Red Blood Cell Size Is Important for Adherence of Blood Platelets to Artery Subendothelium

By Piet A. M. M. Aarts, Piet A. Bolhuis, Kjell S. Sakariassen, Robert M. Heethaar, and Jan J. Sixma

The hematocrit is one of the main factors influencing platelet adherence to the vessel wall. Raising the hematocrit causes an increase of platelet accumulation of about an order of magnitude. Our studies concern the role of red cell size. We have studied this effect using an annular perfusion chamber, according to Baumgartner, with human umbilical arteries and a steady-flow system. Normal human red blood cells (MCV 95 cu μ) increased platelet adherence sevenfold, as the hematocrit increases from 0 to 0.6. Small erythrocytes from goats (MCV 25 cu μ) caused no increment in adherence in the same hematocrit range. Rabbit erythrocytes (MCV 70 cu μ) caused an intermediate increase in adherence. Red blood cells from newborns (MCV 110–130 cu μ) caused a larger increase in platelet adherence than normal red cells at hematocrit 0.4. These results were further confirmed with large red blood cells from two patients. Experiments with small red cells (MCV 70 cu μ) of patients with iron deficiency showed that platelet adherence was similar to normal red cells, provided the red cell diameter was normal. Small red blood cells of a patient with sideroblastic anaemia caused decreased adherence. These data indicate that red cell size is of major importance for platelet adherence. Red cell diameter is more important than average volume. However, for size differences in the human range, the hematocrit remains the dominant parameter.

THE ADHERENCE of blood platelets to the subendothelium of the vessel wall is an early and critical event in the formation of a hemostatic plug or thrombus.1,2 A widely used method to study platelet adherence to the vessel wall in vitro is the perfusion technique of Baumgartner.3 For unknown reasons, the hematocrit is one of the main factors influencing platelet transport towards the vessel wall in this system.4,5 We report that the volume of red blood cells is one of the main determinants for the enhancement of platelet adherence.

MATERIALS AND METHODS

Blood Collection

Fresh blood was drawn in 1/9 volume of 110 mM trisodium citrate. Blood from healthy donors was obtained from a Red Cross Blood Bank. Human blood, with red cell size deviating from normal, was drawn from patients who were treated in our clinic. Patients with iron-deficient polycythemia and sideroblastic anemia were chosen because of a known lower red cell size. Three patients with polycythemia, treated with phlebotomy, became iron deficient and were studied in three separate runs of perfusions with the blood obtained from the phlebotomy treatment. Only one patient with sideroblastic anaemia could be studied. In this case, two separate runs of perfusions with blood from two phlebotomies were performed. The phlebotomies were performed in this case because of secondary hemachromatosis.

One patient suffering from asthmatic bronchitis with respiratory insufficiency and one patient with alcoholic liver disease were accidentally found by routine examination in our clinical laboratory to have exceptionally large blood cells. In both cases, two separate perfusion runs with blood from two separate blood collections were performed.

Blood from newborns was obtained by draining from the umbilical cord after birth into 110 mM trisodium citrate, yielding 40–60 ml blood for each sample. Data were obtained from 3 perfusion runs using the mixed red blood cells from 2 or 3 newborns for each run.

Goat blood was obtained by venous puncture from three different healthy adult goats. Six perfusion runs, using blood from one goat each run, were performed.

Rabbit blood was obtained by cardiac puncture, yielding about 100 ml blood for each rabbit. For each perfusion run, the blood of 2–3 rabbits was used, at a total of 6 perfusion runs. The whole blood of the patients, newborns, and animals was stored overnight at 4°C, and the red cells were washed prior to perfusion experiments.

Preparation of the Perfusates

Platelet-rich plasma (PRP) was obtained from whole donor blood by centrifugation (10 min. 190 g, 20°C). To 1 volume of PRP, 1 volume of a Kreb's Ringer solution (4 mM KCl, 107 mM NaCl, 20 mM NaHCO3, and 2 mM Na2SO4) containing 19 mM citrate (plasma concentration) and 27 mM glucose was added, which gave a final pH of 6.1. Platelets were pelleted (10 min, 500 g, 20°C) and resuspended in Kreb's Ringer (with citrate and glucose) at pH 6.1 to a concentration of 107 platelets/ml. Labeling with 11C or 11In and aspirin treatment was carried out simultaneously in this suspension.7 To 9 ml platelet suspension, 10 μCi Na35CrO4 (Amersham International Ltd., Amersham, U.K.) or 10 μCi 11In-oxine (Byk, Malinckrodt, Petten, The Netherlands) was added, and, to enhance labeling efficiency, 45 μl oxine (Brit Drug House, Pool, U.K.) (6.5 mg/ml dissolved in 90% ethanol) was added.8 This platelet suspension, containing 10 μM aspirin, was incubated for 15 min at 37°C. Free radiolabel and aspirin were removed by 2 washes (10 min, 500 g, 20°C) with Kreb's Ringer buffer, pH 6.1.7 Platelets exhibited the same adherence behavior with both labeling procedures. The aspirin treatment was performed to prevent thrombus formation. It does not interfere with platelet adherence. The platelets were resuspended in the remaining platelet-poor plasma to a concentration of 190,000 platelets/μl plasma.

The red blood cells were washed once on a cellulose column according to Beutler8 and two times by centrifugation (3,000 g, 10
the steady-flow system of Sakariassen et al. For human arteries we obtained with renal arteries. Perfusion experiments were performed with red cells of a healthy human donor was used. Plasma from AB-positive blood group as the patient was used. Plasma from AB-positive blood was used with red cells of newborns because their blood group was unknown.

Perfusion Experiments

Perfusion experiments with the perfusion chamber according to Baumgartner were performed by recirculation of 15 ml perfusate in the steady-flow system of Sakariassen et al. For human arteries we used umbilical arteries rather than the post-mortem renal arteries that were used in previous studies. The advantage of the use of these vessels is the increased reproducibility, because of the less variable thickness of the vessel wall. Arteries were isolated from the human umbilical cord, obtained immediately after birth, stored in buffer (0.2 M Tris, pH 7.4) and used within 24 hr after 1-hr treatment with 0.1 mM aspirin. Aspirin treatment was done to inhibit prostacyclin production. Everting the vessel segment and short exposure to air was sufficient to remove all endothelial cells. Detailed studies comparing the effect of shear rates and von Willebrand factor concentration showed no difference with the data obtained with renal arteries. Perfusion experiments were performed at a calculated vessel wall shear rate of 800 sec⁻¹ for 3 or 5 min at 37°C. Perusates were precircubated for 5 min at 37°C prior to perfusion.

Determination of Platelet Adherence

Platelet adherence was determined by ⁵¹Cr or ¹¹¹In counting of the artery segments in a gamma-counter (Trigamma 600, Baird Atomic, Inc., Bedford, MA). As a control, morphometric evaluation performed by light microscopy was used. Fixation, embedding, and sectioning of the vessel segments were performed according to Baumgartner. Staining was according to Huber.

Determination of Red Blood Cell Size

Mean cellular volume (MCV) was determined on a Coulter counter (Model-S, Coulter Electronics Ltd, Harpender, England). Average cell diameter was determined with a phase-contrast microscope (Leitz Wetzlar dialux 20EB, E. Leitz GmbH Wetzlar, F.R.G.) with calibrated eye piece ocular (magnification 1,000 x) based on the measurement of 100 cells.

RESULTS

The dependence of blood platelet adherence on the hematocrit is shown in Fig. 1. The larger red blood cells of men and rabbits enhanced blood platelet adhesion, whereas the red blood cells of goats, which are about the same size as human platelets, did not. Microscopic controls after perfusion showed that red blood cells from goats and rabbits did not adhere to the human subendothelium or agglutinate with the platelets or with each other. Platelet count after perfusion with goat and rabbit red cells was not significantly decreased, as reported for human red cells.

Goat and rabbit red cells exhibited more hemolysis than human red cells. Free hemoglobin level in the plasma after perfusion was 900 µg/ml for goat red cells, 800 µg/ml for rabbit red cells, and 300 µg/ml for human red cells. Unsheared perfusates had free hemoglobin levels of 180 µg/ml, and fresh human citrate plasma contained about 70 µg/ml. However, recombination of the platelet-poor plasma of these hemoglobin-enriched perfusates with fresh human platelets and fresh human red cells at hematocrit 0.4 gave platelet adherence in the normal range for human red cells. Therefore, the decreased platelet adherence with goat and rabbit red cells is not caused by substances released by hemolysis. Goat platelets in human plasma in the presence of human red cells showed only little affinity for human subendothelium (<10⁵ platelets/sq cm). Competition of goat platelets still present in the washed goat red cells with the labeled human platelets is therefore not likely to influence our experiments.

Confirmation of the importance of the red blood cell size for platelet adherence to subendothelium was obtained with perfusion experiments with human red blood cells that were either larger or smaller than normal. As large red blood cells, we used red blood cells from newborns, which had a mean cell volume of 120 cu µ and mean diameter of 11 µ, and red blood cells of 2 patients in whom an increased red cell volume was accidentally found by routine examination—one with alcoholic hepatitis and the second unexplained in a patient with chronic respiratory disease and secondary polycythemia. For experiments with large blood cells, we used perfusion times of 3 min instead of 5 min to avoid saturation effects. Large red blood cells...
enhanced platelet adhesion more strongly than normal cells (Table 1).

Small red blood cells were obtained from patients with controlled polycythemia vera in whom iron deficiency had been induced by repeated phlebotomy. The mean cell volume of the cell was decreased (65 cu μ), but the average cell diameter (8μ) had remained unchanged. Red cells from such patients caused normal enhancement of platelet adhesion. Red blood cells from a patient with sideroblastic anemia, with a MCV of 60 cu μ and an average diameter of 6 μ, were studied on two separate occasions. Platelet adhesion was less enhanced by these red blood cells than by normal cells (Table 1). Perfusates with red cells of patients and newborns did not exhibit visual hemolysis, so free hemoglobin determination after perfusion was not performed.

DISCUSSION

Several observations concerning the motion of red blood cells may be used to explain the enhanced platelet transport. According to Keller, red blood cells rotate in the shear field of the flow and cause local small turbulence, leading to a mixing effect and a faster movement of surrounding molecules and small particles. By this mechanism, red cells can enhance the transportation of platelets. Keller calculated that the mixing effect of rotating rigid spheres would be proportional to the squared radius of the rotating sphere and to the local shear rate. Obviously, this calculation is only an approximation of the effect induced by the disc-shaped red blood cells, especially at the applied hematocrits. However, it indicates that particle radius and shear rate are characteristic parameters, in agreement with the experimental data shown here.

A second explanation is based on the observation by Goldsmith that tracer red blood cells in concentrated ghost suspensions undergo an erratic radial displacement. The radial displacement is caused by continuous collision of passing cells and will induce displacement for platelets and plasma as well. The transport rate will increase proportionally to the shear rate (collision frequency) and the volume of the red blood cell, but not to the diameter of the cell, as we report here.

A third explanation may be sought in the presence of a so-called "skimming layer." In flowing blood, the red cells occupy mainly the center of the vessel, resulting in a skimming layer of platelet-rich plasma at the vessel wall. If the size difference between platelets and red cells is large, as it is for human red cells, platelets might be expelled into the skimming layer region. The increased platelet concentration at the vessel wall would then result in an increased platelet adhesion. The small red cells from goats, almost equal in size with the platelets, are expected to be equally mixed with the platelets. In this case, the local platelet concentration near the vessel wall would not be influenced by the increasing hematocrit. However, when no active red-cell-induced platelet transport mechanism occurs, the platelet concentration in the skimming layer can only increase by a factor of 2.5 (raising the hematocrit from 0 to 0.6), as recently observed in arterioles by Tangelder, and thus, this cannot account for the increase in platelet adhesion by a factor of 6–7.

As observed by Tschopp and Baumgartner, adenosine diphosphate (ADP) released from damaged red cells cannot be responsible for red-cell-dependent platelet adhesion, which is consistent with our observation that replacement of red cells by ghosts results in unchanged platelet adhesion. This does not exclude the possibility that ADP released from red blood cells under more turbulent conditions may play a role in hemostasis.

Preliminary results of our recent work indicate an influence of red cell deformability on platelet adherence. Artificial manipulation of red cell deformability causes significant changes in platelet adherence. Differences of red cell deformability, as measured by viscometry, is present in the studied red cell types. However, differences in deformability between the red cells

<table>
<thead>
<tr>
<th>Red Blood Cells</th>
<th>MCV (cu μ)</th>
<th>Average Cell Diameter (μ)</th>
<th>Perfusion Time</th>
<th>Platelet Adherence (10⁹/sq cm) (mean ± SD)</th>
<th>Significance of Difference With Normal Red Cells** (Paired t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron-deficient polycythemia</td>
<td>65</td>
<td>8</td>
<td>5 min</td>
<td>5.2 ± 1.4 (9)</td>
<td>4.8 ± 1.2 (11)  NS</td>
</tr>
<tr>
<td>Sideroblastic anemia</td>
<td>60</td>
<td>6</td>
<td>5 min</td>
<td>3.2 ± 0.8 (11)</td>
<td>4.8 ± 1.2 (11)  p &lt; 0.01</td>
</tr>
<tr>
<td>Newborns</td>
<td>120</td>
<td>11</td>
<td>3 min</td>
<td>6.8 ± 2.0 (21)</td>
<td>3.8 ± 1.3 (21)  p &lt; 0.001</td>
</tr>
<tr>
<td>Alcoholic liver disease</td>
<td>120</td>
<td>10</td>
<td>3 min</td>
<td>7.7 ± 1.3 (7)</td>
<td>5.2 ± 1.0 (8)  p &lt; 0.01</td>
</tr>
<tr>
<td>Asthmatic bronchitis</td>
<td>110</td>
<td>9</td>
<td>3 min</td>
<td>3.6 ± 0.8 (9)</td>
<td>2.6 ± 0.6 (11)  p &lt; 0.01</td>
</tr>
</tbody>
</table>

*Number of perfusions in parentheses. 
**Simultaneously performed.
cells of patients, newborns, goats, rabbits, and the normal controls studied in one run of perfusions were not systematic and far too small to account for the observed differences in platelet adherence presented in this article.

From this study we conclude that platelet adherence induced by red cells is caused by flow-controlled red cell motion, in which the size is a critical parameter.

Although the results can be best explained by the mixing concept of Keller, it is obvious that fully free rotation of red cells will not occur at the applied hematocrits. It is not unlikely, however, that some degree of red cell rotation, according to Keller’s concept, will be present at higher red cell concentrations, and this may be sufficient to enhance platelet adherence.

REFERENCES

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