Generation of platelet-derived microparticles in patients undergoing cardiac surgery is not affected by complement activation

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

Methods: Prospectively, 71 patients were included who underwent elective coronary artery bypass grafting with cardiopulmonary bypass. The patients were randomly allocated to one of the 3 groups: uncoated oxygenator, UnModified Surface (n = 25) or oxygenator coated with either BioPassive Surface (n = 25) or BioActive Surface (n = 21). Platelet-derived microparticles and terminal complement complexes were determined before bypass and after induction of anesthesia, 15 minutes after the start of cardiopulmonary bypass, at the end of cardiopulmonary bypass, and 30 minutes after administration of protamine sulfate.

Results: Demographic and cardiopulmonary bypass data were similar for the 3 groups. At the end of cardiopulmonary bypass, platelet-derived microparticle numbers were decreased in all 3 groups. No significant differences were observed among the groups at any sampling point. At the end of cardiopulmonary bypass, terminal complement complex concentrations were increased in all groups ($P < .001$), and significant differences among the groups were present ($P = .002$).

Conclusions: Despite significant complement activation, no increase in numbers of circulating platelet-derived microparticles was found in the systemic blood of patients undergoing cardiac surgery with cardiopulmonary bypass. Thus complement activation in vivo does not necessarily affect generation of platelet-derived microparticles.

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On activation and during apoptosis, platelets and other cells bud off small parts of their plasma membrane, the so-called microparticles. Extensive in vitro studies have been reported on platelet-derived microparticles (PMPs).[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 PMPs.[1,3,4] PMPs possess platelet factor 3 activity; that is, they facilitate coagulation through exposure of negatively charged phospholipids, thereby providing binding sites for activated coagulation factors V (factor Va), VIIIa, IXa, and XIa[1,2,5,6] and enabling the formation of tenase and prothrombinase complexes.[1,2,7]

TABLE 1. Demographic and CPB data

	Surface modification of the oxygenator		
	UMS	BPS	BAS
Patient no.	25	25	21
Male/female	25/0	23/2	20/1
Age (y)	62.0 ± 7.1[1]	59.9 ± 9.7	61.4 ± 9.9
Weight (kg)	87.5 ± 12.1	87.1 ± 14.8	83.1 ± 9.8
Body surface area (m^2)	2.04 ± 0.17	2.06 ± 0.18	1.99 ± 0.12
Hemoglobin (mmol/L)	8.5 ± 0.6	8.4 ± 0.7	8.3 ± 0.5
Platelet count (10^9/L)	227 ± 67.4	219 ± 69	233 ± 53
Cardiopulmonary bypass (min)	93.5 ± 21.8	85.1 ± 21.4	95.4 ± 24.2
Aortic crossclamping time (min)	58.5 ± 15.9	55.1 ± 17.2	58.6 ± 19.7

All data are presented as means ± SD. No significant differences were found among the groups for any of the variables.

Increased numbers of PMPs have been reported in the circulation of patients with diabetes,[8,9] patients undergoing cardiopulmonary bypass (CPB),[10,11] and patients with acute coronary ischemia,[12] heparin-induced thrombocytopenia,[13] myocardial infarction,[14] uremia,[15] idiopathic thrombocytopenic purpura,[16] disseminated intravascular coagulation,[17] plasmapheresis,[18] or meningococcal septic shock,[19] which are all diseases that have been associated with a thromboembolic tendency. Increased numbers of PMPs have been found also in the pericardial fluid of patients undergoing surgical intervention with CPB.[20]

Despite the widespread presence of PMPs in the circulation, however, the mechanisms causing their release in vivo are still unknown. Complement activation is markedly increased during CPB, whereas coatings are known to reduce complement activation. Generation of PMPs induced by complement activation was anticipated. Therefore in the present study the relationship between in vivo complement activation and the concentrations of circulating PMPs was evaluated in patients undergoing cardiac surgery with CPB with noncoated oxygenators and coated oxygenators.

Materials and Methods

Patients

On approval of the Medical Ethics Committee of the Academic Medical Center and obtainment of signed informed consent, we prospectively included 71 patients who underwent elective coronary artery bypass grafting with CBP. Inclusion criteria were age 21 to 75 years, elective coronary artery bypass surgery, ejection fraction of greater than 30%, body surface area of greater than 1.66 m^2, and a preoperative hemoglobin value of greater than 7.5 mmol/L. Exclusion criteria were combined valve surgery or aneurysmectomy, redo operations, insulin-dependent diabetes mellitus, creatinine plasma level of greater than 300 μmol/L, preoperative intra-aortic balloon pumping, preoperative use of nonsteroidal anti-inflammatory drugs, preoperative immunosuppressive therapy of greater than 24 hours' duration, allergic reactions, and chronic obstructive pulmonary disease. The patients were randomly allocated to one of 3 groups (Table 1).

CPB

All extracorporeal bypass circuits consisted of a hollow-fiber oxygenator (Affinity; Medtronic, Minneapolis, Minn). The oxygenator was uncoated (ie, 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 noncoated components of the extracorporeal circuit were identical for all patients and included a soft-shell venous reservoir, 2 additional reservoirs, an arterial line filter, a tubing system (Medtronic), and a roller pump as an arterial blood pump (3M Sarns, Ann Arbor, Mich). 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 through the soft-shell venous reservoir during the bypass procedure. The extracorporeal system was primed with 500 mL of Ringer lactate solution, 1 L of Haemaccell (Behring, Malburg, Germany), 100 mL of mannitol 20% (wt/vol), 50 mL of sodium bicarbonate 8.4% (wt/vol), and 200 mL of aprotinin (2×10^6 KIU Trasylol; Bayer, Leverkusen, Germany). Magnesium sulfate, 4 mmol/10 kg (ie, 24 $\geq \times \leq$ 32 mmol) and 10,000 IU of bovine heparin (Leo 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) before cannulation of the aorta. CPB was initiated when the activated clotting time was 480 seconds or greater. During CPB, the activated clotting time was maintained at greater than 480 seconds by means of administration of additional heparin when required. Moderate hypothermia (30°C-34°C) was used for all patients. Myocardial protection was achieved by using cold (4°C-8°C) crystalloid cardioplegia solution (St Thomas). Shed blood in the surgical field was processed with a cell saver (HaemoLite 2 plus; Haemonetics Corp, Braintree, Mass). 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 sulfate at a 1:1 ratio.

Collection of Blood Samples

Arterial blood samples were obtained before induction of anesthesia, 15 minutes after the start of CPB, at the termination of CPB, and 30 minutes after protamine administration. For comparison between plasma samples, PMP numbers, platelet counts, and terminal complement complex (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 ethylenediamine tetraacetic acid (Becton Dickinson, San Jose, Calif) and analyzed on a Celldyn 4000 (Abbot, Mijdrecht, The Netherlands).

TCCs
Arterial blood (2 mL) was anticoagulated with 10 mmol/L ethylenediamine tetra-acetic acid. Cell-free plasma aliquots (1 mL) were prepared by means of centrifugation (11 minutes at 1100*g* and 4°C) and stored at −80°C until use. TCC measures the TCC bound to the S-protein, representing the soluble nonlytic form of TCC. TCC was determined by means of enzyme-linked immunoassay (Quidel, San Diego, Calif).

PMPs
Arterial blood (4.5 mL) was collected into 3.2% trisodium citrate (Becton Dickinson). Blood cells were removed by means of 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 (17,570*g* and 20°C) to pellet the microparticles, as described previously.[19-22] After removal of 225 μL of (MP-free) plasma, the 25-μmL microparticle-enriched plasma was diluted with 225 μmL of phosphate-buffered saline (PBS; 154 mmol/L NaCl and 1.4 mmol/L phosphate, pH 7.4) containing 10.9 mmol/L trisodium citrate. Microparticles were resuspended and centrifuged (30 minutes at 17,570*g* and 20°C). Again, 225 μL of the supernatant was removed, and microparticles were resuspended in the remaining 25 μL and diluted 4-fold with PBS/citrate buffer, of which 5 μL was used per flow cytometric determination.

Flow Cytometric Analysis
Microparticle samples were analyzed in a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). PMPs were identified on forward scatter, side scatter, and binding of both phycoerythrin-labeled annexin V (PharMingen, San Jose, Calif) and fluorescein isothiocyanate–labeled anti-CD61 (glycoprotein IIIA; clone Y2/51, IgG1; Dako A/S, Glostrup, Denmark) to distinguish PMPs from events caused by noise. To identify annexin V–positive microparticles, a fluorescence threshold was placed in a microparticle sample prepared without addition of calcium to correct for autofluorescence. To identify CD61-positive events, microparticles were incubated with a similar concentration of isotype-matched control antibody (fluorescein isothiocyanate–labeled IgG1; Becton Dickinson) to set the fluorescence threshold. Microparticles (5 μL) were diluted in 35 μL of PBS containing 2.5 mmol/L $CaCl_2$ (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 was added. The mixtures were incubated in the dark (15 minutes at room temperature). Subsequently, 200 μL of PBS/calcium buffer was added, and the suspensions were centrifuged (30 minutes at 17,570*g* and 20°C). Finally, 200 μL of (MP-free) suspension was removed. The microparticles were diluted in 300 μL of PBS/calcium buffer before flow cytometry, and all samples were analyzed for 1 minute. To estimate the number of PMPs per liter of plasma, the number of events (N) found in the upper right (marker and annexin V positive) quadrant of the flow cytometric analysis (FL1 vs FL2) was used in the following formula: $Number/L = N \times [100/5] \times [355/150] \times [10^6/250]$.

Statistics
Data were analyzed by using SPSS, release 11.0 (SPSS, Chicago, Ill). Demographic and CPB data are reported as means with SDs. Outcome data (PMPs, platelets, and TCCs) were corrected for hemodilution (hemoglobin) and are presented as medians with interquartile ranges. For all outcome variables, statistical analyses were performed on the change of that variable relative to the baseline value (t = 0) per patient. Statistical significance ($P < .05$, 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 noncoated oxygenators. Comparisons over time within treatment groups were made by applying the Wilcoxon signed-rank test to the (paired) observations at baseline and at the end of CPB. Comparisons between (treatment) groups were made by applying the Kruskal-Wallis test to the change between the end of CPB and baseline. The observations at 15 minutes after the start of CPB and at 30 minutes after protamine sulfate 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 value, platelet count, and surgical data (CPB time and aortic crossclamping time). No significant differences were found among the groups for any of the variables tested (Table 1). There were no differences among the groups in hemoglobin concentrations and platelet counts at any sampling time. Hemodilution was similar in the 3 coating groups: the decreases in hemoglobin concentrations and platelet counts after the 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 administration of the cardioplegia solution for myocardial protection. In addition, in all 3 coating groups, a similar decrease in platelet count was observed after administration of protamine sulfate.

PMPs and Platelet Counts During CPB
The numbers of PMPs at baseline were similar among the 3 coating groups (Figure 1, *A*). Fifteen minutes after the start of CPB, the PMP numbers decreased in all 3 groups, despite correction for hemodilution. During CPB, the PMP numbers slightly recovered, but at the end of bypass, they were still less than before the start of CPB. At the end of bypass, there was a significant decrease of PMPs in the UMS group ($P < .001$) and the BPS group ($P = .028$) when compared with baseline values. After administration of protamine sulfate, PMP numbers again slightly decreased in all groups. Significant differences were not observed among the groups at any sampling point.

In contrast to PMP numbers, platelet counts significantly increased during CPB when corrected for hemodilution

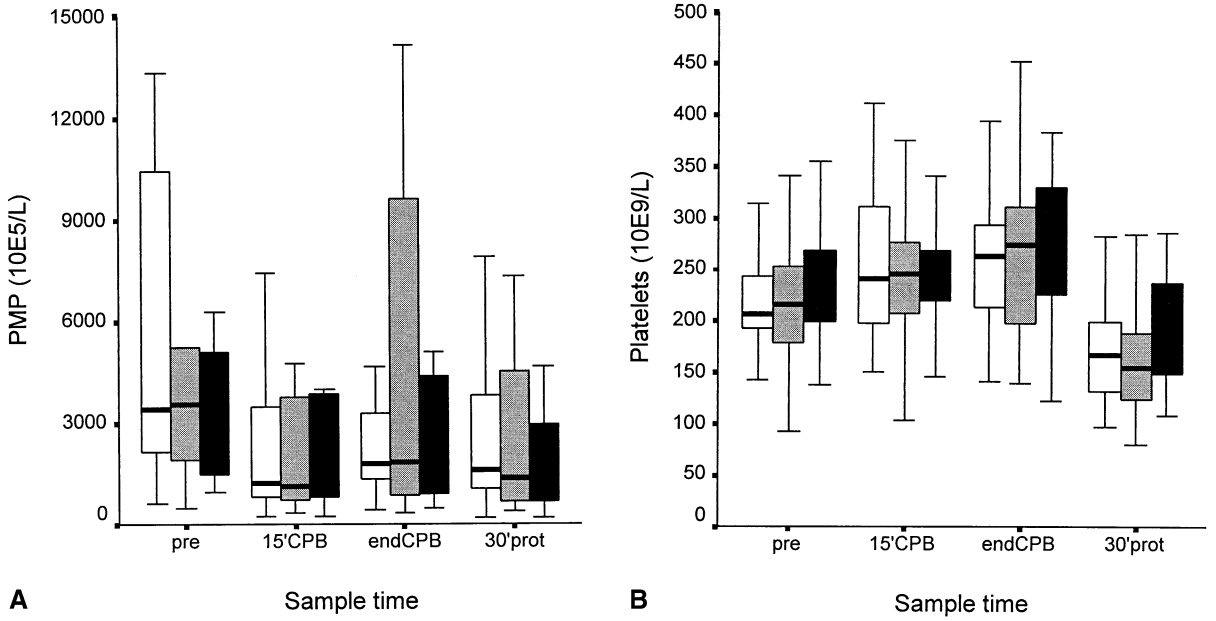

Figure 1. Systemic concentrations of PMPs and platelet counts in patients undergoing CPB. Concentrations of PMPs and platelet counts before bypass and after induction of anesthesia (baseline value; *pre*), 15 minutes after the start of bypass *(15'CPB)*, at the end of bypass and after release of the aortic crossclamp *(end CPB)*, and 30 minutes after administration of protamine sulfate *(30'prot)* are shown. The measurements were made in 25 patients treated with an uncoated oxygenator (ie, without Surface Modification, UMS group, *open bars*), 25 patients treated with an oxygenator coated with a BioPassive Surface (BPS group, *shaded bars*), and 21 patients treated with an oxygenator coated with a BioActive Surface (BAS group, *striped bars*). PMP concentrations *(A)* and platelet counts *(B)* were determined as described in the "Methods" section. No significant differences were present among the 3 oxygenator groups. Medians and interquartile ranges are presented.

(Figure 1, *B*; end of CPB: UMS group, $P = 0.004$; BPS group, $P < .001$, and BAS group, $P = .003$).

Complement Activation During CPB

Before CPB, complement activation was not detectable or hardly detectable (Figure 2). Fifteen minutes after the start of CPB, plasma concentrations of TCCs increased in all 3 coating groups, indicating complement activation. TCC concentrations further increased during CPB and remained high after protamine sulfate administration. At the end of CPB, TCC concentrations were significantly increased in all 3 groups ($P < .001$) when compared with baseline values. At the end of CPB, significant differences among the coating groups were present ($P = .002$). At the other sample times, no significant differences among 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 because earlier in vitro studies clearly showed that complement activation triggers the release of PMPs.[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 might contribute to microparticle release in other patient populations or that it might affect the release of microparticles in concert with other stimuli.

Earlier studies reported that CPB triggers the formation of PMPs both in vitro[26] and in vivo.[10,11] In contrast, we found an intraoperative decrease 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 rather afterward. Because pericardial blood contains relatively high numbers of cell-derived microparticles itself and is highly activated with regard to fibrinolysis and coagulation, one could 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

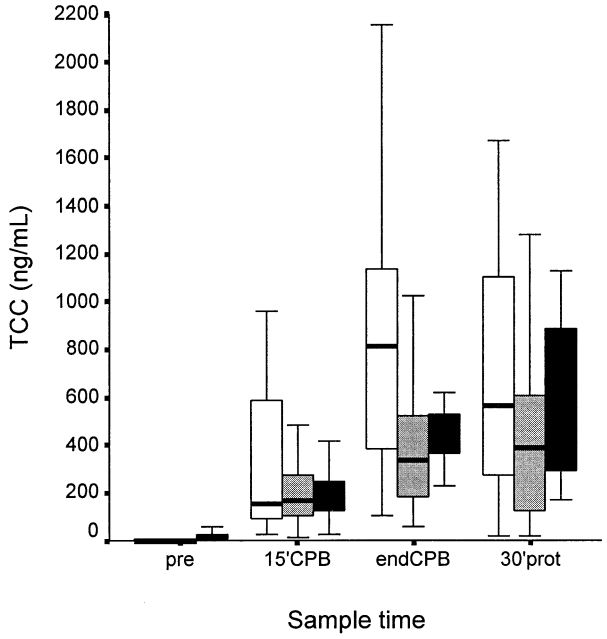

Figure 2. Concentrations of systemic TCCs in patients undergoing CPB. TCC concentrations before bypass and after induction of anesthesia (baseline value, *pre*), 15 minutes after the start of bypass *(15'CPB)*, at the end of bypass and after release of the aortic crossclamp *(end CPB)*, and 30 minutes after administration of protamine sulfate *(30'prot)* are shown. The measurements were made in 25 patients treated with an uncoated oxygenator (ie, without Surface Modification (UMS group, *open bars*), 25 patients treated with an oxygenator coated with a BioPassive Surface (BPS group, *shaded bars*), and 21 patients treated with an oxygenator coated with a BioActive Surface (BAS group, *striped bars*) and performed as described in the "Methods" section. Medians and interquartile ranges are presented.

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 colleagues[30] reported that platelets from the systemic circulation of patients undergoing CPB showed normal reactivity in vitro, had no loss of surface glycoprotein complexes Ib-IX and IIb-IIIa, and had hardly degranulated.

In contrast, pericardial blood contains relatively high numbers of PMPs and erythrocyte-derived microparticles.[20,27] Whether local complement activation (ie, in this wound blood) contributes to the release of PMPs cannot be excluded.

In the present study this shed blood was processed by a cell saver before being returned to 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 after bypass.

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

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