Clinical aspects of blood activation in open-heart surgery
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Generation of platelet-derived microparticles in patients undergoing cardiac surgery is not affected by complement activation

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ABSTRACT

Objective: The mechanisms causing the presence of platelet-derived microparticles (PMP) in the circulation are unknown. In vitro, platelets release PMP in response to complement activation. This study evaluates the relationship between complement activation and levels of circulating PMP in patients undergoing cardiac surgery.

Methods: Prospectively 71 patients were included who underwent elective coronary artery bypass grafting assisted by cardiopulmonary bypass (CPB). The patients were randomly allocated to one of the three groups: uncoated oxygenator, UnModified Surface (UMS; n=25) or oxygenator coated with either BioPassive Surface (BPS; n=25) or BioActive Surface (BAS; n=21). PMP and terminal complement complexes (TCC) were determined before bypass after induction of anaesthesia, 15 minutes after start CPB, at the end of CPB and 30 minutes after administration of protamine sulphate.

Results: Demographic and CPB data were similar for the three groups. At the end of CPB, PMP numbers were decreased in all three groups. No significant differences were observed between the groups at any sampling point. At the end of CPB, TCC concentrations were increased in all groups (p<0.001), and significant differences between the groups were present (p=0.002).

Conclusions: Despite significant complement activation, no increase in numbers of circulating PMP was found in systemic blood of patients undergoing cardiac surgery assisted by CPB. Thus, complement activation in vivo does not necessarily affect generation of PMP.
INTRODUCTION

On activation and during apoptosis platelets and other cells bud off small parts of their plasma membrane, the so-called microparticles (MP). Extensive in vitro studies have been reported on platelet-derived microparticles (PMP)\(^1,2\). When platelets are stimulated in vitro with agonists such as a combination of α-thrombin and collagen, the complement complex C5b-9 or the Ca\(^{2+}\)-ionophore A23187, they release large numbers of PMP\(^1,3,4\). PMP possess ‘platelet factor 3 activity’, i.e. they facilitate coagulation via exposure of negatively charged phospholipids, thereby providing binding sites for activated coagulation factors V (factor Va), VIIIa, IXa, XIa\(^1,2,5,6\) and enabling the formation of tenase- and prothrombinase complexes\(^1,2,7\).

Increased numbers of PMP have been reported in the circulation of patients with diabetes\(^8,9\), undergoing cardiopulmonary bypass (CPB)\(^10,11\), suffering from acute coronary ischaemia\(^12\), heparin-induced thrombocytopenia\(^13\), myocardial infarction\(^14\), uremia\(^15\), idiopathic thrombocytopenic purpura\(^16\), disseminated intravascular coagulation (DIC)\(^17\), plasmapheresis\(^18\), or meningococcal septic shock\(^19\), which are all diseases that have been associated with a thromboembolic tendency. Also in the pericardial fluid of patients undergoing CPB surgery elevated numbers of PMP have been found\(^20\).

Despite the widespread presence of PMPs in the circulation, however, still the mechanisms causing their release in vivo are unknown. Complement activation is markedly increased during CPB, while coatings are known to reduce complement activation. Generation of PMP induced by complement activation was anticipated. Therefore, in the present study the relationship between in vivo complement activation and the concentrations of circulating PMP was evaluated in patients undergoing cardiac surgery assisted by CPB with non coated oxygenators (UMS group) and coated oxygenators (BPS and BAS groups).
MATERIALS AND METHODS

Patients

Upon approval of the Medical Ethics Committee of the Academic Medical Center, and signed informed consent, we prospectively included 71 patients who underwent elective coronary artery bypass grafting assisted by CBP. Inclusion criteria were age 21 to 75 years, elective coronary artery bypass surgery, ejection fraction > 30%, body surface area > 1.66 m² and preoperative hemoglobin > 7.5 mmol/L. Exclusion criteria were combined valve surgery or aneurysmectomy, redo operations, insulin dependent diabetes mellitus, creatinine plasma level > 300 μmol/L, preoperative intra-aortic balloon pumping, preoperative use of non steroid anti-inflammatory drugs, preoperative use of warfarin, preoperative immunosuppressive therapy > 24 hrs, allergic reactions and chronic obstructive pulmonary disease. The patients were randomly allocated to three groups (Table 1).

Cardiopulmonary bypass

All extracorporeal bypass circuits consisted of a hollow fiber oxygenator (Affinity®, Medtronic, Minneapolis, MN). The oxygenator was uncoated, i.e. without surface modification, UnModified Surface (UMS; n=25) or coated with either BioPassive Surface (BPS; n=25) (Trillium®, Medtronic) or BioActive Surface (BAS; n=21) (Carmeda®, Medtronic). The additional, non coated, components of the extracorporeal circuit were identical for all patients, and included a soft-shell venous reservoir, two additional reservoirs, an arterial line filter, tubing system (Medtronic) and a roller pump as arterial blood pump (3M Sarns, Ann Arbor, MI). One of the additional reservoirs was used to collect the shed blood, which was processed by a cell saver before being returned into the systemic circulation after termination of the bypass procedure. The other reservoir was used to collect left vent (systemic) blood, which was returned into the systemic circulation via the soft-shell venous reservoir during the bypass procedure. The extracorporeal system was primed with 500 mL Ringerlactate solution, 1 L Haemacell (Behring, Malburg, Germany), 100 mL mannitol 20% (w/v), 50 mL of sodium bicarbonate 8.4% (w/v) and 200 mL aprotinin (2 x 10⁸ KIU Trasylol; Bayer, Leverkusen, Germany). Magnesium sulphate 4 mmol/10 kg (i.e. 24 < x < 32 mmol) and 10,000 IU bovine heparin (Leo
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Pharmaceutical Products, Weesp, The Netherlands) were added to the priming solution. The total priming volume was 1.85 L. All patients received 300 IU/kg heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) before cannulation of the aorta. CPB was initiated when the activated clotting time (ACT) was > 480 s. During CPB, the ACT was maintained above 480 s by administration of additional heparin when required. Moderate hypothermia (30-34 °C) was used for all patients. Myocardial protection was achieved using cold (4-8 °C) crystalloid cardioplegia solution (St. Thomas). Shed blood in the surgical field was processed by a cell saver (HaemoLite 2 plus, Haemonetics Corp., Braintree, MA). Processed blood was returned into the systemic circulation of the patients immediately after CPB. After weaning from CPB and decannulation, heparin was neutralized with protamine sulphate at a 1:1 ratio.

Collection of blood samples

Arterial blood samples were obtained before induction of anesthesia, 15 minutes after start CPB, at termination of CPB and 30 minutes after protamine administration. For comparison between plasma samples, PMP numbers, platelet counts and TCC concentrations were corrected for hemodilution by hemoglobin concentration.

Cell count

Blood samples for hemoglobin and platelet counts were collected in 5 mL glass vacutainer tubes containing EDTA (Becton Dickinson (BD), San Jose, CA), and analyzed on a Celldyn 4000 (Abbot, Mijdrecht, The Netherlands).

Terminal complement complex (TCC)

Arterial blood (2 mL) was anticoagulated with 10 mM EDTA. Cell-free plasma aliquots (1 mL) were prepared by centrifugation (11 minutes at 1100 g, 4 °C) and stored at -80 °C until use. TCC measures the terminal complement complex bound to the S-protein, representing the soluble, non-lytic form of TCC. TCC was determined by ELISA (Quidel, San Diego, CA).
Platelet-derived microparticles (PMP)

Arterial blood (4.5 mL) was collected into 3.2% trisodium citrate (BD). Blood cells were removed by centrifugation for 20 minutes at 1550 g and room temperature, and plasma aliquots (250 μL) were snap frozen in liquid nitrogen and stored at -80 °C until use. After thawing plasma aliquots on melting ice, plasma was centrifuged for 30 minutes (17570 g, 20 °C) to pellet the microparticles (MP) as described previously. After removal of 225 μL of (MP-free) plasma, the 25 μL MP-enriched plasma was diluted with 225 μL of phosphate-buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4), containing 10.9 mmol/L trisodium citrate. MP were resuspended and centrifuged (30 minutes at 17570 g and 20 °C). Again, 225 μL of the supernatant was removed and MP were resuspended in the remaining 25 μL and diluted fourfold with PBS/citrate buffer, of which 5 μL was used per flow cytometric determination.

Flow cytometric analysis

MP samples were analyzed in a FACSCalibur flow cytometer with CellQuest software (BD). To distinguish PMP from events due to noise, PMP were identified on forward scatter (FSC), side scatter (SSC), and binding of both PE-labeled annexin V (PharMingen, San Jose, CA) and FITC-labeled anti-CD61 (glycoprotein IIIA; clone Y2/51, IgG1 from Dako A/S (Glostrup, Denmark)). To identify annexin V-positive MP, a fluorescence threshold was placed in a MP sample prepared without addition of calcium to correct for autofluorescence. To identify CD61-positive events, MP were incubated with a similar concentration of isotype matched control antibody (FITC-labeled IgG1; BD) to set the fluorescence threshold. MP (5 μL) were diluted in 35 μL PBS containing 2.5 mmol/L CaCl2 (pH 7.4) and 5 μL of 500-fold prediluted normal mouse serum (Central Laboratory of the Netherlands Red Cross Bloodtransfusion Service, Amsterdam, The Netherlands). After incubation for 15 minutes at room temperature, annexin V (5 μL) plus anti-CD61 (5 μL) or IgG1-control antibody were added. The mixtures were incubated in the dark (15 minutes, room temperature). Subsequently, 200 μL PBS/calcium buffer were added and the suspensions centrifuged (30 minutes at 17.570 g, 20 °C). Finally, 200 μL of (MP-free) suspension were removed. The MP were diluted in 300 μL PBS/calcium buffer before flow cytometry, and all samples were analyzed for 1 minute. To estimate the
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number of PMP/L plasma, the number of events (N) found in the upper right (marker- and annexin V positive) quadrant of the flow cytometry analysis (FL1 versus FL2) was used in the formula: Number/L = N x [100/5] x [355/150] x [106/250].

Statistics

Data were analysed using SPSS, release 11.0 (SPSS Inc., Chicago, IL). Demographic and CPB data are reported as means with standard deviations. Outcome data (PMP, platelets and TCC) were corrected for hemodilution (hemoglobin) and are presented as medians with interquartile ranges. For all outcome variables, statistical analyses were performed on the change (Δ) of that variable relative to the baseline value (t = 0) per patient. Statistical significance (p<0.05, 2-sided) is indicated. The sample size was chosen on the basis of preliminary observations, indicating that approximately 25 patients per group would be sufficient to achieve statistically significant differences in blood-activation between coated and non coated oxygenators. Comparisons over time within treatment groups were made by applying the Wilcoxon Signed Ranks Test to the (paired) observations at baseline and at end CPB. Comparisons between (treatment) groups were made by applying the Kruskal-Wallis Test to the change between end CPB and baseline. The observations at 15 minutes CPB and at 30 minutes protamine sulphate are presented for descriptive purposes.

RESULTS

Clinical results

The coating groups (UMS, BPS and BAS) were compared for preoperative parameters, including body surface area, age, sex, hemoglobin, platelet count and surgical data (CPB time and aortic cross clamping time). No significant differences were found between the groups for any of the variables tested (Table 1). There were no differences between the groups in hemoglobin concentrations and platelet counts at any sampling time. Hemodilution was similar in the three coating groups, the decrease in hemoglobin concentration and platelet count after start of the CPB procedure were most likely due to hemodilution of the systemic patient blood by the priming volume of the extracorporeal circuit, and by administration of the cardioplegia solution for myocardial protection. In
addition, in all three coating groups a similar decrease in platelet count was observed after administration of protamine sulphate.

Table 1. Demographic and CPB data

<table>
<thead>
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<th>Surface modification of the oxygenator</th>
<th>UMS</th>
<th>BPS</th>
<th>BAS</th>
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<tr>
<td>Patient number</td>
<td>25</td>
<td>25</td>
<td>21</td>
</tr>
<tr>
<td>Male / female</td>
<td>25 / 0</td>
<td>23 / 2</td>
<td>20 / 1</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.0 ±7.1</td>
<td>59.9 ± 9.7</td>
<td>61.4 ± 9.9</td>
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<tr>
<td>Weight (kg)</td>
<td>87.5 ± 12.1</td>
<td>87.1 ± 14.8</td>
<td>83.1 ± 9.8</td>
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<tr>
<td>Body surface area (m²)</td>
<td>2.04 ± 0.17</td>
<td>2.06 ± 0.18</td>
<td>1.99 ± 0.12</td>
</tr>
<tr>
<td>Hemoglobin (mmol/L)</td>
<td>8.5 ± 0.6</td>
<td>8.4 ± 0.7</td>
<td>8.3 ± 0.5</td>
</tr>
<tr>
<td>Platelet count (10⁹/L)</td>
<td>227 ± 67.4</td>
<td>219 ± 69</td>
<td>233 ± 53</td>
</tr>
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<td>Cardiopulmonary bypass (min)</td>
<td>93.5 ± 21.8</td>
<td>85.1 ± 21.4</td>
<td>95.4 ± 24.2</td>
</tr>
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<td>Aortic cross clamping (min)</td>
<td>58.5 ± 15.9</td>
<td>55.1 ± 17.2</td>
<td>58.6 ± 19.7</td>
</tr>
</tbody>
</table>

¹All data are presented as mean ± SD. No significant differences were found between the groups for any of the variables.

PMP and platelet counts during CPB

The numbers of PMP at baseline were similar between the three coating groups (Figure 1A). Fifteen minutes after the onset of the CPB, the PMP numbers decreased in all three groups, despite correction for hemodilution. During CPB, the PMP numbers slightly recovered but, at the end of bypass, were still less than before start CPB. At the end of bypass there was a significant decline of PMP in the UMS group (p<0.001) and in the BPS group (p=0.028), when compared to baseline values. After administration of
protamine sulphate, PMP numbers again slightly decreased in all groups. Significant differences were not observed between the groups at any sampling point.

In contrast to PMP numbers, the platelet counts significantly increased during CPB when corrected for hemodilution (figure 1B; end of CPB: UMS group p=0.004, BPS group p=0.001, BAS group p=0.003).

**Figure 1.** Systemic concentrations of platelet-derived microparticles (PMP) and platelet counts, in patients undergoing cardiopulmonary bypass (CPB). Concentrations of PMP and platelet counts before bypass after induction of anaesthesia (baseline value; ‘pre’), 15 minutes after start bypass (15’CPB), at the end of bypass after release of the aortic cross clamp (end CPB), and 30 minutes after administration of protamine sulphate (30’prot). The measurements were made in 25 patients treated with an uncoated oxygenator i.e. without Surface Modification (UnModified Surface [UMS: white bars], 25 patients treated with an oxygenator coated with a BioPassive Surface [BPS: grey bars] and 21 patients treated with an oxygenator coated with a BioActive Surface [BAS: striped bars]. PMP concentrations (panel 1A) and platelet counts (panel 1B) were determined as described in Methods. No significant differences were present between the three oxygenator groups. Medians and interquartile ranges are presented.
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Figure 2. Concentrations of systemic terminal complement complexes (TCC), in patients undergoing cardiopulmonary bypass (CPB). TCC concentrations before bypass after induction of anaesthesia (baseline value; ‘pre’), 15 minutes after start bypass (15’CPB), at the end of bypass after release of the aortic cross clamp (end CPB), and 30 minutes after administration of protamine sulphate (30’prot). The measurements were made in 25 patients treated with an uncoated oxygenator i.e. without Surface Modification (UnModified Surface [UMS: white bars]), 25 patients treated with an oxygenator coated with a BioPassive Surface [BPS: grey bars] and 21 patients treated with an oxygenator coated with a BioActive Surface [BAS: striped bars], and performed as described in Methods. Medians and interquartile ranges are presented.

Complement activation during CPB

Before CPB, complement activation was not or hardly detectable (Figure 2). Fifteen minutes after the onset of CPB, plasma concentrations of TCC increased in all three coating groups, indicating complement activation. TCC concentrations further increased
Complement activation and platelet-derived microparticles during CPB, and remained high after protamine sulphate administration. At the end of CPB, TCC concentrations were significantly increased in all three groups (p<0.001), when compared to baseline values. At the end of CPB significant differences between the coating groups were present (p=0.002). At the other sample times no significant differences between the coating groups were found.

DISCUSSION

This study shows that despite considerable activation of the complement system in vivo, circulating PMP numbers did not increase. These results were not anticipated, since earlier in vitro studies clearly showed that complement activation triggers the release of PMP.1,4,23-25 Our present data, however, suggest that other mechanisms must be involved in the release of microparticles in vivo. On the other hand, we cannot exclude that complement activation may contribute to microparticle release in other patient populations, or may affect the release of microparticles in concert with other stimuli.

Earlier studies reported that CPB triggers the formation of PMP, both in vitro26 and in vivo.10,11 In contrast, we found an intraoperative decline in PMP numbers. Most likely, this lack of increase is due to the many -relatively recent- improvements of the extracorporeal circuits.27-29 Alternatively, in the present study the pericardial blood was not reinfused during the bypass procedure, but afterwards. Since pericardial blood contains relatively high numbers of cell-derived microparticles itself and is highly activated with regard to fibrinolysis and coagulation, one may hypothesize that by not returning this blood into the systemic circulation during bypass, one of the triggers for (systemic) blood activation has been eliminated. The same holds for platelet activation. Whereas earlier studies reported extensive platelet activation during CPB, more recent studies showed less platelet activation.27,30 For instance, Kestin and colleagues30 reported that platelets from the systemic circulation of patients undergoing CPB showed normal reactivity in vitro, had no loss of surface glycoprotein complexes Iib-IX and IIb-IIIa, and had hardly degranulated.

In contrast, pericardial blood contains relatively high numbers of PMPs and erythrocyte-derived MP.20,27 Whether local complement activation, i.e. in this wound blood, contributes to the release of (P)MP, can not be excluded.
In the present study, this shed blood was processed by a cell saver before being returned into the systemic circulation after the bypass procedure had been terminated. This treatment excludes the possibility that systemic blood activation during the bypass procedure is caused by reinfusion of the processed shed blood. On the other hand, we cannot exclude that this reinfusion contributes to blood activation post-bypass.

Taken together, although the release of MP in vitro from platelets is initiated by complement activation, we found no evidence for a relationship between complement activation and the concentrations of PMP in systemic blood from patients undergoing CPB. We conclude that complement activation in vivo does not importantly affect the generation of PMP.

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