they were using a different technique for measuring platelet associated von Willebrand factor. The decrease of platelet associated von Willebrand factor was parallel to the decrease of GPIb expression on the platelet surface (r = 0.967).

Changes of metabolic variables such as lactate accumulation, glucose consumption and decrease in pH confirm that the functional impairment to a large extent is due to a deteriorated platelet metabolism.

There is currently some controversy regarding the expression of GMP-140 following platelet activation during cardiopulmonary bypass. Some studies have shown no22 or very little23 expression of GMP-140. Metzlaar et al24 found a slight increase in the percentage of GMP-140 positive cells. Similar results were reported by Rinder et al.25 In our study we did not find any spontaneous increased expression of GMP-140. However, at the end of the cardiopulmonary bypass there was a tendency for the platelets to respond more to ADP stimulation compared to the start of the operation, measured as expression of GMP-140 percentage positive cells (Table 4). These findings are in agreement with our other findings that the platelets showed an increased tendency to bind ligand proteins, fibrinogen and von Willebrand factor after stimulation with ADP and ristocetin.

<table>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Before Heparin</td>
<td>Post Heparin</td>
</tr>
<tr>
<td>ADP 0.5 µmol/L (5 min)</td>
<td>Fibrinogen</td>
<td>58.2 ± 6.2</td>
<td>63.4 ± 4.2</td>
</tr>
<tr>
<td>Ristocetin 5 g/L (10 min)</td>
<td>vWF</td>
<td>91.1 ± 1.5</td>
<td>88.8 ± 1.7</td>
</tr>
<tr>
<td>ADP 5 µmol/L (60 min)</td>
<td>GMP-140</td>
<td>19.8 ± 2.4</td>
<td>22.9 ± 2.3</td>
</tr>
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</table>

*P = 0.0077 versus Post Heparin.
respectively, when comparing the response before and at the end of the surgery. Flow cytometric studies using an antibody (PAC-1) specific for the activated form of GP IIb/IIIa has shown that this receptor is activated during cardiopulmonary bypass. Thus, it is most likely that an increased binding of fibrinogen to platelets could be expected during this type of surgery.

In conclusion our studies have shown that flow cytometric measurements can be a useful tool for rapid evaluation of the status of stored platelet concentrates and that these techniques might also be useful for evaluation of platelet activation in vivo and platelet function in acute ill patients.

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References