Anti-GPVI (HY101) activates platelets in a multi-color flow cytometry panel.

A short method communication.

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Abstract

The platelet transmembrane receptor GPVI can be assessed together with other platelet membrane markers in a whole blood multi-color flow cytometry panel. The advantage of combining multiple antibodies in a single tube is the possibility of distinguishing multiple platelet subgroups. In this short communication we describe an activation problem encountered with anti-GPVI, clone HY101. Activation of platelets was seen after the addition of anti-GPVI in a flow cytometry panel, highlighted by the expression of the activation markers CD62P, PAC-1, CD63 and CD107a. This was also confirmed by platelet aggregation studies.

Keywords: anti-GPVI, HY101, platelet, flow cytometry

Introduction

Glycoprotein (GP)-VI is transmembrane protein, a member of the immunoglobin superfamily and one of the important platelet collagen receptors [1]. The expression of GPVI is restricted to platelets and megakaryocytes. GPVI is associated with dimeric Fc-receptor γ chain [2], which contains an immunoreceptor tyrosine-based activation motif (ITAM). Upon ligand binding, GPVI dimerization leads to phosphorylation of the tyrosine residues and further signal transduction ensues [3]. Finally, phospholipase Cγ2 becomes activated and calcium (Ca^{2+}) level rises. Next to collagen, several other endogenous GPVI ligands exist, for example fibrin and adiponectin [4,5]. The synthetic triple-helical collagen-related peptide (CRP), an exogenous ligand, consists of the glycine–proline–hydroxyproline (GPO)n tandem repeats that mediate binding of collagen to GPVI [6]. GPVI contains a cleavage site which enables shedding of the extracellular domain via the desintegrin and metalloproteinase 10 (ADAM10) upon activation [7].

GPVI on the platelet membrane can be assessed by flow cytometry. The availability of commercial anti-GPVI is still scarce. In the present work we would like

to highlight an activation problem with anti-GPVI, clone HY101, directed against an epitope in the extracellular domain of GPVI. This antibodycoupled to a fluorochome, was used in a multi-color platelet flow cytometry protocol assessing both resting and activated state.

Methods

Flow cytometry

Peripheral blood was collected in a citrate tube (Monovette plastic 3.0 mL, 3.2% citrate, 106 mM (Sarstedt, Numbrecht, Germany); 10:1 blood:citrate ratio) after drawing a discard tube. Pre-analytical processing was performed according to Södergren et al [8]. In brief, blood samples were kept for 1 hour at room temperature before adding a mix of fluorochrome coupled Abs and HEPES buffer. Brilliant Stain Buffer (BD) was used in one experiment. For platelet activation experiments, agonists, Ca^{2+} and GPRP were added to the mix. Samples were incubated for 10 minutes at room temperature. Incubation was stopped by adding 1 mL of HEPES-Ca^{2+} buffer and analysed within 1 hour. The following platelet surface markers were included: CD41a phycoerythrin (PE, clone: HIP8, BD), CD42b BV711 (clone: HIP1, BD), CD42a APC-Vio® 770 (clone: REA209, Miltenyi Biotec, Bergisch Gladbach, Germany), PAC-1 fluorescein isothiocyanate (FITC, binds active conformation of αIIbβ3, BD), CD62P BV786 (anti-P-selectin, α-granule release, clone: AK-4, BD), CD107a PE-CF594 (anti-lysosomal-associated membrane protein 1 (LAMP-1), clone: H4A3, BD), CD63 BV510 (anti-LAMP-3, clone: H5C6, BD). This mix was tested both with and without anti-GPVI BV421 (clone: HY101, BD). In one analysis we also used anti-GPVI PE (HY101; BD) instead of anti-GPVI BV421. A BV421 Mouse IgG1, k isotype control was also included in the experiment (BD).

Anti-GPVI BV421 (0,2 mg/mL; 2 µL or 0,4 µg in a final volume of 60 µL; +-1.000.000 platelets) was tested at different dilutions: 1/10, 1/20, 1/30.

An experiment in which paraformaldehyde (1% final concentration) was used to fix platelets before adding anti-GPVI BV421 was also executed.

For platelet activation α-thrombin (T6884, Sigma-Aldrich, St. Louis, Missouri, US) and synthetic cross-linked collagen-related peptide (CRP-XL; CambCol Laboratories, Littleport, Cambridgeshire, UK) were used at final concentrations of 5 U/mL and 5 µg/mL respectively. Fibrin polymerisation was blocked with GPRP (G5779, Sigma-Aldrich, 2 mM final concentration). Analysis was done on the FACS ARIA II (Becton Dickinson (BD), Franklin Lakes, NJ, US). Compensation was executed with BD CompBeads (ref: 552843) and Miltenyi MACS CompBeads anti-REA (130-104-693). Acquisition was stopped after obtaining 10,000 platelets in a temporary gate. Platelets were selected on a forward/side scatter plot and, after removing doublets, the gate was set on the CD41 positive events.

Platelet aggregation

Platelet aggregation experiment was performed using light transmission aggregometry (Chrono-log 700, STAGO). Blood was drawn on a CPDA-tube (1/10; citrate, phosphate, dextrose, adenine) and centrifuged at 330 x g for 20min. Eptifibatide (4µg/mL) and apyrase (1 U/mL) were added to platelet rich plasma(PRP) and centrifuged 800 x g for 10min. Platelet pellets were washed once in modified Tyrode's buffer (135 mM NaCl, 12 mM $NaHCO_3$, 3 mM KCl, 0.3 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM D-glucose, 10 mM Hepes, 0.35 % BSA, pH 7.4, 37 °C) containing eptifibatide (4 µg/mL) and apyrase (1 U/mL) at 1000 x g for 10 min. Platelets were re-suspended at a density of 2.5 x 10^5/µl in modified Tyrode's buffer.

Light transmission of washed human platelets (52.2 x 10^6), stirred at 1200rpm, 37°C, was recorded after addition of 2mM $CaCl_2$ and GPVI Ab. A BV421 Mouse IgG1 isotype control was also tested.

Results

Flow cytometry

Results are depicted in Figure 1.

In the flow cytometry panel without anti-GPVI BV421 no expression of platelet activation markers was seen in resting state. When anti-GPVI was added in resting state, activation was demonstrated with every platelet activation marker, reflecting α-granule release, lysosomal release and αIIbβ3 activated form. Compared to the positive control (addition of thrombin and CRP-XL), platelet activation marker expression was less pronounced in the resting panel with anti-GPVI.

Activation diminished with lower concentrations of anti-GPVI (Supplementary Figure 1), although CD62P was still activated at 53,1% for a 1/30 dilution. Mean fluorescence intensity at different dilutions and in comparison with the isotype control is shown in Supplementary Figure 2.

Replacing anti-GPVI BV421 with anti-GPVI PE also resulted in platelet activation (Supplementary Figure 3). Adding the Brilliant Stain Buffer did not have an impact on results (Supplementary Figure 4).

Fixation with paraformaldehyde 1% before addition of anti-GPVI abolished activation of platelets demonstrated by negative activation markers (Supplementary Figure 5).

Platelet aggregation

Results are depicted in Figure 2.

Platelet aggregation was performed using a high (6.67µg/mL) and a low (0.2µg/mL) anti-GPVI Ab concentration. In agreement with the flow cytometry experiment, only high concentration of anti-GPVI Ab induced platelet aggregation.

Discussion and conclusions

A whole blood multi-color platelet flow cytometry panel is a valuable tool to map platelet phenotype in a rather easy and rapid way. The advantage of combining multiple Abs in a single tube is the possibility of distinguishing multiple platelet subgroups. Platelets being easily activated, it is paramount to thoroughly validate the technique used beforehand to avoid any (pre-) analytical problems [9].

In this short communication we describe an activation problem encountered with anti-GPVI, clone HY101, in a whole blood platelet flow cytometry panel. This activation issue was confirmed with a platelet aggregation study. Neither replacing anti-GPVI BV421 (HY101) with anti-GPVI PE (HY101), nor the addition of Brilliant Stain Buffer, recommended when using >1 brilliant dye, had an impact on flow cytometry results. Fixating the platelets before addition of anti-GPVI, abolished the activation problem, but complicates the staining protocol, especially for agonist activation assays.

The anti-GPVI monoclonal antibody, recognizing the extracellular domain of GPVI, was generated for the first time by Chen et al [10]. The authors state that binding of HY101 on platelets does not interfere with collagen or convulxin signalling, suggesting a binding region distinct from the ligand binding site. However, the article did not mention experiments studying the ability of HY101 to aggregate nor activate platelets.

Trifiro et al [11] state that "HY101 is a neutral monoclonal Ab that neither activates GPVI nor inhibits ligand binding to GPVI." It is however not clear if this was tested by the authors. In a paper looking at aggregation and shedding properties of different anti-GPVI Abs, HY101 was unfortunately not included [12]. In this article, the majority of Abs identified against the extracellular domain of GPVI (n = 8) were able to induce aggregation, except for the 11A7 clone. Fab fragments from the same Abs, except for 11A7, did not induce aggregation but were able to inhibit collagen- and CRP -induced aggregation. Another study, describing a GPVI deficient patient, used anti-GPVI HY101 to evaluate membrane GPVI expression. Other platelet membrane markers including the activation marker CD62P were analysed. Again, it is not clear if anti-GPVI was added to the cocktail of Abs. If not, platelet activation in resting state or inhibition of activation in collagen-stimulated conditions by anti-GPVI HY101 would not have been detected in the control samples. The majority of papers mentioned above did not test anti-GPVI HY101 induced aggregation nor activation, possibly explaining why the activating property of HY101 has not been described yet.

The mechanism of platelet activation by the monoclonal anti-GPVI Ab is not clear. Given that HY101 clone is described as neutral and activates platelets in a dose-dependent manner, it is very likely that platelets are activated by clustering FcγRIIa receptor. Why some monoclonal Abs cluster FcγRIIa receptor while others do not is not known [13]. This cluster effect diminishes with lower concentrations of Ab. By assessing aggregation in the presence of IV.3 (FcγRIIa blocker) the article by Al-Tamimi et al concluded a direct agonist role independent of the FcγRIIa receptor for the GPVI antibodies studied [12].

Recently, Biocytex (Stago, Asnières, France) developed a quantification assay using anti-GPVI 1G5 clone. It is believed that this assay should be used to establish international standards for GPVI expression at the platelet surface [14]. However the same

clone (1G5) has been described to induce aggregation and GPVI shedding [12]. In our opinion, this is not a suitable clone for multi-color platelet flow cytometry panels using platelet agonists. A non-activating GPVI antibody like anti-GPVI clone 11A7 could be a good alternative for utilization in multi-color platelet agonist experiments. To our knowledge, this clone is not commercially available.

Also of interest, Jung et al [15] described GPVI dimer-specific antibodies, m-Fab-F and 204-11 Fab. They imply that GPVI dimer is the collagen-binding form of GPVI. These Fab fragments inhibit/block however collagen binding.

Another alternative for avoiding anti-GPVI induced activation, is the "fix-first method" described by Spurgeon et al [16] where the antibody cocktail is added after formaldehyde fixation. The disadvantage of this technique is a possible decrease in staining intensity in for example anti-CD62P. PAC-1 staining may be abolished after formaldehyde fixation.

Declaration of interests

'The authors report no conflicts of interest'.

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