CONCISE REPORT

Human Platelet Surface Binding of Endogenous Secreted Factor VIII-von Willebrand Factor and Platelet Factor 4

By James N. George and Adam A. Onofre

Washed human platelets in buffers containing either 2 mM Ca<sup>2+</sup> or 4 mM EDTA were stimulated by human α-thrombin to induce secretion. The binding of two endogenous secreted proteins, factor-VIII-related protein (VIII-R) (von Willebrand factor) and platelet factor 4, was measured by reacting thrombin-treated and control platelets with specific antibodies to these proteins, then quantifying antibody binding with <sup>125</sup>I-staphylococcal protein A.

Since platelet activities involving endogenous granule-secreted proteins, such as VIII-R, coagulation factor V<sup>2</sup> and fibrinogen, occur at the cell surface, plasma membrane binding and concentration of these proteins could increase the efficiency of the hemostatic process. Previous studies have demonstrated the binding of exogenous fibrinogen<sup>3,5</sup> and VIII-R<sup>6</sup> to the surface of activated platelets and the appearance of endogenous fibronectin<sup>7</sup> and factor VIII<sup>8</sup> on the platelet surface following activation. Recently, we demonstrated that the major platelet granule glycoprotein, GP-G, becomes exposed on the platelet surface following thrombin-induced secretion.<sup>9,10</sup> This has been further defined to occur by calcium-mediated membrane binding.<sup>11</sup> We have extended our studies of platelet surface binding of endogenous secreted proteins to two other well-characterized proteins: factor VIII-related protein (von Willebrand factor) and platelet factor 4 (PF4), the heparin-neutralizing protein. Both of these granule proteins were associated with the platelet membrane surface by a calcium-dependent mechanism after thrombin-induced secretion. This ability to bind endogenous secreted proteins to the plasma membrane surface may provide a mechanism by which the platelet can concentrate and organize its secreted proteins for subsequent physiologic reactions.

Preparation of <sup>125</sup>I-SPA and <sup>125</sup>I-BSA

Staphylococcal protein A (SPA) (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), 100 µg, was dissolved in 50 µl Tyrode's buffer, pH 7.4, and labeled with 2 mCi Na<sup>125</sup>I (New England Nuclear Co., Boston, Mass.) using "Enzymobeads" (Bio-Rad Laboratories, Richmond, Calif.) containing lactoperoxidase and glucose oxidase. After incubation the beads were removed by centrifugation and the <sup>125</sup>I-SPA (specific activity approximately 15 µCi/µg) was isolated by G-25 sephadex chromatography. Radioiodinated bovine serum albumin (BSA) was prepared by the same method, and the control experiment with <sup>125</sup>I-BSA was performed as described below for antibody binding (approximate specific activity of <sup>125</sup>I-BSA: 5 µCi/µg).

Platelet Secretion and Antibody Binding

Blood samples were obtained from normal volunteers, with informed consent, who had not ingested aspirin or other compounds that may affect platelet secretion for at least 7 days. Blood was drawn through a 19-gauge needle into a "miniset" vein infusion set (Travenol Laboratories, Inc., Deerfield, Ill.) into a plastic syringe containing one-sixth volume of anticoagulant, after discarding the first 10 drops, and placed in ice to minimize platelet secretion. The anticoagulant solution was ACD containing PGE<sub>1</sub><sup>9</sup>, Platelet-rich plasma (PRP) was prepared by centrifugation at 150 g for 20 min at 4°C; platelets were isolated by centrifugation at 1000 g for 20 min at 4°C, and the pellet was resuspended in RCD-PGE<sub>1</sub>-0.35% BSA, pH 6.5.<sup>10</sup> The platelets were labeled with <sup>51</sup>Cr (25 µCi for platelets isolated from 50 ml blood) for 20 min at room temperature (Na<sub>2</sub>CrO<sub>4</sub>, Amersham Corp., Arlington Heights, Ill.). Then platelets were washed 3 times at 4°C in RCD-PGE<sub>1</sub>-BSA (1000 g for 10 min at 4°C) and resuspended to 2 x 10<sup>5</sup>/ml in either Tyrode's-BSA—2 mM CaCl<sub>2</sub> or Tyrode's-BSA—4 mM EDTA, pH 7.4. At this step the <sup>51</sup>Cr cpm/platelet was determined with a dual-channel scintillation counter (model 1191, Tracer Analytic, Inc., Atlanta, Ga.) and an automatic particle counter ("Thrombocounter-C," Coulter Electronics, Inc., Hialeah, Fla.), and this value was subse-
comparing all pairs of means using the BMDP computer program statistical package.

Statistical comparisons were performed by a repeated measures analysis of variance followed by Duncan's multiple range test for the values for the antibody-incubated samples in each experiment.

Following this incubation, platelets were washed 4 times in RCD, resuspended in 0.5 ml aliquots. Using release of $^{37}$Cr to quantify platelet lysis, we have previously reported that lysis was equal with thrombin-treated and control platelets under these conditions. No $^{37}$Cr was lost from platelets after formaldehyde fixation. Antibody (20 µg) or nonimmune IgG (100 µg) were added and the samples were incubated for 20 min at $37^\circ$C. Then platelets were washed 3 times in RCD and resuspended in 0.5 ml aliquots. Using release of $^{37}$Cr to quantify platelet lysis, we have previously reported that lysis was equal with thrombin-treated and control platelets under these conditions. No $^{37}$Cr was lost from platelets after formaldehyde fixation. Antibody (20 µg) or nonimmune IgG (100 µg) were added and the samples were incubated for 20 min at $37^\circ$C. Then platelets were washed 3 times in RCD, resuspended to 0.5 ml, and incubated with 0.2 µg of $^{125}$I-SPA for 60 min at $37^\circ$C. Following this incubation, platelets were washed 4 times in RCD and the $^{37}$Cr activity (to determine platelet number) and $^{125}$I activity measured. The data were expressed as $^{125}$I cpm/10$^4$ platelets. The value for $^{125}$I-SPA cpm/10$^4$ platelets for a simultaneous control sample (no IgG) with EDTA and no thrombin was subtracted from the values for the antibody-incubated samples in each experiment. Statistical comparisons were performed by a repeated measures analysis of variance followed by Duncan's multiple range test for comparing all pairs of means using the BMDP computer program statistical package.

Measurement of Platelet Secretion of $^{14}$C-5HT

For these experiments, platelets were prepared as described above except that $^{37}$Cr was omitted and platelets were labeled instead of $^{14}$C-5HT. Platelet-secreted serotonin was determined from the supernatant fluid after thrombin incubation and formaldehyde fixation.

RESULTS

Thrombin-induced platelet secretion, quantified by $^{14}$C-5HT release, was equal in either the presence or absence of calcium: 51% with 2 mM Ca$^{2+}$, 49% with 4 mM EDTA (average values of four experiments). To determine if protein in the incubation medium became nonspecifically associated with the platelet surface related to thrombin treatment or the presence of calcium, a control study was performed by adding 0.4 µg of $^{125}$I-BSA to the platelet suspensions simultaneously with thrombin (or with buffer in control samples) and measuring platelet-associated $^{125}$I-BSA after formaldehyde fixation and three subsequent washes. There was no difference in binding of $^{125}$I-BSA to the platelets related to thrombin or Ca$^{2+}$ (cpm/10$^4$ platelets, average of two experiments): thrombin-Ca$^{2+}$, 0.69; control-Ca$^{2+}$, 0.64; thrombin-EDTA, 0.64; control-EDTA, 0.68). The average percent of added $^{125}$I-BSA remaining with the platelets in all of these samples was 0.57%. The association of $^{125}$I-BSA with platelets was the same in the absence of formaldehyde treatment.

Binding of secreted proteins to the platelet membrane surface was determined by incubating the platelets with a specific antibody, then measuring

---

**Table 1: Platelet Surface Binding of Endogenous Factor VIII-R (von Willebrand Factor) and Platelet Factor 4 Following Thrombin-Stimulated Secretion**

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>Buffer Containing</th>
<th>$^{125}$I-BSA cpm/10$^4$ Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Thrombin-Treated)</td>
</tr>
<tr>
<td>A. Control samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No IgG (3)</td>
<td>Ca$^{2+}$</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>11</td>
</tr>
<tr>
<td>Nonimmune IgG (3)</td>
<td>Ca$^{2+}$</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>15</td>
</tr>
<tr>
<td>B. Platelet-secreted proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII-R (von Willebrand factor) (5)</td>
<td>Ca$^{2+}$</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>47</td>
</tr>
<tr>
<td>Platelet factor 4 (5)</td>
<td>Ca$^{2+}$</td>
<td>219</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>122</td>
</tr>
<tr>
<td>C. Nonplatelet protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII-coagulant protein (3)</td>
<td>Ca$^{2+}$</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>12</td>
</tr>
</tbody>
</table>

Washed platelets were stimulated with 1 U/ml thrombin in the presence of either 2 mM Ca$^{2+}$ or 4 mM EDTA (thrombin omitted in control samples). Subsequent antibody binding was measured with $^{125}$I-BSA and the data are the mean values for $^{125}$I cpm/10$^4$ platelets for the number of experiments shown in parentheses. For all experiments, the average number of platelets per sample was 3.3 x 10$^4$. Platelets bound more antibody to PF4 ($p < 0.01$) and VIII-R ($p < 0.05$) in the presence of thrombin and Ca$^{2+}$ than with any of the other three conditions. For the other groups, there was no difference among the four incubation conditions.
bound antibody by subsequent incubation with 125l-SPA. Control studies (Table 1) demonstrated no difference in the binding of 125l-SPA to platelets that had been previously incubated with nonimmune IgG or with no IgG, and also no difference among these samples related to the presence of thrombin or calcium. This “background” binding was approximately 1.0% of added 125l-SPA cpm. Significantly more PF4 and VIII-R were bound to the platelet surface after thrombin-induced secretion in the presence of Ca++ than after secretion in an EDTA buffer or with control (no thrombin) platelets (Table 1). In every experiment, more antibody bound to the platelets in the presence of thrombin and calcium than with any of the other three conditions. Since VIII-R and PF4 were also identified on the surface of control platelets, it was apparent that exogenous thrombin was not required for the exposure of these proteins. This exposure of VIII-R and PF4 may be related to platelet secretion stimulated by the trauma of the centrifugation, which was minimized but not prevented by the inclusion of PGE, in the platelet washing buffers. This could be mediated by endogenous secreted calcium, since EDTA was not included in the washing buffers. An antibody to a nonplatelet protein, VIII-C, was not bound to the platelet surface with any of the incubation conditions.

DISCUSSION

Factor VIII-R (von Willebrand factor) is synthesized by endothelial cells and megakaryocytes and circulates in plasma in a complex with a genetically and immunologically distinct protein, factor VIII-coagulant protein (VIII-C).

While VIII-C has no known association with platelets, VIII-R is the critical mediator of platelet adhesion to subendothelium. Our experiments demonstrated that platelets bind secreted VIII-R to their membrane surface by a calcium-mediated reaction, but no VIII-C was associated with the platelet surface. Nachman and Jaffe have previously demonstrated an association of VIII-R with the plasma membrane of fresh platelets unstimulated by thrombin. Their data may be comparable to our control experiments, since in both instances platelets were subjected to multiple washes in the absence of EDTA and some platelet secretion probably occurred.23 The surface receptor for VIII-R may be membrane glycoprotein Ib.26 The localization and concentration of secreted VIII-R on specific membrane receptors could facilitate platelet–endothelium interaction and would be more efficient than simple release of all VIII-R into the surrounding plasma.

PF4 is a platelet-specific protein that is secreted from α-granules. Although its heparin-neutralizing activity has been recognized for 33 yr and its structure is well defined, the physiologic function of PF4 is unknown.2 If it acts as a procoagulant substance by neutralizing endogenous heparin and thereby protecting newly generated factor Xa and thrombin, this function would be most effective on the platelet surface where Xa binds and catalyzes thrombin formation.2 Our experiments demonstrated that platelets bind secreted PF4 to their membrane surface by a calcium-mediated reaction. Earlier studies have identified platelet membrane-associated heparin-neutralizing activity.27 For both VIII-R and PF4, the membrane-bound protein may be only a small fraction of the total secreted protein but could be critical for physiologic reactions.

These studies suggest the possibility that surface binding may be a mechanism for cells to localize and concentrate their endogenous secreted proteins. This may be especially effective in blood platelets, since the granules concentrate in the center of the cell prior to release and the secreted proteins must traverse the narrow canalicular system of invaginated plasma membrane prior to emerging on the cell surface.28

ACKNOWLEDGMENT

We thank Dr. George Barnwell for performing the statistical analyses, Drs. Shirley Levine, Leon Hoyer, and Rodger McEver for their advice and review of the manuscript, and Judi Skinner and Williade Ramp for their secretarial assistance.

REFERENCES

10. George JN, Lyons RM, Morgan RK: Membrane alterations caused by platelet aggregation and secretion, in Rotman A, Meyer
The abbreviations used in this article are: ACD, acid citrate dextrose; RCD, Ringer’s citrate dextrose; PGE₁, prostaglandin E₁; PRP, platelet-rich plasma; BSA, bovine serum albumin; IgG, immunoglobulin G; PF4, platelet factor 4; VIII-R, factor VIII-related protein (von Willebrand factor); VIII-C, factor VIII coagulant protein; SPA, staphylococcal protein A.
Human platelet surface binding of endogenous secreted factor VIII-von Willebrand factor and platelet factor 4

JN George and AR Onofre