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

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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-IIIa 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 immunoassay, and glycoproteins released from platelets, also by immunoassay. 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 glycocalcin; not the vWF binding site)</td>
<td>Dr Michael Berndt</td>
</tr>
<tr>
<td>3G6</td>
<td>Human platelet membrane GPIb (glycocalcin; not the vWF binding site)</td>
<td>Dr Bert Adelman</td>
</tr>
<tr>
<td>FMC25</td>
<td>Human platelet membrane GPIb-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 GPIb-IIIa complex (little effect on platelet aggregation)</td>
<td>Dr Michael Berndt</td>
</tr>
<tr>
<td>WM18</td>
<td>Human platelet membrane GPIb-IIIa complex</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 la 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 ¼ 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 E1 (PGE1, Sigma Chemical Co, St Louis). After resting for one hour at 22°C, the blood was centrifuged at 150 g for 15 minutes at 22°C and the platelet-rich plasma was removed. The platelets were washed twice with centrifugation at 2,000 g for ten minutes at 22°C, resuspended at 50,000 platelets/µL in Tyrode’s buffer, pH 7.3, with 50 mmol/L NaHCO3, 0.4 mmol/L NaHPO4, 0.1% glucose, 0.35% bovine serum albumin, and assayed by flow cytometry for platelet surface glycoproteins (see the next section). The second aliquot of the blood was centrifuged at 150 g for 15 minutes at 22°C and the supernatants were removed for ELISA determination of platelet release of glycocalcin (see later), the lysates were thawed and, to remove any cellular debris, centrifuged at 22°C for four times at 8,000 g (conditions that result in only a small degree of sedimentation of actin filament-associated GPIb).4

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 al17 the platelet surface GPIb-IIIa complex was assessed on platelets activated in a Ca2+-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 Ca2+-containing buffer. All platelet samples were then washed twice in Tris-buffered saline by centrifugation at 2,000 g for ten 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 GPIb-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.5 In parallel assays, 6D1 was replaced by monoclonal antibody WM23 or 3G6 and in other 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 containing 1% Triton X-100 (Sigma), 50ng/mL PGE1, and either 5 mmol/L EDTA or 2 mmol/L CaCl2 was assayed. As described earlier, the supernatants used for assays with 6D1 were EDTA-containing, whereas the supernatants used for assays with 10E5 and WM18 were Ca2+-containing.

ELISA determination of total platelet content of GPIb and the GPIb-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.7 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 containing 1% 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
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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 1.0:0.9:2.0 (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/10⁸ 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
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 GPIb content and glyocalcicin 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/10⁸ platelets. As shown in the lower portion of Table 2, the glyocalcicin 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/10⁸ 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).

**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 glyocalcicin portion of the α chain of platelet surface GPIb was not confined to its vWF binding site. In addition to the previously reported⁴ 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 glyocalcicin 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 GPIb² because (α) three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on...
The effects of thrombin on platelet glycoproteins are likely to be important in modulating the interactions of platelets with the vessel wall. N Engl J Med 311:1084, 1984