Decreased Platelet Thrombogenicity in Association With Increased Platelet Turnover and Vascular Damage

By M. R. Buchanan, C. J. Carter, and J. Hirsh

Platelet turnover is increased when platelets interact with prosthetic surfaces and damaged vessel wall. To determine whether the resulting increase in young platelets is associated with an increased tendency to thrombosis, we induced a state of increased platelet turnover in rabbits by inserting a sterile cannula into the abdominal aorta and tested for platelet thrombogenicity by measuring the deposition of circulating platelets onto a second injury site in the carotid arteries. Platelet half-life was decreased and platelet turnover was increased after the aortic cannulation, although the circulating platelet count remained unchanged. Platelet thrombogenicity determined 20 hr after cannulation was significantly decreased when compared to sham-operated animals. Ear bleeding studies demonstrated that the platelets circulating in cannulated animals were hemostatically less effective than those in sham-operated animals. This effect was intrinsic to the platelet and was associated with a platelet function defect. These data suggest that platelets exposed to a damaged or foreign surface interact with the surface and then circulate in a less reactive state.

There is both clinical and experimental evidence that platelet survival is decreased and turnover increased when blood is exposed to prosthetic surfaces or extensive areas of vascular damage. The increase in platelet turnover is associated with an increase in the proportion of young platelets in the circulation. Since young platelets are metabolically and functionally more active than old platelets, it has been suggested that patients with vascular disease and increased platelet turnover may have an increased risk of developing further thromboembolic complications. This possibility is supported by reports by Steele and associates that patients with coronary artery disease and valvular heart disease who have increased platelet turnover have a higher frequency of thromboembolic events than those with a normal platelet turnover.

To test this, we investigated the thrombogenic and hemostatic potential of platelets circulating during a state of experimentally induced increased platelet turnover in rabbits.

MATERIALS AND METHODS

Acid-citrate-dextrose consisted of 25 g C₆H₅Na₃O₇, 2H₂O, 15 g C₆H₈O₇·H₂O, and 20 g C₆H₁₂O₆ in 1 liter deionized water at pH 4.5, milliosmolarity 450. Sodium citrate (C₆H₅Na₃O₇·2H₂O) was made up to a 3.8% solution. Heparin (Hepalean, porcine mucosa) was obtained from Harris Laboratories, Brantford, Ontario.

From the Department of Pathology, McMaster University Medical Centre, Hamilton, Ontario, Canada.

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Address reprint requests to Dr. M. R. Buchanan, Department of Pathology, Room 4N48, McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8S 4J9.

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Calcium-free Tyrode’s solution consisted of 5 ml Stock 1, 2 ml Stock 2, 100 mg glucose, and 350 mg bovine serum albumin (Pentex, Miles Laboratories, Ill.) in 100 ml deionized water, pH 6.25, milliosmolarity 290. Stock 1 contained 160 g NaCl, 4 g KCl, 20 g NaHCO₃, and 1.16 g NaH₂PO₄·H₂O in 1 liter deionized water. Stock 2 contained 20.33 g MgCl₂·6H₂O in 1 liter deionized water. Calcium-free and albumin-free Tyrode’s was the same as above except that the albumin was excluded.

Chromium-51 (⁵¹Cr) was obtained as Na₂⁵¹CrO₄ in saline, 1 mCi/ml, 300–400 mCi/mg, from New England Nuclear, Boston, Mass. ¹⁴C-serotonin (¹⁴C-5HT) was obtained as 5-hydroxy-(2-¹⁴C) tryptamine creatinine sulphate, 58 mCi/mmole, from Amersham Searle, Arlington Heights, Ill.

Preparation of ⁵¹Cr-Labeled Cells

(A) ⁵¹Cr-labeled platelets. Donor rabbit platelets were labeled in vitro with ⁵¹Cr by a method previously described in detail.¹¹ Briefly, donor rabbits were exsanguinated through a carotid artery and the blood was collected into acid-citrate-dextrose. The platelets were isolated by differential centrifugation, washed with calcium-free Tyrode’s solution, and incubated in calcium-free and albumin-free Tyrode’s containing ⁵¹Cr (90 µCi/donor rabbit). The platelets were resuspended in acid-citrate-dextrose platelet-poor plasma at a concentration of $2.5 \times 10⁸$ platelets/mm³, and 3-ml aliquots were injected into each recipient rabbit. These platelets are referred to as “homologous ⁵¹Cr platelets.”

(B) ⁵¹Cr-labeled red cells. Donor rabbit red cells obtained from 8.7 ml whole blood in 1.5 ml acid-citrate-dextrose were labeled with 500 µCi ⁵¹Cr according to the method of Dacie and Lewis.¹² The labeled cells were resuspended in 10 ml of the phosphate-buffered saline and incubated at 22° C until use.

(C) ¹⁴C-labeled platelets. The rabbits’ own circulating platelets were labeled with ¹⁴C-5HT by injecting 10 µCi/kg ¹⁴C-5HT into each rabbit according to the method of Henson and Harker.¹³

Primary Injury Model (Aortic Cannulation)

Rabbits were anesthetized with sodium pentobarbital (MTC Pharmaceuticals, Hamilton, Ontario), and an 8-in sterile polyethylene cannula (PE-190 Intramedic) was inserted into the abdominal aorta via the femoral artery and tied in situ. These rabbits are referred to as “cannulated” rabbits. In control rabbits, one femoral artery was tied off, and these rabbits are referred to as “sham-operated” rabbits.

Platelet Survival Determinations

Cannulated and sham-operated animals were injected with homologous ⁵¹Cr-labeled platelets 30 min after operation. One-milliliter samples of whole blood were collected into 0.5 ml of ACD and transferred into a 10 x 75 mm glass tube. Samples were collected at 2, 4, 8, 20, 24, 28, 44, and 48 hr after operations. All samples were counted for 10 min in a 1085 Nuclear Chicago gamma counter. ⁵¹Cr-whole blood recovery was expressed as a percent of the 2-hr sample, which was arbitrarily considered as 100%. The data were analyzed using a computer-assisted semilogarithmic regression analysis and expressed as platelet half-life ($t/2$).

The proportion of labeled platelets remaining in the systemic circulation after infusion, i.e., platelet recovery, was calculated from the platelet activity/ml of the 2-hr sample, multiplied by the estimated blood volume, and divided by the platelet ⁵¹Cr activity injected. Platelet turnover/ml/hr was calculated from the mean peripheral platelet count determined on samples collected at 4, 8, 24, and 48 hr after injection, divided by the platelet survival time in hours, and corrected for recovery. This method is described in detail by Harker.¹⁴

Measurement of Platelet Thrombogenicity

Cannulated and sham-operated animals were injected either with homologous ⁵¹Cr platelets 30 min after operation or were injected with ¹⁴C-5HT 18 hr after operation. Twenty hours after operation, both carotid arteries were isolated. Each vessel was injured by applying 2 serafine clamps onto the vessel 1 cm apart and left for 10 min. The clamps were then removed and blood flow was restored. Sixty minutes later, a citrated blood sample was collected from each rabbit by a cardiac puncture, and then the rabbit was heparinized (200 U/kg; Connaught Laboratories, Toronto, Ontario) and killed. Both carotid arteries were removed, slit longitudinally, and washed clean of blood in 20 ml of saline. The vessel wall segments were placed intimal side down into 1 ml of NCS tissue solubilizer (Amersham Corporation, Arlington Heights, Ill.) for 60 min to dissolve the radioactive platelets adherent to the
intimal surface. The nonsolubilized section of vessel was then removed, and the radioactivity remaining in the solubilizer was measured in a liquid scintillation analyzer (Model PW 4510A; Phillips Electronics Inc. Ltd., Toronto, Ontario). The nonsolubilized section of vessel wall was laid flat on transparent acetate film and then photocopied. The area of vessel wall was determined by cutting out the copy and weighing it. This was compared to weights of standard 100 sq mm pieces of paper and expressed as sq mm.

Control pieces of vessel were obtained proximal to the injury site and treated in an identical manner.

Platelet-specific activity of platelets prepared from the citrated blood samples was determined, and the radioactivity of the solubilized portion of vessel wall was expressed as platelet/10 sq mm of vessel wall.

**Blood Volume Loss Studies**

Twenty hours after rabbits were cannulated or sham-operated, each rabbit was injected with $^{51}$Cr-labeled red cells. The rabbits were then anesthetized with sodium pentobarbital and one ear was immersed into a saline bath maintained at 37°C. After 10 min of warming in the bath, the ear was punctured with a BP #11 scalpel blade. The red cell blood loss was determined by measuring the amount of $^{51}$Cr-labeled red cells that escaped into the saline bath over the succeeding 10-min period. This method is described in detail elsewhere in the literature.13

**Measurement of Platelet Thrombogenecity in Normal Recipient Animals of Platelets Obtained From Cannulated and Sham-Operated Animals**

Eighteen hours after operation, cannulated and sham-operated animals were injected with $^{14}$C-5HT. Two hours later, both groups of animals were exsanguinated. The platelets from each group were isolated, washed twice in 10 ml calcium and albumin-free Tyrode's, and resuspended in 24 ml of normal platelet-poor plasma. Three-milliliter aliquots of either of these two $^{14}$C-5HT platelet pools were then injected into two separate groups of normal recipient rabbits. One hour later, both carotid arteries of each recipient animal were isolated and injured in the manner described above. The number of $^{14}$C-5HT platelets adherent to the injured surface 1 hr after injury was then determined.

**In Vitro Platelet Aggregation Studies With Cannulated and Sham-Operated Rabbit Platelets**

Whole blood (22.5 ml) was collected into sodium citrate (0.38% final concentration) from the central ear artery of each unanesthetized rabbit. Platelet-rich plasma was prepared from each sample, and the platelet count adjusted to $4 \times 10^6$ cu mm with autologous platelet-poor plasma. Platelet aggregation studies were performed in a dual-channel aggregation module that was coupled to a recorder, Model PF10HO-D (Payton Associates, Scarborough, Ontario). Platelet aggregation was induced by different concentrations of acid-soluble collagen and adenosine diphosphate (ADP). Six hours later, each rabbit was anesthetized, cannulated or sham-operated, and then allowed to recover. Twenty hours after operation, another 22.5 ml whole blood was collected into citrate and the aggregation studies were repeated using the identical concentrations of the aggregating agents.

Platelet aggregation was expressed as maximum aggregation or the maximum deflection of the light transmission curve from the initial baseline level.

**RESULTS**

Platelet $t_1/2$, determined within the first 48 hr of operation, was 12 hr in cannulated rabbits and 38 hr in sham-operated rabbits, $p < 0.001$ (Table 1). The circulating platelet count before and after $^{51}$Cr-platelet injection was not significantly different between the cannulated and sham-operated animals, although the platelet count increased in both groups after the injection of homologous $^{51}$Cr-platelets (Table 1). Platelet turnover in cannulated animals was significantly greater than in sham-operated animals ($p < 0.001$).

The thrombogenecity of platelets in cannulated and sham-operated animals was compared by measuring the number of autologous $^{14}$C-5HT or homologous $^{51}$Cr-
Table 1. Platelet Half-Life ($t'/2$), Platelet Count, Platelet Recovery, and Platelet Turnover in Cannulated and Sham-Operated Rabbits

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<th>Cannulated Rabbits</th>
<th>Sham-Operated Rabbits</th>
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<tr>
<td>$t'/2$ (hr)</td>
<td>12 ± 6*</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>Platelet count before</td>
<td>208,000 ± 21,000</td>
<td>218,000 ± 20,000</td>
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<tr>
<td>$^{31}$Cr-platelet injection</td>
<td>(NS)</td>
<td></td>
</tr>
<tr>
<td>Mean platelet count after</td>
<td>237.500 ± 12,800</td>
<td>251.560 ± 18,357</td>
</tr>
<tr>
<td>$^{31}$Cr-platelet injection</td>
<td>(NS)</td>
<td></td>
</tr>
<tr>
<td>$^{51}$Cr platelet recovery</td>
<td>68 ± 4</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>(% injected)</td>
<td>(NS)</td>
<td></td>
</tr>
<tr>
<td>Platelet turnover/ml/hr</td>
<td>29,106 ± 1,569*</td>
<td>9,735 ± 710</td>
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Platelet $t'/2$ was calculated by a computer-assisted semilogarithmic regression analysis. Platelet counts were determined both before and 4, 8, 24, and 48 hr after the injection of $^{31}$Cr-platelets. $^{51}$Cr-platelet recovery was determined on the 2-hr sample, and platelet turnover was calculated from the mean peripheral platelet count determined on samples collected after the $^{51}$Cr-platelet injection and corrected for recovery. Data given as mean ± SEM; n = 12.

Table 2. Platelet Adhesion to the Carotid Injury Site (Platelets/10 sq mm Vessel Wall, Mean ± SEM) of Autologous $^{14}$C-5HT-Labeled Platelets and Homologous $^{51}$Cr-Labeled Platelets in Cannulated and Sham-Operated Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Cannulated Rabbits</th>
<th>Sham-Operated Rabbits</th>
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<tr>
<td>$^{14}$C-platelets</td>
<td>414,949 ± 104,845*</td>
<td>940,650 ± 60,544</td>
</tr>
<tr>
<td>(n = 16)</td>
<td></td>
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<tr>
<td>$^{51}$Cr-platelets</td>
<td>87,125 ± 19,197*</td>
<td>239,226 ± 60,545</td>
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<td>(n = 16)</td>
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* $p < 0.001$
Table 3. Comparison of Hemostatic Effectiveness of Platelets Obtained From Cannulated and Sham-Operated Animals 20 hr After Operation: Red Cell Volume Loss

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<th>Cannulated Rabbits</th>
<th>Sham-Operated Rabbits</th>
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<tbody>
<tr>
<td>Red cell volume loss</td>
<td>73 ± 13*</td>
<td>20 ± 4</td>
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</tbody>
</table>

*p < 0.001.

Blood loss experiments were performed to measure the function of unlabeled platelets in both cannulated and sham-operated rabbits. Red blood cell loss was 73 µl over a 10-min period in the cannulated animals and 20 µl in the sham-operated animals (p < 0.001), demonstrating that the circulating platelets in the cannulated animals were hemostatically less effective than those platelets in sham-operated animals (Table 3).

In order to determine whether this effect was intrinsic to the platelet or was associated with changes in plasma components that may alter platelet function, the experiments were repeated using washed platelet suspensions labeled with 14C-5HT in vivo in cannulated and sham-operated rabbits, which were then injected into normal recipient animals with an induced carotid injury.

In normal rabbits injected with 14C-5HT platelets obtained from sham-operated animals, 238,396 platelets/10 sq mm of vessel wall were adherent to the injured carotid surface. In contrast, only 81,618 platelets/10 sq mm of homologous 14C-5HT platelets obtained from cannulated donors were adherent to the injured surface, p < 0.001 (Table 4).

Collagen-induced platelet aggregation in vitro was significantly decreased 18 hr after cannulation (p < 0.01), whereas ADP-induced platelet aggregation was unaffected (Fig. 1). Collagen- and ADP-induced platelet aggregation in vitro was not affected by the sham operation.

DISCUSSION

Young platelets have been shown to be metabolically more active,5–7 more effective in shortening the bleeding time,8,14,16 and more adherent to collagen fibers than old platelets.4 These observations have led to the suggestion that patients with thrombosis and increased platelet turnover may possess a population of highly reactive platelets and so may be at higher risk to further thromboembolic events.

We have shown that platelets exposed to a thrombotic stimulus that is associated with increased platelet turnover are less reactive when tested either in an experimental thrombus or an experimental blood loss model in vivo and less reactive to a collagen stimulus when tested in vitro.

Table 4. 14C-Platelet Adhesion (Platelets sq mm Vessel Wall, mean ± SEM) to the Carotid Injury Site in Normal Recipient Rabbits by Platelets Obtained From Cannulated or Sham-Operated Donor Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Cannulated Rabbits</th>
<th>Sham-Operated Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet adhesion</td>
<td>81.618 ± 11,388*</td>
<td>238.396 ± 33.557</td>
</tr>
</tbody>
</table>

*p < 0.001.
There are a number of possible explanations for these results. First, it is possible that immature platelets released into the circulation as a consequence of the increased platelet turnover have defective platelet function. This is unlikely, since it has been shown by other investigators that young platelets released into the circulation are hemostatically more effective. A second possibility is that insertion of the cannula results in changes in plasma proteins that influence platelet function. This possibility was excluded by demonstrating that washed platelets from cannulated rabbits were also less thrombogenic. A third possibility is that platelets that have been exposed to one stimulus undergo changes that render them refractory to subsequent stimulation. The interaction of platelets with the cannula could influence their function either by altering the platelet membrane or by inducing the platelets to undergo the release reaction. George et al. have suggested that platelets lose glycoprotein fragments from their surface when they interact with various stimuli, and these glycoproteins could be important for maintaining platelet function. Reimers et al. have demonstrated that thrombin degranulated platelets are hemostatically less effective yet survive normally in vivo. Finally, there are a number of clinical studies that indicate that platelets that have been exposed to prosthetic surfaces, such as a pump oxygenator in cardiopulmonary bypass surgery, develop a release reaction defect and are hemostatically less effective. In our experiments, ADP-induced platelet aggregation was normal, whereas collagen-induced platelet aggregation was impaired, suggesting that the platelets acquired a defect in the release reaction.

In conclusion, we have demonstrated that an increase in platelet turnover induced by a mechanical thrombotic stimulus was associated with decreased rather than increased thrombogenicity. It is likely that this impairment in platelet function is caused by interaction with the initial thrombogenic stimulus, which renders them less reactive to subsequent stimuli.
ACKNOWLEDGMENT

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REFERENCES

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