Cardiopulmonary Bypass Induces Leukocyte-Platelet Adhesion

By Christine S. Rinder, Jayne L. Bonan, Henry M. Rinder, Joseph Mathew, Roberta Hines, and Brian R. Smith

Cardiopulmonary bypass (CPB) has been demonstrated to activate platelets, producing an increased number of circulating platelets that have undergone α-granule release and express granule membrane protein-140 (GMP-140) on their surface. In vitro, GMP-140 mediates activated platelet adhesion to neutrophils (PMN) and monocytes, causing the formation of leukocyte-platelet conjugates. Using a newly developed assay that measures the percentage of circulating platelets that have undergone α-granule release and activate platelets, producing an increased number of circulating platelets causes significant platelet activation-dependent granule external membrane protein (PADGEM). In vitro studies have demonstrated that GMP-140 on activated platelets mediates binding to monocytes and neutrophils (PMN), but not to lymphocytes. To date, no studies have investigated the occurrence or perturbation of leukocyte-platelet conjugate formation in vivo. In addition, the functional consequences of leukocyte-platelet adhesion is currently uncertain. It is reasonable-to postulate that adhesion of activated platelets to leukocytes may cause subsequent leukocyte activation, localization of the leukocyte-platelet conjugate to damaged endothelium, or modulation of coagulation pathways. One clinical situation that significantly activates platelets in a "controlled" time course and that can be studied for leukocyte-platelet adhesion is cardiopulmonary bypass (CPB). In this report, using a flow cytometry assay to determine the percentage of leukocyte subsets with bound platelets in whole blood, we demonstrate that platelet activation on CPB is temporally accompanied by increased monocyte and PMN adhesion to platelets and by activation of phagocytic cells as evidenced by increased expression of the Leu-CAM integrin, CD11b.

MATERIALS AND METHODS

Antibodies. All monoclonal antibodies (MoAbs) were used as purified whole IgG or IgM. All experiments included irrelevant isotype-specific mouse MoAb as negative controls. The MoAb 1E3 (gift of Dr. K. Ault, Maine Cytometry Research Institute, South Portland, ME) is specific for GMP-140/PADGEM. P2 (AMAC, Westbrook, ME) recognizes GPIb/IIa. Anti-CD45 (HLE, Becton-Dickinson Immunochemistry Systems, San Jose, CA) recognizes a CD45 isoform present on PMN, monocytes, and lymphocytes, but not erythroid cells or platelets. The MoAbs MAA (Leu-M1) and D12 (Leu-15, Becton-Dickinson) recognize CD15 and CD11b, respectively.

Patient studies. After institutional approval by the Human Investigation Committee of the Yale University School of Medicine, 17 consecutive patients undergoing elective surgery requiring CPB were studied. All patients underwent CPB for a mean of 101 ± 29 minutes (SD) using a COBE CML EXEL membrane oxygenator at comparable flow rates. Two patients took aspirin during the 7 preoperative days, but each had a normal template bleeding time at the time of surgery. Whole blood samples were taken from the radial artery catheter; preliminary studies demonstrated no difference between sampling from the radial artery and from the proximal right atrium. A 200-μL sample of whole blood was immediately fixed in 1% (final concentration) paraformaldehyde in phosphate-buffered saline (PPBS). Samples were taken at the following time points: before start of surgery, after systemic heparinization before bypass, 10 minutes after the start of bypass, on bypass before rewarming, at termination of bypass (before protamine administration), 2 to 4 hours post-bypass, and 18 hours post-bypass. Patient samples were studied for (1) platelet activation (GMP-140 expression); (2) quantitative leukocyte surface adhesion protein expression; and (3) percentage of leukocyte-platelet conjugates.

Fluorescence labeling. All whole blood samples were fixed for 60 minutes at 4°C, then washed three times in Tyrodes-Hepes (TH) buffer (Hepes 5 mmol/L, NaCl 140 mmol/L, KCl 2.7 mmol/L, dextrose 5.5 mmol/L, NaH2PO4,0.42 mmol/L, and NaHCO3, 12 mmol/L, pH 7.4). Samples were divided into three parts: one for platelet surface GMP-140 measurement, one for leukocyte surface markers, and one labeled for leukocyte-platelet conjugates. For determination of the percentage of circulating platelets expressing GMP-140, 100 μL of sample was incubated with fluorescein isothiocyanate (FITC)-anti-GPIb/IIa (P2, AMAC) and biotinylated anti-GMP-140 (1E3) MoAbs for 20 minutes, washed, and resuspended in 100 μL TH buffer. The sample was then incubated with saturating concentrations of phycoerythrin (PE)-avidin (Becton-Dickinson) for 20 minutes, washed, and resuspended in 300 μL TH for FACS analysis.
Leukocyte surface markers were measured by incubating sepa—

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rate 100-μL samples with saturating concentrations of PE-anti-CD11b (D12, Becton-Dickinson) and FITC-anti-CD15 (MMA, Becton-Dickinson) for 20 minutes. The samples were then washed and resuspended in 300 μL TH buffer for FACS analysis. For the percentage of leukocyte-platelet conjugates, a separate 100-μL sample was incubated with saturating concentrations of FITC-conjugated anti-CD45 and biotinylated anti-GPIIb/Illa (P2, AMAC) MoAbs for 20 minutes, then washed and resuspended in 100 μL TH buffer. PE-avidin labeling and preparation for FACS analysis were performed as above.

Flow cytometry. Samples were analyzed on a FACScan flow cytometer (Becton-Dickinson) with data stored in list mode files. The determination of the percentage of platelets expressing GPI-140 was performed as previously described.13 The measurement of leukocyte surface CD11b and CD15 was performed by live-gating on leukocyte-sized events, using forward- versus side-scatter parameters to differentiate between monocyte and PMN subsets. Mean PE and FITC fluorescence for each subset was then analyzed for CD11b and CD15, respectively. As noted above, isotype- and fluorochrome-matched control MoAbs were used in each experiment to determine “nonspecific” background MoAb binding. This background varied less than 5% for each experiment.

The determination of leukocyte-platelet conjugates was performed as recently described.15 In brief, live-gating on leukocyte-sized events was performed to exclude single platelets using a combination of forward- and side-scatter and positive anti-CD45 fluorescence. Leukocyte subsets, that is, monocytes, PMN, and lymphocytes, were distinguished from one another on the basis of these characteristics. An isotype-matched (PE-conjugated) control MoAb was used to set a threshold (99% of events below threshold) for positive platelet marker fluorescence. The percentage of platelet-marker-positive events (PE-anti-GPIIb/Illa) represents the percentage of leukocytes with at least one bound platelet.

Previous studies with fractionated cell populations have demonstrated that this fluorescence assay can detect leukocytes with only a single platelet bound per leukocyte,4 and that either GPIb or GPIIIa/Illa as the platelet marker yields similar results for the percentage of leukocyte-platelet binding. However, it is known that surface expression of GPIb after platelet activation may actually decline by fluorescent methods, because of protease degradation or sequestration of GPIb in the open canalicular system, rendering GPIb inaccessible to fluorescent antibodies.6,7 In contrast, GPIib/IIIa may increase after platelet activation with release of intracellular GPIIb/Illa. Nonetheless, these changes are sufficiently small for the sensitivity of the assay that the fluorescent threshold (using either GPIb or GPIIIa/Illa) for determining the percentage of leukocyte-platelet conjugates was unaffected by platelet activation. In previous CPB studies, both GPIb and GPIIIa have increased in their surface expression, but with less change in total GPIIIa/Illa.8 Therefore, GPIIIa/Illa was used as the platelet marker for this study. Inert bead and image analysis studies have previously established that measured leukocyte-platelet conjugates are not the result of simultaneous passage of unbound platelets and leukocytes through the detection chamber, confirming that this assay measures only platelets bound to leukocytes, and that after paraformaldehyde fixation, measured conjugates remain stable for hours, confirming that the fixation protocol stabilizes conjugates versus free cells.

RESULTS

Platelet activation. The percentage of free circulating activated platelets, ie, expressing surface GMP-140, increased over baseline within 10 minutes on CPB (bars in Fig 1). This percentage continued to increase significantly (P < .01) during CPB, peaking at 19% ± 2.5% (mean ± SEM for the 17 patients) of circulating platelets just before rewarming. The percentage of GMP-140-positive platelets at 2 to 4 hours post-bypass then declined, but did not return to pre-CPB levels. By 18 hours post-bypass, the percentage of circulating activated platelets returned to a value not significantly different from baseline.

Platelet-leukocyte binding. For the 17 patients, the percentage of leukocytes with bound platelets was comparable between leukocyte subsets before the start of surgery, with 15% ± 1.5% (mean ± SEM) of PMN and lymphocytes and 18% ± 1.5% (mean ± SEM) of monocytes binding platelets. These values are similar to those previously observed in healthy normal controls (data not shown). The percentage of circulating monocyte-platelet conjugates increased with the onset of CPB and continued to increase, peaking at the end of CPB, with a mean of 44% ± 4.5% (SEM) of circulating monocytes having bound platelets (P < .01). At 2 to 4 hours after termination of CPB, platelet-monocyte binding had decreased to 36% ± 4.3% (mean ± SEM) of circulating monocytes, and by 18 hours after CPB, had returned to baseline values.

The percentage of circulating PMN-platelet conjugates peaked after only 10 minutes (P < .05) on CPB, much
earlier than monocytes; in addition, the peak percentage of bound PMN was 23% ± 3.8% (mean ± SEM), only half of the peak monocyte-platelet binding. This percentage of PMN-platelet conjugates slowly declined over the remainder of CPB, and returned to baseline 2 to 4 hours after termination of CPB.

In sharp contrast to monocytes and PMN, the percentage of circulating lymphocyte-platelet conjugates significantly decreased during CPB (P < .05), reaching its nadir, 11% ± 1.6% (mean ± SEM), on CPB before rewarming. After termination of CPB, platelet-lymphocyte binding then increased to pre-bypass values.

Monocyte and PMN activation. Surface expression of CD11b on monocytes (Fig 2) gradually but significantly increased (P < .01) during and after CPB, peaking at 2 to 4 hours after termination of bypass at three times the baseline value. At 18 hours after CPB, monocyte CD11b expression remained significantly higher than baseline. CD11b expression on PMN also increased significantly (P < .01) to a value three times higher than baseline, but, unlike monocytes, PMN CD11b increased immediately after the onset of CPB and peaked earlier than monocytes (before rewarming). PMN CD11b then declined to baseline post-CPB, unlike monocytes, which still had significantly elevated CD11b expression post-CPB. CD15 quantitative expression on monocytes and PMN was also monitored. Only a slight increase in mean surface expression over time on CPB was observed for this moiety (50% increase compared with 300% for CD11b).

The quantitative relationship between changes in platelet-leukocyte binding and simultaneous changes in leukocyte CD11b expression over time for individual patients was examined to determine whether there was a temporal and quantitative correlation of monocyte and/or PMN CD11b expression and increased circulating platelet-leukocyte conjugates. Using linear regression analysis, at the point of peak platelet-monocyte binding (end of CPB), the increase in platelet-monocyte conjugates correlated significantly with the increase in monocyte CD11b expression at that time point (r = .6, P = .01). Similarly, at peak CD11b expression on monocytes, occurring 2 to 4 hours after CPB, the increase in platelet-monocyte binding at that time point again correlated with the increase in monocyte CD11b (r = .66, P < .01). By contrast, the increase in platelet-PMN binding, compared with the increase in PMN CD11b at their respective peaks (10 minutes after start of CPB and before rewarming), did not correlate for either time point (P > .05 for both time points).

The two patients on preoperative aspirin had similar patterns of platelet activation, leukocyte-platelet conjugate formation, and CD11b expression as the remainder of the study group when both groups were analyzed separately.

DISCUSSION

GMP-140 has been identified as a platelet glycoprotein that mediates in vitro binding of activated platelets to monocytes and PMN. We and other investigators have previously demonstrated that CPB activates platelets and causes an increase in the percentage of circulating platelets expressing GMP-140. This study confirms that finding and relates the in vivo time course of platelet GMP-140 expression to its known in vitro function of mediating leukocyte adhesion. This is the first clinical study to examine alterations in circulating leukocyte-platelet adhesion and their relation to both platelet and leukocyte surface adhesion and “activation antigen” receptor changes.

For this study, unmanipulated blood drawn from an arterial catheter was immediately fixed in paraformaldehyde. Prior studies by our laboratory have shown that this procedure appears to provide a reasonable approximation of measuring “in vivo” platelet-leukocyte conjugates. Although we cannot eliminate the possibility that the conjugate formation seen is partially an artifact of blood drawing or of the stability of the assay over time, the dependence of activated platelet-leukocyte adhesion on both divalent cations and specific epitopes of the GMP-140 molecule in normal whole blood, and the reproducible change observed over time in this study, strongly suggest that these results reflect the in vivo situation.

Ten minutes after the start of CPB, platelet activation,
and both monocyte-platelet and PMN-platelet conjugate formation, increased. However, monocyte-platelet binding increased over a longer duration and to a greater absolute value than PMN-platelet binding, consistent with in vitro data that indicates monocytes have a competitive advantage over neutrophils for adhesion to activated platelets. The time course and peak of free circulating, activated platelets also corresponded to the course and extent of monocyte-platelet binding, but less so with PMN-platelet conjugate formation, further emphasizing this specific monocyte advantage. In contrast, lymphocyte-platelet conjugates decreased on bypass. This finding is consistent with in vitro observations that lymphocytes do not bind GMP-140-positive platelets, and furthermore, as has been shown in vitro, suggests that platelet activation and subsequent adhesion to monocytes and PMN can compete platelets off lymphocytes. It is also possible that increased phagocyte-platelet binding on CPB is partly due to upregulation of the receptor on monocytes and PMN which binds GMP-140. This receptor has not been completely characterized, but there is evidence to suggest that sialylated forms of CD15 and/or Lewis' may be partial counterligands for GMP-140. Although CD15 expression on monocytes and PMN increased slightly by the end of CPB, the increase in leukocyte-platelet conjugates on CPB appears best explained by the rapid increase in circulating GMP-140-positive platelets, both because of temporal pattern and quantitative alteration, as well as prior in vitro observations.

CD11b (also known as complement receptor 3) increased over time on bypass on monocytes and PMN, but with different time courses for the two cell types. PMN expression of CD11b peaked immediately on CPB, a pattern more indicative of activation by the extracorporeal circuit, whereas monocyte expression increased more gradually (albeit to a similar peak value). The increased monocyte CD11b expression correlated with the percentage of monocyte-platelet conjugates, whereas PMN CD11b showed no correlation with PMN-platelet adhesion. CD11b upregulation on monocytes could be due to several mechanisms: direct contact activation of leukocytes by the CPB apparatus, complement activation, or perhaps as a consequence of leukocyte-platelet adhesion. In the setting of CPB, it is likely that all three mechanisms for upregulation of leukocyte CD11b may be operative.

The complications that may occur after CPB include bleeding, pulmonary and other organ dysfunction. These may be due to a combination of cellular and soluble factors (eg, complement) changes. The findings in this study of increased leukocyte-platelet conjugates on CPB add an additional factor to consider in post-CPB dysfunction. One possible consequence of increased leukocyte-platelet adhesion in vivo is that such conjugates may be physically sequestered in the pulmonary or other localized vascular beds, producing local vasoactive changes and inflammation, as noted in some animal models. Increased leukocyte CD11b may also be particularly relevant to CPB-induced injury. Infusion of anti-CD11b has been shown to prevent reperfusion injury in several animal models, and the addition of MoAbs to CD11b to monocytes in vitro results in marked interference with leukocyte adherence to endothelial cell monolayers. Therefore, upregulation of CD11b on monocytes and PMN during CPB may contribute to tissue injury directly, especially in the postarrested myocardium. In addition, since CD11b also functions as the receptor for the inactivated complement component C3b, classical and contact activation of the complement system on CPB could be regulated in part by leukocyte CD11b, as could aspects of the contact-dependent soluble coagulation system, since CD11b has also been shown to be a receptor for fibrinogen and factor X. Further dissection of the pathophysiology of CPB with respect to leukocyte-platelet adhesion and its functional consequences will likely require study of both in vitro and in vivo model systems.

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