Thrombosis Research 130 (2012) 92–98

Contents lists available at SciVerse ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers

Jeroen F. van Velzen [a,*], Britta A.P. Laros-van Gorkom [c], Gheorghe A.M. Pop [d], Waander L. van Heerde [b]

[a] Department of Immunology, University Medical Centre Utrecht, Utrecht, The Netherlands
[b] Department of Clinical Medicine Laboratory for Hematology, Radboud University Nijmegen Medical Centre, The Netherlands
[c] Department of Haematology, Radboud University Nijmegen Medical Centre, The Netherlands
[d] Department of Cardiology, Heart centre, Radboud University Nijmegen Medical Centre, The Netherlands

ARTICLE INFO

Article history:
Received 30 November 2011
Received in revised form 1 February 2012
Accepted 21 February 2012
Available online 16 March 2012

Keywords:
Multicolor flow cytometry
Platelets
Gating strategy
Thrombocytopathy
Thrombocytopenia

ABSTRACT

Introduction: Flow cytometry allows the analysis of multiple antigens in a single tube at a single cell level. We present a rapid and sensitive two tube flow cytometric protocol for the detection of multiple platelet antigens and activation markers gated on a pure platelet population.
Materials and methods: The presence of platelet specific antigens was analyzed in citrated whole blood of normal platelets and from patients diagnosed with platelet abnormalities. Quiescent platelets as well as stimulated platelets were analyzed using a gating strategy based on ubiquitously expressed platelet membrane markers.
A ubiquitously expressed platelet marker was combined with antibodies against the activated alpha2b-beta3 (PAC-1), Lysosomal Activated Membrane Protein (CD63) and P-selectin (CD62P).
Results: We were able to detect the platelet antigens CD36, CD41, CD42a, CD42b and CD61 in one single tube. Our approach allowed the single tube determination of PAC-1, CD63 and CD62P after activation of platelets by thrombin, collagen, ADP and PAR-1, and determination of platelet abnormalities.
Conclusions: Our two tube multi-parameter screening protocol is suited for the analysis of platelet antigens expressed on quiescent and activated platelets and allows the detection of aberrancies as found in blood of patients with thrombocytopathy such as Glanzmann Thrombasthenia, storage pool disease with diminished granule content and patients treated with clopidogrel and acetylsalicylic acid.

Introduction

Platelets play an important role in the regulation of hemostasis. Platelets will adhere to the damaged vessel wall, and form aggregates. Moreover, after adhesion and activation, platelets propagate coagulation due to expression of negatively charged phospholipids at the outer leaflet of their plasma membrane. Improper function of platelets due to a diminished platelet number or an anomaly in one of the receptors or (intracellular) organelles might result in a bleeding disorder.

Flow cytometry allows the simultaneous detection of surface antigens in a sensitive and specific manner. Till now only two or three antibody combinations have been used in several studies [1–8]. The most important antigens are the VWF binding receptor glycoprotein (GP) complex Ibα/β-IX-V (CD42a-d), the collagen binding receptor GPIIIb (CD36) and the platelet aggregation receptor alpha2b-beta3 (CD41/CD61). CD41 and CD61 are both or separately aberrant in Glanzmann Thrombasthenia (GT), an autosomal inherited platelet disorder [9,10]. GPIX (CD42a) or GPIbα (CD42b) can be aberrant in the bleeding disorders Bernard-Soulier syndrome [11], May Hegglin anomaly and Sebastian syndrome [12]. CD36 interacts with CD9 and is involved in platelet adhesion, aggregation and also in pathogen recognition (for example in malaria) [13].

Platelet responsiveness to different agonists can be studied with antibodies against activation markers. Receptor activation will ultimately result in secretion of internal granules [14] and cytoskeletal rearrangements which mediates signal transduction [15]. The most dominant platelet activation markers are P-selectin (CD62P), activated alpha2b-beta3 complex (PAC-1) and lysosomal associated membrane protein (CD63). CD62P is located in the α-granule membrane. The receptor domains of PAC-1 are both present at the plasma membrane and membranes of the α-granules [16]. CD63 is located in the membrane of dense granules and is translocated to the plasma membrane after activation [17].

Abbreviations: GP, Glycoprotein; GT, Glanzmann Thrombasthenia; α-SPD, alpha storage pool disease; δ-SPD, Delta storage pool disease; ADP, Adenosine diphosphate; PBS, Phosphate Buffered Saline; PAR-1, protease-activated receptor-1; GPRP, peptide Gly-Pro-Arg-Pro; PFA, paraformaldehyde; FITC, Fluorescein IsoThioCyanate; PE, Phyco-Erythrin; ECD, PhycoErythrin-Texas Red; PE-Cy5, PhycoErythrin-cyanine5; PE-cyanine7, PhycoErythrin-Cy7; PAR-1, Protease Activated Receptor-1.

* Corresponding author at: Department of Immunology, University Medical Centre Utrecht , Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Tel.: +31 88 7557608.
E-mail address: j.vanvelzen@umcutrecht.nl (J.F. van Velzen).

0049-3848/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.thromres.2012.02.041

Aberrant expression of CD62P has been reported in patients with α-storage pool disease (α-SPD) [5,18]. Aberrant expression of PAC-1 and CD63 have been reported in Gray Platelet syndrome (α-SPD) [5,19,20], Hermansky-Pudlak syndrome [21] and Wiskott-Aldrich syndrome (δ-SPD) [5,22]. PAC-1 expression can also be decreased by drug treatment with clopidogrel, tirofiban and abciximab [23-28]. Clopidogrel blocks the ADP (Adenosine diphosphate) receptor P2Y12, resulting in a diminished expression of PAC-1 after activation with ADP. Abciximab, a Fab fragment of an immunoglobulin directed to activated alpha2b-beta3, and tirofiban, a synthetic non-peptide inhibitor, competes with PAC-1 as they all are directed to the activated alpha2b-beta3 complex.

We used 1. Different gating strategies as the selection of a pure platelet population is a prerequisite to screen for platelet abnormalities or small subpopulations [5].

2. Standardisation for platelet preparation with and without and activation using PFA (final concentration 1%) [2,29] 3. used time limits between blood drawing and measurement as PFA could effect platelet expression in time [30,31]. 4. Two single tube, five antibody protocols for evaluation of platelet membrane GP's and activation markers.

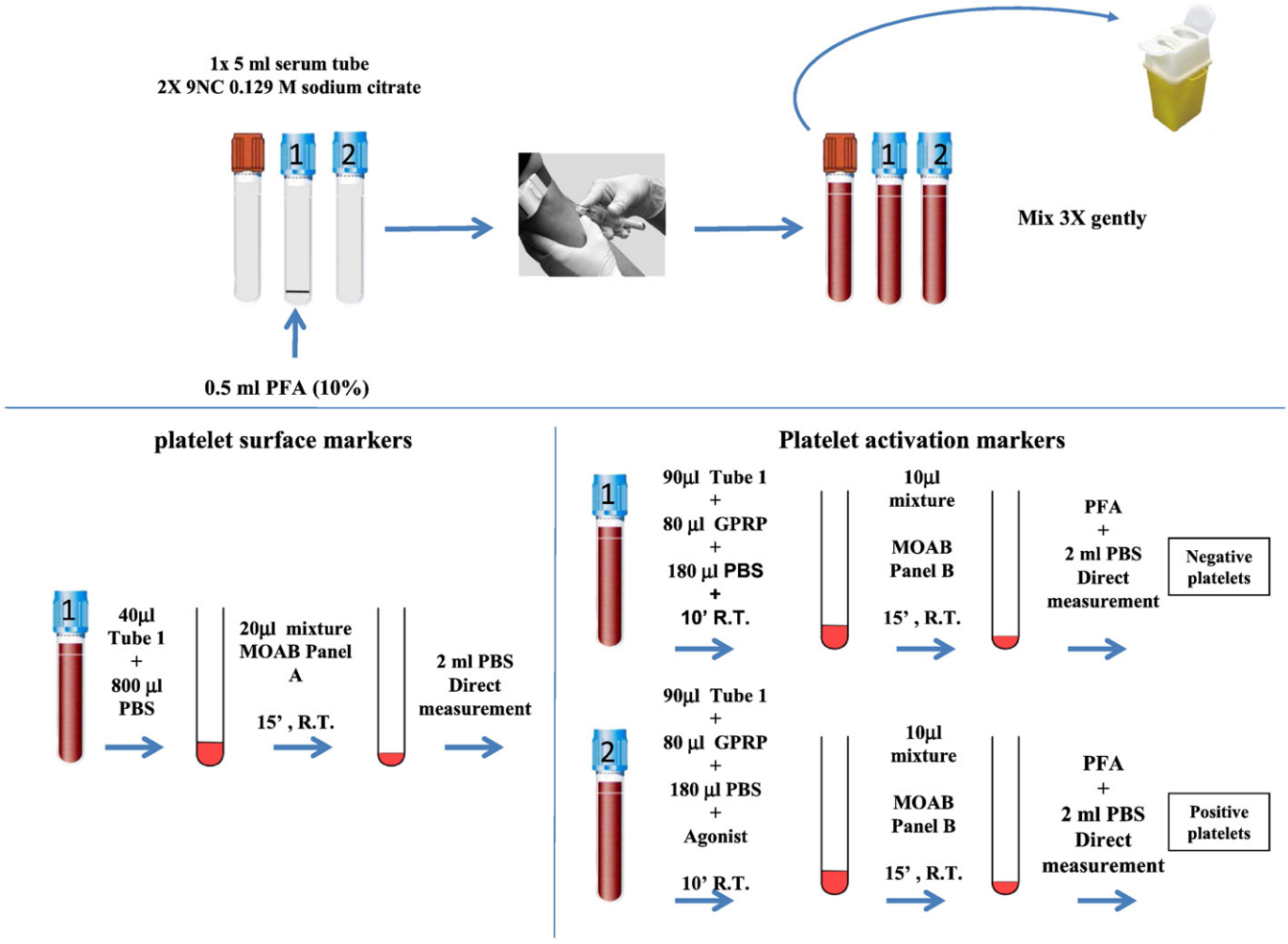
Materials and methods

Monoclonal antibodies and reagents

CD36 (GPIV) AlloPhycoCyanin (CB38 clone), CD62P PhycoErythrin-Cy5 (AK-4 clone) and PAC-1 Fluorescein IsoThioCyanate (BALB/c clone) were obtained from Becton Dickinson Pharmingen (San Jose, CA). CD41 PhycoErythrin-Texas Red (P2 clone), CD42a Fluorescein IsoThioCyanate (SZ1 clone), CD42b PhycoErythrin (SZ2 clone), CD61 PhycoErythrin-Cy7 (SZ21 clone) CD63 PhycoErythrin (CLBGRan/12 clone), CD42b PhycoErythrin-Cy7 (SZ2 clone) were purchased from Beckman Coulter Immunotech (Marseille France). The peptide Gly-Pro-Arg-Pro (GPRP), a synthetic tetra-peptide (Biotrend chemicals AG Zurich Germany) was used to inhibit platelet aggregation and fibrin polymerization. 10% paraformaldehyde (PFA) (E.Merck Darmstadt, Germany) was used to fix the platelets. Phosphate Buffered Saline (PBS) (Braun Melsungen AG Melsungen, Germany) was used to dilute the samples. To activate platelets, thrombin (Organon Teknika Durham, United States), PAR-1 (Peptides International Louisville, Kentucky), ADP and collagen type I (Kordia life science Leiden, Holland) were used.

Panel of patient samples

Blood of two patients with a different variant of Glanzmann Thrombasthenia were used to validate the detection protocol for ubiquitously expressed membrane markers and to validate the responsiveness to different agonists. Both had impaired aggregation in response to ADP and collagen and impaired PAC-1 expression using a dual color flow cytometry approach. One patient with an α-SPD had a decreased concentration of platelet factor 4, 0.27 μg in 10^8 platelets (normal values 0.7-1.5 in 10^8 platelets) and beta-thromboglobulin 11.1 IU in 10^4 platelets (normal value 18.6-47.10 IU in 10^4 platelets). Furthermore gray platelets were observed morphologically, combined



Scheme 1. Sample preparation scheme.