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Dimerization of glycoprotein Ibα is not sufficient to induce platelet clearance

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Summary

Background—The mechanism of platelet clearance is not clear. Many antibodies binding the membrane-distal ligand-binding domain (LBD) of glycoprotein (GP)Ibα induce rapid clearance of platelets and acute thrombocytopenia, which requires the bifurcated antibody structure. It was thought that binding of these antibodies induced lateral dimerization or clustering of GPIbα in the plasma membrane, which leads to downstream signaling and platelet clearance. However, many antibodies targeting GPIbβ and GPIX, which are associated with GPIbα in the GPIb-IX complex, do not induce platelet clearance, providing contradicting evidence to the clustering model.

Objectives—To test whether dimerization or clustering of GPIbα is sufficient to transmit the signal that leads to platelet clearance.

Methods—We have recently raised several monoclonal antibodies targeting the mechanosensitive domain (MSD) of GPIbα. Binding of these anti-MSD antibodies was characterized by biochemical methods. Their ability to stimulate platelets and induce platelet clearance in mice was assessed.

Results and conclusion—Infusion of anti-MSD antibodies does not cause thrombocytopenia in mice. These antibodies exhibit no detectable effects on platelet activation and aggregation in vitro. Further biochemical investigation shows that the anti-MSD antibody 3D1 binds two copies of GPIbα on the platelet surface. Therefore, lateral dimerization of GPIbα induced by antibody binding is not sufficient to initiate GPIb-IX signaling and induce platelet clearance. Our results suggest that a factor other than or in addition to clustering of GPIbα is required for inducing platelet clearance.
Introduction

The homeostasis of platelets in the circulation hinges on the balance of platelet production and clearance. A widely adopted model of acute thrombocytopenia through excessive platelet clearance is the infusion of a monoclonal antibody (MAb) targeting the N-terminal ligand-binding domain (LBD) of platelet GPIbα. Within minutes of infusion nearly all the platelets are cleared from the animal [1, 2]. The effect is not dependent on the Fc region of the antibody, as these anti-LBD antibodies can directly induce GPIb-IX signaling and activate platelets, leading to rapid platelet clearance [3, 4]. This is consistent with the reports that the clearing effect of anti-LBD antibodies is largely resistant to intravenous immunoglobulin G (IVIG) treatment [4, 5]. A correlation of IVIG refractoriness and the presence of anti-GPIb-IX antibody is also present in patients with immune thrombocytopenia [6], suggesting that anti-LBD antibodies exert similar effects on human and murine platelets. However, the molecular mechanism by which these anti-LBD antibodies induce GPIb-IX signaling has remained sketchy.

That a F(ab')2 but not Fab fragment can achieve the same clearing effect as the intact anti-LBD MAb indicates that the underlying mechanism requires the bifurcated structure [3, 7]. Such requirement has led to a GPIbα clustering model, in which the anti-LBD MAb induces lateral dimerization or clustering of GPIbα, thereby transmitting a signal into the platelet that subsequently leads to its fast clearance [4, 8]. This clustering model is attractive as it also can account for observations that many anti-LBD MAbs, although sharing no common binding epitopes, appear equally adept at clearing platelets [1, 2, 9, 10], and that its physiological ligand, VWF, is also a multimer [11]. Consistent with this model, it has been reported that binding of VWF caused a fraction of GPIbα to partition into the cholesterol-rich lipid raft domain of the plasma membrane [12, 13], thereby drawing close analogy to the widely accepted signaling mechanism of many immune receptors [14–16].

In platelets GPIbα is in complex with GPIbβ and GPIX [17]. One would expect that a ligand inducing the clustering of GPIbα should concurrently cluster GPIbβ and GPIX, and vice versa. However, unlike anti-LBD MAbs, many anti-GPIbβ and anti-GPIX MAbs do not induce thrombocytopenia in mice [2, 18, 19], which appears to contradict directly the aforementioned clustering model. Is it possible that targeting GPIbα induces different signals from targeting GPIbβ and/or GPIX, or is there something special about targeting the LBD of GPIbα? In addition to the N-terminal LBD, a separate structural domain called mechanosensitive domain (MSD) is present between the macroglycopeptide region and the transmembrane domain of GPIbα [20]. In this paper, we report that an anti-MSD MAb 3D1 does not cause thrombocytopenia in mice although it binds two copies of GPIbα on the platelet surface. Thus, dimerization or clustering of GPIbα is not sufficient to transmit the signal that leads to platelet clearance. A factor other than or in addition to clustering of GPIbα is required for inducing GPIb-IX-mediated signaling and platelet clearance.
Materials and methods

Materials and animals
MAbs 5G6, 3D1, the epitope peptide, and green fluorescent protein-fused lactadherin C2 domain (GFP-LactC2) have been described earlier [21]. Control mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA). W7 (Calbiochem, La Jolla, CA), APC anti-human P-selectin antibody (Biolegend, San Diego, CA), FITC PAC-1 antibody (BD Pharmingen, San Diego, CA), human VWF (Haematologic Technologies, Essex Junction, VT), ionomycin (Sigma-Aldrich, St. Louis, MO), thrombin and ristocetin (Chrono-Log, Havertown, PA) were purchased. Murine whole blood was collected from the hTg mice (transgenic mice expressing only human GPⅠbα) [22] under anesthesia through the retro-orbital plexus, and used to prepare washed platelets as described [23]. All animal procedures have been performed in accordance with institutional guidelines and approval. The informed consent and related protocols for drawing blood from healthy donors were approved by Emory University Institutional Review Boards.

Platelet counts
Fifty micrograms of antibody was injected intravenously into a 6–8-week-old hTg mouse. One hour after injection, a blood sample was collected using heparinized capillary tubes. The platelet number was determined using an automated cell counter [9]. Statistical analysis was performed using the 2-tailed unpaired Student t test, with a P value <0.05 considered statistically significant.

Flow Cytometry
Washed platelets (1.2×10^7/ml) were incubated with noted antibodies, reagents or agonists at RT for 20 min, washed and analyzed by flow cytometry largely as described [21].

Platelet Aggregometry
Agonist-induced platelet aggregation was performed as described [21]. When required, the antibody was added to platelet-rich plasma (PRP) and incubated for 5 min before stimulation with agonists. Human VWF was added to hTg murine platelets to a final concentration of 5 μg/ml before the addition of antibody and agonists.

Results and Discussion
We reported earlier a group of MAbs, represented by 5G6 and 3D1, which recognize residues Lys461-Leu470 in the MSD of human GPⅠbα and do not induce platelet activation [21]. Pre-incubation of platelets with the 10-residue epitope peptide blocked binding of 5G6 or 3D1 [21]. Both MAbs showed similar apparent affinities, in the nanomolar range, for multivalent ovalbumin-conjugated epitope peptide or GPⅠbα in human platelets [21]. Nanomolar affinities for the washed hTg murine platelets that express only human GPⅠbα were also observed for both MAbs (Fig. 1A,B). In contrast, these MAbs exhibit differential binding affinities for the monomeric epitope peptide or GPⅠb-IX complex, with 5G6 binding (~10 nM) much tighter than 3D1 (>1 μM) [21]. Consistently, after the MAb was bound to the platelet, 5G6 binding was slightly reduced by the epitope peptide, whereas 3D1 binding...
was completely inhibited with an apparent IC₅₀ of ~1 μM epitope peptide (Fig. 1C,D). The drastic difference in the binding of 3D1 for monovalent and multivalent ligands indicates that the avidity effect makes a major contribution to the tight binding of 3D1 for platelet GPIbα. In other words, it binds two copies of GPIbα on the hTg platelet.

Like 5G6 reported earlier [21], adding 3D1 to human platelets inhibited constitutive and W7-induced shedding of GPIbα (Fig. 2A). Moreover, 3D1 did not induce aggregation of human PRP or hTg platelets. Nor did it affect ristocetin-induced aggregation of human platelets or hTg platelets mixed with human VWF (Fig. 2B,C). Additional analysis of molecular markers of platelet activation, including αIIbβ3 activation and P-selectin expression, confirmed that 3D1 treatment did not activate platelets (Fig. 2D). To test whether 5G6 or 3D1 induces thrombocytopenia in hTg mice, platelet count was measured immediately before and 1 hour after injection of 5G6, 3D1, mouse IgG or LJ-P3, a mouse anti-human LBD MAb that induces acute thrombocytopenia [9]. Figure 3 shows that, although both 5G6 and 3D1 bound hTg platelets in vivo, neither induced rapid platelet clearance as LJ-P3 did.

In summary, we report for the first time the evidence that an anti-GPIbα MAb binds two copies of GPIbα on the platelet surface but it neither activates platelets nor causes platelet clearance, thereby demonstrating that dimerization or clustering of GPIbα is not sufficient to induce platelet clearance.

The anti-MSD MAbs, like many anti-GPIbβ and anti-GPIX MAbs, do not induce platelet clearance. Unlike LBD, MSD is located near the cell membrane, and it is juxtaposed with GPIbβ and GPIX extracellular domains [20]. Therefore it appears that MAbs binding to the membrane-proximal domains of GPIb-IX have different effects on platelets from many anti-LBD MAbs (Fig. 4). The current model of GPIbα clustering is not adequate to account for this difference. It was reported recently that even some anti-LBD MAbs do not cause platelet activation and clearance [8], with the caveat that divergent binding of these anti-LBD MAbs was assumed but not yet demonstrated. Nonetheless, the existence of anti-MSD MAb 3D1 that binds 2 copies of GPIbα on the platelet but does not cause platelet clearance indicates that dimerization or clustering of GPIbα is not sufficient to induce platelet clearance. Our results suggest that a factor other than or in addition to GPIbα clustering is required for inducing platelet clearance. The molecular nature of this factor, however, awaits further elucidation.

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References


Antibodies (Abs) targeting GPIbα N-terminal domain induce platelet clearance in a dimer-dependent manner.

Study of binding of Abs to GPIbα mechanosensory domain (MSD) and their ability to clear platelets in mice.

An anti-MSD Ab binds 2 copies of GPIbα in platelets but does not induce platelet clearance.

The prevailing clustering model of GPIbα signaling is incorrect or needs revision.
Figure 1. 3D1 bound two copies of GPIIbα on the platelet

(A) Purity of 3D1 as shown in an 8% Bis-Tris SDS gel under non-reducing (n.r.) and reducing (r.) conditions. Molecular weight markers (M) are shown and labeled in kDa on the left. (B) Binding isotherms of 5G6 and 3D1 to washed hTg murine platelets. Platelets were incubated with 5G6 (●) and 3D1 (○) at indicated concentrations for 20 min. Binding of antibodies was detected by flow cytometry using FITC-conjugated goat anti-mouse IgG, and quantitated by the mean fluorescence intensity (MFI). (C, D) Differential inhibition of binding of anti-MSD MAbs by the epitope peptide to hTg murine platelets or human platelets. Washed platelets were treated with 12 nM of 5G6 or 3D1 at RT for 20 min, followed by the addition of the epitope peptide to indicated concentrations and the incubation for additional 20 min. The platelets were then washed once and binding of MAb was measured by flow cytometry and normalized to the MFI value in the absence of peptide treatment as 100%. Data points are shown as mean ± SD (n=3). The inhibition curve of 3D1 was fitted to a one-site competition binding equation.
Figure 2. 3D1 inhibited GPIbα shedding in human platelets and exhibited no detectable effect on platelet aggregation and activation

(A) Overlaid flow cytometry histograms showing the inhibition of constitutive and induced shedding of GPIbα by 3D1. Washed human platelets were treated with 5% DMSO (left panel) or 150 μM W7 dissolved in 5% DMSO (right) in the absence (platelet, green trace) or presence of 12 nM 3D1 (red) or 100 μM GM6001 (GM, black) at RT for 3 h. The platelets were then washed and probed for the surface GPIbα expression level using biotin-labeled WM23 and FITC-conjugated streptavidin as described [21]. Platelets treated only with FITC-conjugated streptavidin (control, gray) were included as a negative control. (B, C) Overlaid platelet aggregation traces showing the lack of effects of 3D1 on (B) human or (C) hTg murine platelets. Left panel, aggregation traces of fresh human PRP or hTg murine platelets after the addition of saline (black trace) or 20 nM 3D1 in saline (red). Right, ristocetin-induced aggregation of human PRP or hTg murine platelets that had been pretreated with either saline (black) or 20 nM 3D1 (red). Human VWF (hVWF) was added to hTg platelets to a final concentration of 5 μg/ml before addition of 3D1 or ristocetin.
Ristocetin to a final concentration of 1.5 mg/ml was added at the time point indicated by arrows to induce aggregation as described before [21]. (D) Overlaid flow cytometry histograms showing that treatment of washed human platelets with 3D1 did not induce platelet activation. Platelet activation is indicated by binding of mAb PAC-1, which is specific for activated integrin αIIbβ3 (left panel), increased P-selectin expression on the platelet surface (middle), and binding of GFP-LactC2, which is specific for exposure of phosphoserine lipids (right). Washed platelets were treated with 20 nM of mouse IgG (control, gray traces) or 3D1 (red) at RT for 10 min and then probed using FITC-conjugated PAC-1, APC-conjugated anti-P-selectin Ab or GFP-LactC2. Platelets treated with 0.5 U/ml thrombin or 1 μM ionomycin were included as positive controls (black traces). The histograms or traces are representative of at least three independent experiments.
Figure 3. Neither 5G6 nor 3D1 induced rapid platelet clearance in hTg mice

(A) Mouse platelet counts at 1 h after infusion of control mouse IgG, 5G6, 3D1 or LJ-P3, expressed as a percentage of those prior to infusion. Results are mean ± SEM (n=4). n.s. indicates not significant. (B) Mouse whole blood was drawn from the retro-orbital plexus at 1hr after infusion of control mouse IgG, 5G6 or 3D1 and then washed platelets were prepared. Binding of antibodies was detected by flow cytometry using FITC-conjugated goat anti-mouse IgG, and quantitated by the MFI. Gray peak: Control mouse IgG. Thin line: 5G6. Thick line: 3D1. Each plot is representative of three independent experiments.
Figure 4. A cartoon of GPIb-IX complex depicting the differential effects of anti-GPIb-IX monoclonal antibodies in inducing platelet clearance

Many anti-LBD MAbs, such as AN51 [8], AP-1 [1], LJ-P3 [9], 6B4 [7], p0p3 [2] and NIT-A [4], induce rapid clearance of platelets in mice while sharing no common epitopes. On the contrary, anti-LBD MAb VM16d did not induce platelet clearance [8]. Moreover, anti-GPIbβ MAbs, RAM.1 F(ab′)2 fragment [18] and Xia.C3 (Emfret), anti-GPIX MAb Xia.B4 [19], anti-MSD MAbs 5G6 and 3D1 (this report), all of which target membrane-proximal domains in GPIb-IX, do not induce rapid clearance of platelets.