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Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex

AD Michelson and MR Barnard
Thrombin-Induced Changes in Platelet Membrane Glycoproteins Ib, IX, and IIb-IIIa Complex

By Alan D. Michelson and Marc R. Barnard

Platelet membrane glycoprotein Ib (GPIb) and the GPIb-IX complex have central roles in the interaction of platelets with the plasma coagulation system, damaged vessel walls, and other platelets. We investigated the effects of thrombin on these glycoproteins. Monoclonal antibodies were used to assess platelet surface glycoproteins by flow cytometry, total platelet glycoprotein content by immunosorbent assay, and glycoproteins released from platelets, also by immunosassay. Five new observations were made with regard to thrombin-induced changes in platelet membrane glycoproteins: (a) The marked decrease in platelet surface binding of antibodies directed at GPIb was not confined to antibodies directed at the von Willebrand factor binding site. (b) There was a marked decrease in platelet surface binding of an antibody directed at GPIX, with maintenance of the 1:1 ratio of platelet surface binding of antibodies directed at GPIb and GPIX. (c) Changes in platelet surface binding of antibodies were not restricted to a distinct subpopulation of platelets. (d) There was no associated platelet release of glycocalcin (a proteolytic fragment of GPIb). (e) There was no associated platelet release of the GPIb-IIIa complex. These thrombin-induced changes may be important in modulating the reactivity of platelets with the damaged vessel wall and with each other.

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**Table 1. Monoclonal Antibodies Used in This Study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>6D1</td>
<td>Human platelet membrane GPIb (vWF binding site on glycocalcin)</td>
<td>Dr Barry Collier</td>
</tr>
<tr>
<td>WM23</td>
<td>Human platelet membrane GPIb (macroglycopeptide portion of glycoprotein; not the vWF binding site)</td>
<td>Dr Michael Berndt</td>
</tr>
<tr>
<td>3G6</td>
<td>Human platelet membrane GPIb (glycoprotein; not the vWF binding site)</td>
<td>Dr Burt Adelman</td>
</tr>
<tr>
<td>FMC25</td>
<td>Human platelet membrane GPIIb-IIIa complex (inhibits platelet aggregation induced by ADP, epinephrine, collagen)</td>
<td>Dr Barry Collier</td>
</tr>
<tr>
<td>10E5</td>
<td>Human platelet membrane GPIIb-IIIa complex (little effect on platelet aggregation)</td>
<td>Dr Michael Berndt</td>
</tr>
<tr>
<td>WM18</td>
<td>Human platelet membrane GPIIb-IIIa complex (with an equal volume of 2% formaldehyde in Tyrode’s buffer)</td>
<td>Dr Michael Berndt</td>
</tr>
<tr>
<td>KC4</td>
<td>Human PADGEM protein</td>
<td>Dr Bruce Furie</td>
</tr>
<tr>
<td>OX6</td>
<td>Rat lymphocyte a antigen</td>
<td>Dr Bruce Woda</td>
</tr>
</tbody>
</table>

*For references, see Materials and Methods.

**Thrombin activation of washed platelets.** Blood was drawn by venipuncture from healthy adult volunteers who had not ingested aspirin within the previous ten days. The blood was drawn directly into a syringe containing 1/4 vol of acid-citrate-dextrose (85 mmol/L trisodium citrate, 71 mmol/L citric acid, 111 mmol/L dextrose, pH 4.5) and 50 ng/mL (final concentration) prostaglandin E,

Flow cytometric assessment of platelet surface glycoproteins. Fixed, washed thrombin-activated and control platelets (50,000/μL) were prepared as described earlier and incubated for 20 minutes at 22°C with a saturating concentration of one of five monoclonal antibodies (6D1, WM23, 3G6, FMC25, or 10E5) to assess platelet surface glycoproteins. In parallel experiments, aliquots of these platelet suspensions were incubated with either monoclonal antibody KC4 or monoclonal antibody OX6 to assess platelet activation and background binding, respectively. As described by George et al, the platelet surface GPIIb-IIIa complex was assessed on platelets activated in a Ca++-containing buffer, whereas the platelet surface GPIb-IX complex was assessed on platelets activated in an EDTA-containing buffer. Thus, the platelet samples that had been prepared in the presence of 2 mmol/L CaCl₂ (see earlier) were incubated with either 10E5 or OX6, whereas the platelet samples that had been prepared in the presence of 5 mmol/L EDTA were incubated with either 6D1, WM23, 3G6, FMC25, or OX6. Platelet surface expression of PADGEM protein was assessed by antibody KC4 on platelets prepared in the Ca++-containing buffer. All platelet samples were then washed twice in Tris-buffered saline by centrifugation at 2,000 g for 10 minutes at 22°C and incubated for 20 minutes at 22°C with a saturating concentration of fluorescein isothiocyanate—labeled goat antimouse IgG antibody (Cooper Biomedical, Malvern, PA). The platelets were then washed twice, resuspended in Tris-buffered saline, and analyzed in a FACS 440 (Becton Dickinson, Mountain View, CA). For each sample, the fluorescence signal from 10,000 individual cells was measured. Forward-light scatter profiles indicated that there was neither platelet clumping nor detectable contamination by other cell types in any of the samples. Histograms were gated for platelets by their characteristic forward and orthogonal light scatter, thereby excluding any possible contribution from other cells or debris. To compare results in linear form, data obtained from fluorescence channels in a logarithmic mode were converted to their linear equivalents (relative specific fluorescence) as previously described.17

**ELISA determination of platelet release of glycocalcin and the GPIIb-IIIa complex.** Supernatants obtained from centrifugation of thrombin-activated and control platelets were assessed for their glycocalcin content by an ELISA using monoclonal antibody 6D1 as previously described.3,19 In parallel assays, 6D1 was replaced by either monoclonal antibody 10E5 or monoclonal antibody WM18. As described earlier, the supernatants used for assays with 6D1 were EDTA-containing, whereas the supernatants used for assays with 10E5 and WM18 were Ca++-containing.

**ELISA determination of total platelet content of GPIb and the GPIIb-IIIa complex.** Lysates obtained after Triton X-100 solubilization of thrombin-activated and control platelets were assessed in the ELISA for their ability to inhibit monoclonal antibody 6D1 binding to platelet surface GPIb. This assay measures the total number of GPIb receptors for vWF because 6D1 binds with equal avidity to GPIb and glycocalcin. The assay was validated by demonstrating a comparable total number of GPIb molecules per platelet in experiments in which the monoclonal antibody to GPIb was changed from 6D1 to WM23 or 3G6 and in other experiments in which, rather than Triton X-100 platelet lysates, the aqueous phase of Triton X-114–solubilized platelets was assessed (A.D. Michelson, M.R. Barnard, E. Carroll: unpublished observations). Serial dilutions of the lysates obtained after Triton X-100 solubilization of thrombin-activated and control platelets were also assessed in the ELISA for their ability to inhibit the binding of either monoclonal antibody 10E5 or monoclonal antibody WM18 to the platelet surface. As described earlier, the lysates used for assays with 6D1 were centrifuged at 22°C for four minutes at 8,000 g (conditions that result in only a small degree of sedimentation of actin filament–associated GPIb).
were EDTA-containing, whereas the lysates used for assays with 10E5 and WM18 were Ca**+-containing. Control assays in which platelets were replaced by buffer only and then treated with 1% Triton X-100 resulted in the detection of no GPIb (n = 3) or GPIIb-IIIa complex (n = 3), thereby demonstrating that the dilutions of Triton used did not interfere with the ELISA.

Platelet counts. Platelet counting was performed on a Coulter Model ZBI (Coulter Electronics, Hialeah, FL).

RESULTS

A flow cytometric method was used to demonstrate the effect of thrombin on platelet surface glycoproteins. As shown in Fig 1, thrombin resulted in a marked reduction in the platelet surface binding of each of three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on GPIb (Table 1). In addition, thrombin resulted in a similar reduction in the platelet surface binding of a monoclonal antibody (FMC25) directed against GPIX (Fig 1). In contrast, thrombin resulted in a marked increase in the platelet surface binding of a monoclonal antibody (10E5) directed against the GPIIb-IIIa complex (Fig 1). Parallel assays using monoclonal antibody KC4 (directed against PADGEM protein) demonstrated that the thrombin-induced changes in the platelet surface binding of monoclonal antibodies directed against GPIb, GPIX, and the GPIIb-IIIa complex were associated with platelet activation (Fig 1).

As determined by relative specific fluorescence, in unactivated platelets the ratio of platelet surface binding of 6D1:FMC25:10E5 (monoclonal antibodies directed at GPIb, GPIX, and the GPIIb-IIIa complex, respectively) was 1.0:0.9:2.9 (means of five separate experiments). These ratios are comparable to the ratio of platelet surface GPIb:GPIX:GPIIb-IIIa complex reported by other investigators using radioligand binding methods.1,13,14 In maximally activated platelets (thrombin, 1.0 U/10⁶ platelets), the ratio of platelet surface binding of 6D1:FMC25:10E5 was 1.0:0.9:29.1 (means of five separate experiments). Similarly, despite the thrombin-induced changes, there was maintenance of the approximately 1:1 ratio of the platelet surface binding of 6D1:FMC25 at all concentrations of thrombin tested (Figs 1 and 2).

The flow cytometric method of analyzing platelet surface glycoproteins is able to detect distinct subpopulations of platelets because each platelet is analyzed individually.16,17 However, the thrombin-induced changes in binding of monoclonal antibodies to platelet surface GPIb, GPIX, and the GPIIb-IIIa complex were not restricted to a distinct subpopulation of platelets, irrespective of whether the thrombin concentration resulted in partial or complete activation of
Thrombin Concentration (U/10^8 platelets) vs. Thrombin Concentration (U/10^8 platelets)

**Table 2. Effect of Thrombin (1.0 U/10^8 Platelets) on the Total Platelet GPIb Content and Release of Glycocalcin as Determined by ELISA Using Monoclonal Antibody 6D1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Percentage of Total GPIb Content of Control Platelets (Mean ± SEM, n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total platelet GPIb content</td>
<td>100.0 ± 18.4</td>
</tr>
<tr>
<td>Control platelets</td>
<td></td>
</tr>
<tr>
<td>Thrombin-activated platelets</td>
<td>97.4 ± 18.7</td>
</tr>
<tr>
<td>Glycocalcin released from platelets†</td>
<td></td>
</tr>
<tr>
<td>Control platelets</td>
<td>0.3 ± 0.05</td>
</tr>
<tr>
<td>Thrombin-activated platelets</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

* Determined from 1% Triton X-100 lysates of platelets.
† Determined from supernatants.

For both control and maximally thrombin-activated platelets, the total platelet GPIb content and glycocalcin released from platelets were determined by ELISA using antibody 6D1. As shown in the upper portion of Table 2, the total platelet content of GPIb did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. As shown in the lower portion of Table 2, the glycocalcin released from platelets was, as expected, very low in the control and did not rise appreciably in the thrombin-activated platelets.

For both control and maximally thrombin-activated platelets, the total platelet content and platelet release of the GPIIb-IIIa complex were determined by ELISA using antibody 10E5. As shown in Fig 3, the total platelet content of the GPIIb-IIIa complex did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. Likewise, the platelet release of the GPIIb-IIIa complex was very low in the control and did not rise significantly in the thrombin-activated platelets. In parallel assays using antibody WM18, there was similarly no thrombin-induced change in the total platelet content of the GPIIb-IIIa complex and no release of the GPIIb-IIIa complex from platelets (data not shown).

**DISCUSSION**

In this study, the effects of thrombin on platelet membrane GPIb, GPIX, and the GPIIb-IIIa complex were investigated. Five new observations were made.

First, the thrombin-induced change in the glycocalcin portion of the α chain of platelet surface GPIb was not confined to its vWF binding site. In addition to the previously reported thrombin-induced marked decrease in binding of a monoclonal antibody directed at the vWF binding site on GPIb, we observed a comparable marked decrease in the binding of two other monoclonal antibodies directed at different epitopes on the glycocalcin portion of the α chain of GPIb. The thrombin-induced decrease in binding of monoclonal antibodies directed at GPIb was not due to occupation of the antibody binding site by thrombin bound to its receptor on GPIb^3 because (α) three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on platelets (Fig 2). This is demonstrated in Fig 2 by the fact that, for all antibodies tested, increasing concentrations of thrombin resulted in a gradual shift of a single peak (to the left for the GPIb-IX complex, to the right for the GPIIb-IIIa complex).

For both control and maximally thrombin-activated platelets, the total platelet content and platelet release of the GPIIb-IIIa complex were determined by flow cytometry. The solid lines represent platelets activated with the indicated concentrations of thrombin and then incubated with the indicated primary monoclonal antibody. The dashed lines represent platelets incubated with an irrelevant primary monoclonal antibody (background). All samples were then incubated with a fluoresceinated goat antimouse antibody. For each panel, the x-axis represents log fluorescence, and the y-axis represents cell number.

For both control and maximally thrombin-activated platelets, the total platelet content and platelet release of the GPIIb-IIIa complex were determined by ELISA using antibody 10E5. As shown in Fig 3, the total platelet content of the GPIIb-IIIa complex did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. Likewise, the platelet release of the GPIIb-IIIa complex was very low in the control and did not rise significantly in the thrombin-activated platelets (Fig 3). In parallel assays using antibody WM18, there was similarly no thrombin-induced change in the total platelet content of the GPIIb-IIIa complex and no release of the GPIIb-IIIa complex from platelets (data not shown).
EFFECTS OF THROMBIN ON PLATELET GLYCOPROTEINS

Platelets (closed triangles, T supernatant). Thrombin-activated platelets (closed circles, T lysate). Platelet GPIb produced similar results and is bound to GPIX in a 1:1 ratio (the GPIb-IX complex).23"4 The present study demonstrated that the thrombin-induced marked decrease in platelet surface GPIb was not the result of cleavage of platelet surface GPIb. The present study together with previous studies by other investigators\(^7\) suggests that platelet surface GPIb is still present on the platelet surface after thrombin activation but its accessibility to multiple probes is greatly decreased because of either (a) the marked clustering of GPIb molecules that occurs on the platelet surface after thrombin activation\(^5\); (b) the marked shape change that occurs in platelets during thrombin activation (less likely because of the associated increased accessibility of surface GPIIb-IIIa complex); or (c) a major conformational change in the GPIb-IX complex (also less likely because such a change would not be expected to result in the same decrease in binding of four monoclonal antibodies [6D1, WM23, 3G6, and FMC25] directed at different epitopes on the GPIb-IX complex and three of these antibodies [WM23, 3G6, and FMC25] recognize their epitope on Western blots, which suggests that they are relatively insensitive to conformational changes in the antigen).

Fifth, unlike \(\alpha\) granule constituents such as platelet factor 4 and \(\beta\)-thromboglobulin,\(^6\) the GPIIb-IIIa complex was not released from platelets during thrombin activation. Although the present investigation did not directly exclude the possibility that uncomplexed GPIIb and/or GPIIIa subunits were released from platelets during thrombin activation, the lack of change in the total platelet content of the GPIIb-IIIa complex, as determined by two different complex-specific monoclonal antibodies, did not support this view.

In conclusion, given the central roles of GPIb and the GPIIb-IIIa complex in platelet adhesion and aggregation,\(^1\) the presently described thrombin-induced changes in these glycoproteins are likely to be important in modulating the reactivity of platelets with the damaged blood vessel wall and with other platelets.

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REFERENCES


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Fig 3. Effect of thrombin (1.0 U/10\(^6\) platelets) on the total platelet content and release of the GPIIb-IIIa complex as determined by ELISA using monoclonal antibody 10E5. The total platelet content of the GPIIb-IIIa complex was determined from 1% Triton X-100 lysates of control platelets (open circles, C lysate) and thrombin-activated platelets (closed circles, T lysate). Platelet release of the GPIIb-IIIa complex was determined from supernatants of control platelets (open triangles, C supernatant) and thrombin-activated platelets (closed triangles, T supernatant). Results are means \(\pm\) SEM, \(n = 3\).