Brief Communication

New horizons in platelets flow cytometry

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Abstract

Platelet flow cytometry is an emerging tool in diagnostic and therapeutic hematology. It is eminently suited to study the expression of platelet surface receptors both qualitatively as well as quantitatively. It can serve as a useful marker for the documentation of in vivo platelet activation, and thus, fore-warn the risk of thromboembolism in patients with diabetes mellitus, coronary syndromes, peripheral vascular diseases, and pre-eclampsia.

This technique can also be extended to study and compare the effect of various antiplatelet drugs on the level of activation of platelets and to establish any dose-effect relationship of these drugs. Topographical localization of platelet granules and study of platelet-platelet and platelet-leukocyte interaction is also possible by this procedure. All these parameters serve as pointers towards the presence of activated platelets in the circulation with its thromboembolic consequences.

This is a simple reliable and cost effective technique which has a wide application in the diagnosis of various inherited and acquired platelet disorders. Study of platelet cluster of differentiation (CD) markers in various inherited disorders i.e. Bernard Soulier's disease, von Willebrand disease, Glanzman's disease, and Grey platelet syndrome may help categories the molecular lesions in these oft under-studied disorders.

Keywords: *flow cytometry, platelets, CD63, CD62*

Introduction

Platelets flow cytometry is an emerging technology in clinical and research hematology (1,2). It assesses the expression of surface receptors, components of granules, bound ligands, and interaction of platelets with other platelets, neutrophils and monocytes (3–5). With the help of monoclonal fluorescent antibodies, it has become now possible to determine the absolute number of antibody labelled cells by flow cytometer (2). This technique is more useful than other methods due to small amount of blood sample and minimum manipulation of the sample during processing (6).

Applications of flow cytometry

Diagnosis of inherited thrombocytopathies i.e. Bernard-Soulier's syndrome, Glanzmann's thrombasthenia, and storage pool disease has become easy through flow cytometry (7). Pathological and chronological evaluation of acute coronary syndromes, acute cerebro-vascular ischemia, peripheral vascular disease, diabetes mellitus, and pre-eclampsia can be serially studied with flow cytometry by detecting activated platelets, platelet derived microparticles, and platelet-leukocyte aggregates in the circulating blood. It can also be used for monitoring platelet functions before and during angioplasty and cardiopulmonary bypass (2,7). Flow cytometric identification of circulating platelet-monocyte aggregates can also serve as a sensitive marker for in vivo platelet activation (8). In the field of therapeutics this technique can be used to evaluate the efficacy of various antiplatelet drugs (9).

Flow cytometry also enables to diagnose heparin induced thrombocytopenia (4). This is the method of choice for the detection of aggregation response to agonists in patients treated with antiplatelet drugs. This is more sensitive than platelet rich plasma aggregation quantified by aggregometry (2,9).

Platelets flow cytometry is an easy, reliable and sensitive technique for:

- evaluation of platelet functional status by studying the; expression of neoepitopes e.g. CD63, CD62p, platelet–platelet aggregates, platelet-derived microparticles, platelet–leukocyte aggregates. Conditions associated with increased expression of these markers are acute coronary syndromes, acute cerebro-vascular ischemia, peripheral vascular disease, diabetes mellitus, and pre-eclampsia.

- diagnosis of inherited platelet disorders; Bernard–Soulier's syndrome, Glanzmann's thrombasthenia, Storage pool diseases.
- diagnosis of heparin-induced thrombocytopenia.
- monitoring of antiplatelet therapy; ADP receptor blockers, GPIIb-IIIa antagonists, cyclooxygenase inhibitors.
- determination of rate of platelet turnover; Reticulated platelet count.
- immuno-platelet counting by immunological technique.
- applications in blood banking and transfusion medicine; quality control of platelet concentrates, identification of leuko–contamination in platelet concentrates, immunophenotyping of human platelet antigen-1a (HPA-1a), detection of maternal, and fetal anti-HPA-1a antibodies, platelet cross match.
- identification of platelet-associated antibodies; neonatal alloimmune thrombocytopenia, dengue hemorrhagic fever (10,11).

Adapted from Michelson et al. (12)

Basis of laboratory diagnosis of many of these disorders is the characteristics alterations in the CD markers. Some of these changes are described below;

αIIbβ3 is normally present on the surface of the resting platelets, after platelet activation conformational changes occur in the αIIbβ3. PAC-1 is a specific monoclonal antibody that binds to confirmationally changed αIIbβ3 after activation (13). PAC-1-negative platelets are considered as resting platelets while its positivity is an indication of platelet activation.

CD62p is found in the α-granules of platelets. Presence of CD62p on the surface of the platelets indicates platelets in activated state while their absence implies resting state of the platelets (14). CD63 is a 53 kDa lysosomal membrane protein detected on the surface of activated platelets after release reaction (15,16).

Number of young or reticulated platelets depends upon the rate of thrombopoiesis as well as their removal from the circulation. Thiazole orange (TO) is a fluorescent dye that binds intracellular ribonucleic acid (RNA) of the platelets. Flow cytometric analysis of thiazole orange positive platelets is a measure of the rate of thrombopoiesis (17).

Indo-1 and Fluo-3 are fluorescent probes used for the measurement of intracellular calcium after platelet activation (6). Changes in platelet membrane actin and myosin can also be measured by flow cytometry (18).

Technical considerations

Principle of flow cytometry

Platelets are labeled with fluorescent monoclonal antibodies. Cell suspension is passed through the flow chamber equipped with a focused laser beam that activates the fluorophore. Fluorescence is measured for the identification of platelets. Intensity of the emitted light is directly proportional to the number of antibodies attached to the platelet receptors/antigens (12).

Selection of anticoagulant for platelets flow cytometry

Suitable and appropriate use of anticoagulant is mandatory for the processing of samples for flow cytometry. Sodium citrate is the anticoagulant of choice for platelet flow cytometric analysis (19,20). Ethylenediaminetetraacetic acid (EDTA) and heparin affect the glycoprotein structure and cause artifactual platelets activation. EDTA also causes swelling of the platelets and produces changes in αIIbβ3 complex (21). EDTA anticoagulated samples also show significantly lower number of antiCD41a (clone 96.2C1) positive platelets than samples collected in citrate tubes (20).

Fixation of platelets

The use, type and concentration of the fixative for platelet fixation are controversial. Processing of the unfixed sample immediately after collection, results in the lowest in vitro activation (22). However if it is not possible to process the sample immediately platelets are fixed to prevent in vitro platelet activation. Immediate fixation is essential for the study of time dependent changes in platelet activation.

Expression of glycoprotein lb-IX-V complex on the surface of platelet starts to decline within 30 seconds after activation, and reaches the nadir in 5 minutes. It returns to its pre-activation level after 45 minutes of activation (23). Cleavage and internalization of GPIb-IX-V complex is the basis of this appearing-disappearing cycle after activation (24). Expression of αIIbβ3 complex on platelets surface membrane also declines with the passage of time (23).

Formaldehyde and paraformaldehyde in

different concentrations have been used as platelet fixative by several researchers. Platelet studies must be done within 2 hours without fixation. After fixation with 0.5–1.0% formaldehyde or paraformaldehyde platelet samples can be analyzed after 2 hours (25).

Cahill et al. (26) reported that fixation of platelets with formaldehyde before labelling with monoclonal antibodies increases the expression of CD62p and CD63 on the surface of platelets. Formaldehyde should therefore not be used as a fixative for platelet activation studies (26).

Hu et al. (25) on the other hand observed no change in the expression of CD62p during resting state of platelets when fixed with 0.2% or 0.5% formaldehyde. Contrary to the findings of Cahill et al. (26) Hu et al. (25) remarked that 1.0% formaldehyde or paraformaldehyde slightly decreased CD62p positivity of the resting platelets. Stimulation with ADP after fixation with formaldehyde causes slight increase in the positivity of CD62p markers (26). This discrepancy can be resolved by fixing the platelets after labelling. It was observed that when fixation was done after labelling the platelets with the antibody, fixative did not change the positivity of CD41, CD62p, and PAC-1 (22).

As with formaldehyde use of paraformaldehyde as a platelet fixative also gives conflicting results. Atar et al. (27) stated that platelet flow cytometric analysis of paraformaldehyde fixed inactivated platelets can be done within 5 days. Platelets fixation with 0.5% paraformaldehyde before antibody labelling shows marked increase in CD62P positivity. CD41 and PAC-1 activity is also increased when compared with unfixed platelets, while CD42b activity is reduced after fixation (22). Fixation of platelets with paraformaldehyde after labelling results in less than 0.2% increase in activation (28). Since red cell lysis releases adenosine diphosphate (ADP) which is a platelet agonist, it is recommended that lysis of red cells and excessive centrifugation should be avoided during platelet studies (20).

Effect of incubation

Platelet markers CD 41 and CD 61 are not affected by incubation or stimulation with ADP. When incubated for 20 minutes, 2 hours, and 3 hours, no statistical difference in CD41 and CD61 was found (29).

Activation of platelets with ADP results in an increase in the number of CD63 and CD62p positive platelets. Persistent increase in the number of CD63 positive platelets reflects the stability of this molecule after activation with ADP (29,30).

In vivo increase in CD63 is also reported by several authors in various clinical conditions characterized by persistent platelet activation. Peripheral artery disease, myocardial infarction, congestive heart failure, acute cerebral infarction, atherosclerotic ischemic stroke, and non-embolic ischemic stroke are some of the common clinical conditions that are frequently associated with increased CD63 activity in vivo (31–36).

Flow cytometry can also provide an avenue to assess the effect of varying doses of antiplatelet drugs on suppressing platelet activation. In patients with persistent thrombotic complications, despite continued administration of antiplatelet drugs may be benefitted if an effective dose is administered which may be determined by titrating the dose and offering a customized dose as prophylactic antithrombotic therapy.

In a study by Saboor et al. (29) it was found that incubation of platelet rich plasma with ADP resulted in an initial increase in CD62p positivity; this rise was however transient. When activated platelets were incubated for 2 and 3 hours, the mean fluorescence percent positivity of CD62p progressively and significantly decreased. Reduction in CD62p positivity was greater after 3 hours than after 2 hours of incubation. Although CD62p positivity also decreased during incubation, its values remained higher after 3 hours of incubation than the base line values. Buggle et al. (30) also found reduction in the CD62p positive platelets at different intervals in stroke patients. Matzdorff et al. (37) also reported decreased number of CD62p positive platelets in their in vitro experiments.

Advantages of flow cytometry

Some of the advantages of flow cytometry over other laboratory procedures that make flow cytometry an ideal method of platelet analysis are:

- Platelets are analyzed in their physiological state.
- Insignificant in vitro platelet activation due to minimum manipulation of the sample.
- Very small amount of blood required.
- Simultaneous determination of resting as well as activated state of the platelets.

- Detection of neoepitopes expression on the surface of platelets.
- Highly sensitive and specific procedure for the detection of resting and activated platelets and platelet derived microparticles.
- Accurate analysis of platelets of patients with severe thrombocytopenia.
- Fluorescent technique eliminates the risk of exposure to radioactive material.

Conclusion

It is concluded that platelet flow cytometry can be utilized for the diagnosis of various platelets disorders, evaluation of platelets functional status, monitoring of antiplatelet therapy and research purposes. Immediate sample processing without fixation, appropriate anticoagulant are the key players of achieving good results of platelets flow cytometry.

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Conflict of interest

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Authors' Contributions

Conception and design: MS
Critical revision of the article for the important intellectual content and final approval of the article: MM
Drafting of the article: SI

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