Prevention of Acquired Transient Defect in Platelet Plug Formation by Infused Prostacyclin

By Thomas W. Malpass, Stephen R. Hanson, Brian Savage, Eugene A. Hessell II, and Laurence A. Harker

Cardiopulmonary bypass in baboons produced transient severe platelet dysfunction (bleeding times prolonged to 27.8 ± 1.4 min compared with 3.9 ± 0.7 baseline) that was associated with a parallel release of platelet α-granule proteins into plasma (platelet factor 4 and β-thromboglobulin levels of 28.8 ± 9.3 and 20.0 ± 1.8 ng/ml, respectively) and their clearance into urine with a reciprocal depletion from circulating platelets. In contrast, platelet-dense granules did not undergo significant release. The bleeding times normalized rapidly following bypass (8.5 ± 1.4 min at 1 hr). The infusion of prostacyclin (PGI₂) into the bubble oxygenator during bypass (40–80 ng/kg/min) prevented the prolongation in bleeding time (p < 0.01 compared with untreated control values) but did not block the release of α-granule proteins. Dosages outside this range were associated with prolonged bleeding times. These results show that transient platelet dysfunction occurring during cardiopulmonary bypass represents activation of platelets independent of α or dense granule release and is blocked by potent short-acting inhibition of platelet function using PGI₂ infused into the oxygenator apparatus at optimal therapeutic doses.

Cardiopulmonary bypass may be complicated by life-threatening hemorrhage due to acquired defects in hemostasis.1–3 While extracorporeal circulation of blood through an oxygenator apparatus affects many aspects of hemostasis, acquired platelet dysfunction appears to be the most significant abnormality produced.1,2,5,7 Since this acquired platelet defect is transient and is postulated to be the consequence of platelet activation, we have used PGI₂ in a baboon model of cardiopulmonary bypass to test the hypothesis that a potent short-acting inhibitor of platelet activation in the extracorporeal circuit will prevent development of the defect in platelet plug formation.8,9

Materials and Methods

Baboon Model of Cardiopulmonary Bypass

Baboons (Papio cynocephalus) weighing 10–17 kg with hematocrits of 40 ± 3, leukocyte counts of 5100 ± 1200/μl, and platelet counts of 302,000 ± 70,000/μl were studied during cardiopulmonary bypass using a roller-head apparatus with a disposable, hard-shell infant-sized bubble oxygenator (Bentley Temptril, Irvine, Calif.). PGI₂ was infused into the oxygenator during bypass in 9 animals, and 6 animals served as untreated controls. Anesthesia was induced with 10 mg/kg ketamine HCl and maintained with halothane after endotracheal intubation. Vascular access was obtained through the femoral artery and vein and internal jugular vein using medical grade Tygon class VI Bardic catheters. Blood pressure measurements and blood samples were obtained through an indwelling internal carotid catheter. All animals were maintained at 35–38°C throughout bypass. The pump was primed initially with 800–1300 ml lactated Ringer’s solution and 150 ml packed RBCs. The animals received an additional 500 2000 ml of lactated Ringer’s solution during the period of bypass. All bypass animals were anticoagulated with porcine intestine heparin (Upjohn, Kalamazoo, Mich.) 300 U/kg initially, and 150 U/kg at hourly intervals thereafter. Extracorporeal circulation at a rate of 50–60 ml/kg/min was maintained for 3 hr in each animal. The oxygenator was ventilated with 100% oxygen at a rate of 1 liter/min. At the end of the procedure, heparin was neutralized with protamine sulfate given at a ratio of 1.5 mg protamine sulfate/100 U heparin.

Immediately prior to use, PGI₂ sodium salt was mixed with cold carbonate-buffered saline (15 mM; pH 11.0) to a final concentration of 3.14 μg PGI₂/ml. In control studies, PGI₂ was infused directly into the femoral veins of unanesthetized chaired baboons not on bypass using a syringe pump (Harvard Apparatus Co., Millis, Mass.). In animals on bypass the mixture was infused into the venous oxygenator inlet line.

Laboratory Procedures

Platelets were counted with an electronic particle counter on blood collected in EDTA.10,11 Electronic platelet volumes were recorded at 22°C in whole blood anticoagulated with sodium citrate or EDTA using a hemodynamically focused cell sizing transducer (Clay Adams UF-100, Parsippany, N.J.) calibrated with spherical latex particles (1.86, 2.0, and 3.05 μm diameter) and analyzed by lognormal modeling.12 Standardized template bleeding times were performed on the shaved volar surface of the forearm. The normal template bleeding time in 67 normal baboons was 4.0 ± 2.0 min.11,12

Platelet aggregation induced by ADP (Sigma, St. Louis, Mo.) and collagen (Hormon, Munich, West Germany) was studied using platelet-rich plasma (PRP). The platelet concentration was adjusted to 300,000/μl and the citrate concentration was held constant at 0.012 M.13,14 Aggregation was assessed by plotting optical transmission with a dual channel aggregometer (Payton Associates, Buffalo, N.Y.) against the log of ADP or collagen concentration. The concentration of inducing agents required to produce 50% of the maximal achievable amplitude (ED₅₀) was compared with the concentration required to produce the same change prior to bypass.

Blood for plasma PF4 and βTG was drawn as 5-ml samples from a catheter in the carotid artery into precooled syringes containing 1 ml of acid citrate dextrose (ACD; NIH Formula A), acetylserine acid (ASA), and prostaglandin E₁ (PGE₁), 30 mM and 1 μM final concentration, respectively.
original determination. The final concentration in the unknown
concentrations, respectively. Samples were centrifuged at 45,000 g
for 20 min at 4°C within 30 min of collection. One milliliter of
the platelet-free plasma was removed and stored at −20°C. Assays for
plasma PF4 and βTG were performed by competitive radioimmuno-
massay.14,15 The mean plasma concentrations of PF4 and βTG in
normal baboons in our laboratory were 2.9 ± 0.6 ng/ml and 6.3 ±
0.4 ng/ml, respectively. The platelet content of PF4 was calculated
by measuring the concentration of PF4 in serum, dividing by the
platelet count and mean platelet volume, and then adjusting for
variability in the plasmacrit: the results were expressed as ng of
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ml of blood into glass tubes and allowing it to clot and stand
overnight at room temperature. To assay PF4 in urine, a 1 ml
sample was tested because of the relatively small amounts of PF4
present in urine. To correct for alterations in binding found in
different urine specimens, a second sample of the same urine was
assayed with the addition of 1 ng PF4 as an internal standard, and
the percent recovery of the added PF4 was used to correct the
original determination. The final concentration in the unknown
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Autologous platelets were doubly labeled with 14C-serotonin and
31Cr according to established technique.16,17 14C-serotonin platelet
removal was compared with 31Cr-platelet removal as percent changed
from baseline values in order to assess the potential for selective release of dense granules compared with platelet destruc-
tion.

Table 1. The Effect of Infused PGI2 on Platelet Behavior in Normothermic Baboons Undergoing Extracorporeal Bubble Oxygenation

<table>
<thead>
<tr>
<th></th>
<th>Time (hr)</th>
<th>PFA (ng/ml)</th>
<th>βTG (ng/ml)</th>
<th>Bleeding Time (min)</th>
<th>Platelet Count (× 10^12/μl)</th>
<th>Urinary PF4 (ng/mg creat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bubble oxygenation</td>
<td>Baseline</td>
<td>3.4 ± 1.5</td>
<td>4.8 ± 0.7</td>
<td>3.9 ± 0.7</td>
<td>302 ± 98</td>
<td>0.2 ± 0.2</td>
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<tr>
<td></td>
<td>0.5</td>
<td>28.8 ± 9.3</td>
<td>20.0 ± 1.8</td>
<td>21.7 ± 4.6</td>
<td>213 ± 44</td>
<td>0.9 ± 0.8</td>
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<tr>
<td></td>
<td>1</td>
<td>23.3 ± 3.8</td>
<td>19.6 ± 1.5</td>
<td>22.3 ± 4.9</td>
<td>224 ± 48</td>
<td>11.1 ± 12.6</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>21.1 ± 2.5</td>
<td>19.8 ± 1.9</td>
<td>25.2 ± 3.8</td>
<td>153 ± 23</td>
<td>17.3 ± 0.2</td>
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<tr>
<td></td>
<td>2</td>
<td>20.4 ± 1.3</td>
<td>19.4 ± 2.1</td>
<td>22.1 ± 4.7</td>
<td>181 ± 34</td>
<td>15.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>17.6 ± 1.1</td>
<td>19.0 ± 2.0</td>
<td>26.7 ± 2.4</td>
<td>174 ± 32</td>
<td>3.5 ± 0.9</td>
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<td>3</td>
<td>19.5 ± 2.5</td>
<td>18.6 ± 1.9</td>
<td>27.8 ± 1.4</td>
<td>164 ± 26</td>
<td>6.0 ± 0.2</td>
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<tr>
<td></td>
<td>1 (after bypass)</td>
<td>3.3 ± 0.7</td>
<td>15.2 ± 1.9</td>
<td>8.5 ± 1.4</td>
<td>211 ± 78</td>
<td>0.6 ± 0.2</td>
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<tr>
<td>Bubble oxygenation with PGI2 infusion</td>
<td>Baseline</td>
<td>5.1 ± 1.2</td>
<td>3.2 ± 0.9</td>
<td>4.0 ± 0.4</td>
<td>302 ± 42</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>19.8 ± 5.2</td>
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<td>4.7 ± 0.4</td>
<td>168 ± 22</td>
<td>23.0 ± 7.2</td>
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<td>3</td>
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<td>14.0 ± 0.6</td>
<td>5.2 ± 0.9</td>
<td>165 ± 26</td>
<td>10.0 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1 (after bypass)</td>
<td>9.4 ± 2.7</td>
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<td>6.3 ± 1.8</td>
<td>176 ± 16</td>
<td>5.7 ± 0.3</td>
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Values represent the mean ± 1 SE.

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for 20 min at 4°C within 30 min of collection. One milliliter of
the platelet-free plasma was removed and stored at −20°C. Assays for
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from baseline values in order to assess the potential for selective release of dense granules compared with platelet destruc-
tion.

Total platelet ATP and ADP were measured directly using perchloric acid extraction and high performance liquid chromatography.18 A Waters model ALL-204 liquid chromatograph (Waters Associates, Milford, Maine) was used for all analyses. The levels of ATP and ADP were 3.4 ± 0.1 (± 1 SE) and 3.5 ± 0.1 μmoles/10
11 platelets, respectively, (ATP/ADP = 0.97 ± 0.01) in 10 normal
baboons.

p Values were obtained by the paired and nonpaired t test when
observations were normally distributed or by the paired sample
Wilcoxon signed rank test or the Wilcoxon Mann-Whitney rank
sum test when the distribution was not normal.

RESULTS

Untreated Bubble Oxygenator Studies

Immediately following the initiation of cardiopulmonary bypass in six baboons, the platelet count dropped (Table 1, Fig. 1) due to dilution by the oxygenator priming solution. The platelet count decreased to a mean of 49% ± 3% (± 1 SE).* but in no animal did the platelet count decrease to levels that prolong the bleeding time, i.e., less than 100,000 platelets/μl. In control animals not on bypass, the addition of 500 ml of priming buffer to the circulation via the AV cannula in 2 animals and 1000 ml added in another 2 animals decreased the platelet counts as expected (210,000/μl to 140,000/μl, 187,000/μl to 135,000/μl, 202,000/μl to 92,000/μl and 375,000/μl to 180,000/μl, respectively) without significant prolongation of the bleeding time (baseline 4.1 ± 1.5
1 min and 5.2 ± 1.3 min at 30 min after infusion; p > 0.5). Platelet volume measurements did not change (p > 0.5), and there was no detectable plasma hemog-
lobin in any of the untreated or treated animals.

In the group of 6 untreated bypass animals, the mean template bleeding time became prolonged to greater than 20 min within 0.5 hr after the initiation of bypass and reached a maximum of 27.8 ± 1.4 min at 3 hr of bypass. Open cutoff sites for vascular access oozed continuously throughout the period of bypass.

*In the results, variances about the mean are given as ± 1 SE.
After discontinuation of bypass, the bleeding time shortened rapidly (Table 1, Fig. 1) and was 8.5 ± 1.4 min 1 hr after bypass. Five control animals were studied before and after heparinization without bypass to determine the effect of heparinization alone on the bleeding time. Five and 30 min after 300 U/kg heparin, the bleeding times remained normal (3.9 ± 1.3 min preheparin versus 4.7 ± 1.5 and 4.0 ± 1.4 postheparin; p > 0.4).

There was substantial release of platelet α-granule constituents into plasma during bypass (Table 1). Urinary levels of PF4 also increased a 100-fold with a peak at 90 min of 17.3 ± 0.2 ng/mg creatinine, and platelets showed partial depletion of platelet content of PF4 (1.09 ± 0.31 ng/μl × 10^6 preceding and 0.74 ± 0.23 following bypass; p < 0.01). Plasma PF4 levels returned to normal more rapidly than βTG following bypass, consistent with the differences in renal clearance of these proteins.14

During the period of bypass, the normalized ratio of ^14C-serotonin:31Cr-labeled platelets did not decrease significantly (p > 0.4), suggesting that there was no significant loss of dense granule serotonin.16 Platelet ATP/ADP ratios were measured in 2 baboons during and following bypass (0.97 and 1.17 baseline, 0.93 and 1.14 at 30 min, 0.97 and 1.19 at 180 min, and 1.00 and 1.16 at 30 min after bypass). There were small parallel decreases in the total platelet content of both ATP and ADP. ATP levels in μmole/10^11 platelets were 3.9 and 3.9 baseline, 3.2 and 3.0 at 30 min, 3.2 and 3.2 at 180 min, and 3.5 and 3.2 at 30 min after bypass. ADP levels were 4.0 and 3.4; 3.5 and 2.6; 3.3 and 2.7; and 3.5 and 2.8, respectively, for the same intervals. In vitro platelet aggregation became abnormal to both ADP and collagen. The ED_{50} for collagen concentration increased from 0.52 ± 0.11 to 8.70 ± 2.90 μg/ml at 90 min (p < 0.01), and the ED_{50} for ADP concentration increased from 0.88 ± 0.12 μM at baseline to 3.27 ± 0.75 μM at 90 min (p < 0.01).

**Bubble Oxygenator Studies With PGI2 Infusion**

Sixteen animals not on bypass were studied during PGI1 infusion at rates of 40, 100, 200, and 300 ng/kg/min to determine separately the effects of the drug on blood pressure and bleeding times (Fig. 2). The mean baseline bleeding time was 4.0 ± 1.0 min, and baseline arterial pressures averaged 141 ± 16 mm Hg. Significant effects on bleeding time and arterial blood pressure were observed for PGI1 infusion rates of 100 ng/kg/min or greater. PGI1 infusions in 4 control animals not on bypass had no significant effect on the plasma levels of α-granule proteins, e.g., plasma PF4 was 3.2 ± 2.5 ng/ml baseline and 1.4 ± 0.7 ng/ml while infusing 100 ng/kg/min PGI1 (p > 0.2).

Changes in platelet count in the group of PGI1-treated animals were similar to the untreated animals.

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**Fig. 1.** Changes in platelet behavior during cardiopulmonary bubble oxygenator bypass. The bleeding time (solid circles) increases abruptly following the initiation of bypass and prolongs progressively during the first 1.5 hr of bypass. Plasma PF4 (open circles) rises rapidly following the initiation of bypass and peaks at 0.5 hr and then shows a gradual decline. Plasma βTG (closed triangles) shows similar changes following the initiation of bypass with a similar peak at 0.5 hr. Error bars represent ± 1 SE.

**Fig. 2.** Changes in bleeding time and blood pressure in normal baboons not on bypass receiving PGI1 infusion. Administration rates of 40, 100, 200, and 300 ng/kg/min are shown on the horizontal axis. A significant change in bleeding time (solid circles) and blood pressure (open circles) occurs at PGI1 infusion rates of 100 ng/kg/min or greater. Error bars represent ± 1 SE.
Fig. 3. Comparison of bleeding time changes in PGI₂-treated and untreated groups of animals during cardiopulmonary bypass. The mean template bleeding time in animals receiving PGI₂ (solid circles) was markedly shorter than in untreated control animals (open circles). Error bars represent ± 1 SE.

(Table 1: p > 0.2). However, the mean template bleeding time in the 5 animals treated with 40–80 ng PGI₂/kg/min was strikingly shorter than in the untreated animals (6.7 ± 1.6 versus 22.3 ± 4.9 min at 1 hr after the start of bypass. Fig. 3; p < 0.01). Surgical sites of vascular access similarly showed striking cessation of bleeding compared to untreated animals. In contrast, an additional 2 animals receiving PGI₂ at less than 30 ng/kg/min and 2 animals receiving greater than 100 ng/kg/min all had bleeding times greater than 25 min.

Alpha-granule release in the treated animals was at a slower rate than that observed in the untreated animals; plasma levels of βTG increased more slowly in the treated animals and rose progressively throughout bypass to reach the peak value at 3 hr, while the values in untreated animals peaked early (p < 0.01 at 30 min into bypass). PF4 clearance into urine and depletion from platelets also occurred later in the treated group (Table 1). In the PGI₂-treated animals, urine PF4 peaked to 25.4 ± 10.9 ng/mg creatinine and platelet content of PF4 was 0.95 ± 0.15 ng/μl × 10⁶ preclosure and 0.65 ± 0.10 postclosure in this group (p > 0.15 compared to untreated animals). The changes in platelet aggregation induced by ADP and collagen also became abnormal in the treated group of animals. The EDₕ₀ for collagen increased from 0.96 ± 0.18 μg/ml to 7.70 ± 3.2 μg/ml at 90 min into bypass, and the EDₕ₀ for ADP increased from 1.20 ± 0.30 to 3.80 ± 0.69 μM at 90 min (p < 0.01 and p < 0.01 compared to baseline, respectively). As in the untreated animals, dense granule release did not occur as judged by ¹⁴C-serotonin and ⁵¹Cr-platelet kinetics.

DISCUSSION

All animals undergoing cardiopulmonary bypass with bubble oxygenation demonstrated marked prolongation in bleeding time within 30 min of bypass that nearly normalized within an hour following bypass and was not caused by thrombocytopenia or heparinization. The prolongation in bleeding time was associated with a marked parallel rise in both plasma and urine PF4 and plasma βTG and a reciprocal partial depletion of α-granule contents. Dense granules did not undergo significant release. The defect in platelet plug formation was reversible without reformation of the lost α-granule contents, suggesting that platelet dysfunction and α-granule release were independent consequences of platelet activation. These results reproduced the observations in patients undergoing cardiopulmonary bypass. This transient defect in platelet function may be similar to the refractory period following stimulation by ADP in vitro.

In order to test the hypothesis that the acquired functional defect is caused by platelet activation within the extracorporeal circuit we infused PGI₂, a potent short-acting inhibitor of platelet activation, directly into the oxygenator apparatus. The template bleeding time was maintained near normal throughout bypass in animals undergoing bypass with infused PGI₂. In these studies it was important to administer enough drug to inhibit platelet activation within the extracorporeal circuit but at doses sufficiently low to avoid both hypotension and defective platelet function due to excessive systemic PGI₂ itself. The therapeutic “window” that empirically produced this desired effect in baboons was 40–80 ng/kg/min. Interestingly, preservation of the capacity of platelets to form hemostatic plugs by infusing PGI₂ did not prevent α-granule release. This observation further evidences the independent nature of the activation processes underlying transient platelet dysfunction and α-granule release. These results suggest that the infusion of PGI₂ into the oxygenator apparatus may be useful in reducing abnormal bleeding in patients undergoing cardiopulmonary bypass. Moreover, these data emphasize the importance of determining the effective therapeutic window in designing such studies in man.

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