CHAPTER 2B

Flow-cytometric analysis of platelet function

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Introduction

There are many ways to evaluate platelet activity (1). All these methods are directed at detecting soluble metabolites derived from activated platelets in blood, plasma, serum or urine, or at detecting (minute) changes in blood platelets themselves *during* activation in vitro or *after* activation in vivo or in vitro. Upon activation, the shape, granularity and outer surface of a blood platelet change in a variety of ways. Some of these changes can be detected by flow cytometry with the help of fluorescent-dye-conjugated antibodies (2). In essence, fluorescence cytometry is thus able to detect the ultrastructural *anatomical* consequences of *functional* alterations of blood platelets. In fact, it is an *in*direct platelet function assay. For matter of convenience and in line with other authors (1,2), we will use the terms platelet function and platelet activity in the context of blood platelet fluorescence cytometry throughout this thesis.

Flow cytometry

Flow cytometry allows for the rapid analysis of thousands of individual particles (cells or cell fragments) as they pass through a beam of a laser light (3;4). The transmitted, reflected or refracted light is then measured by light-sensitive detectors, each detecting a specific wave length. In this way, up to eight different characteristics of a passing particle can be measured simultaneously, depending on the wavelength and direction of the light bundle (figure 1).

Platelets can be specifically detected in citrate-anticoagulated whole blood by their forward and sideward scatter pattern. The forward scatter intensity (laser light *transmitted through* a blood platelet) is proportional to the size of a platelet, and the sideward scatter intensity (laser light *dispersed* by a blood platelet) is dependent on shape and granularity of the blood platelet (figure 2). In addition, platelets in whole blood can be labelled by specific fluorescent-dye-conjugated (monoclonal) antibodies. These antibodies can be directed at epitopes specific for blood platelets, or even *activated* blood platelets. In this way several epitopes can be detected simultaneously in one platelet.

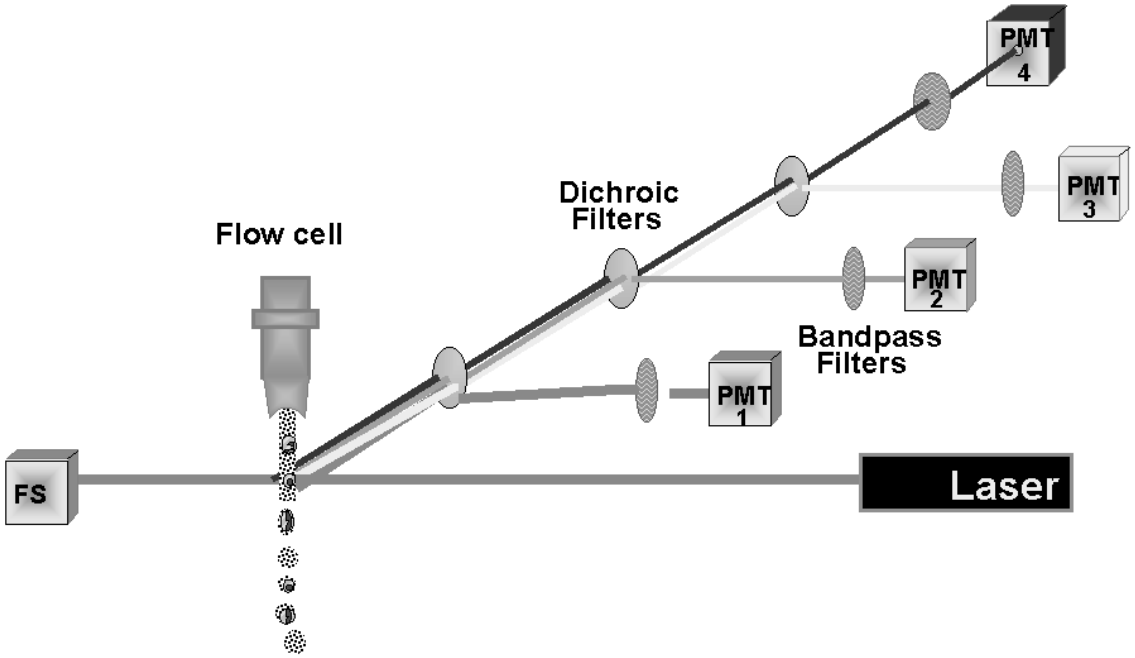

Figure 1

Schematic drawing illustrating the principles of flow cytometry. Light emitted by a laser passes through a cell or blood platelet suspended in a tiny buffer stream and is detected by a light sensitive transducer (FS). Scattered light (with the same wave length) is detected by another transducer. The intensity of this light is much lower, necessitating a photomultiplier transducer (PMT1). Light caputured by a fluorescent label on the cell membrane is emitted with another wavelength and detected by wavelength specific photomultiplier transducers (PMT2, PMT3, PMT4).

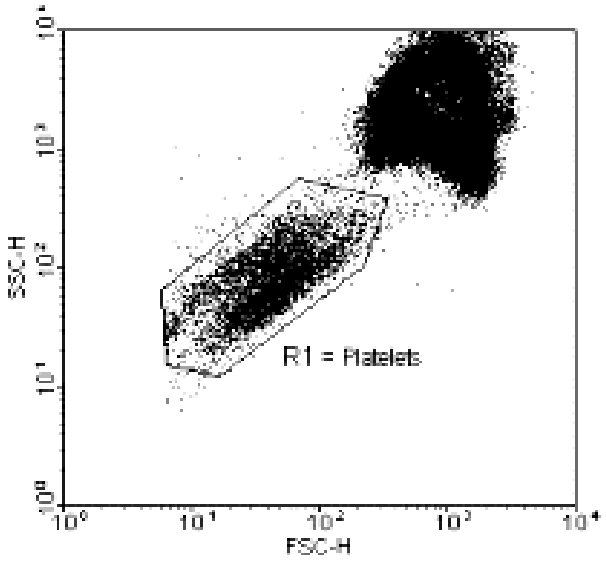

Figure 2

Graph showing the pattern of forward scatter on the x-axis (FSC-H) and sideward scatter on the y-axis (SSC-H) of whole blood. Every dot in the cloud in the encircled region (R1) represents one blood platelet. The cloud in the right upper quadrant represents white blood cells and erythrocytes.

Platelet activation

Initial platelet adhesion to exposed subendothelial matrix is mediated primarily by collagen and Von Willebrand Factor (VWF). The platelet membrane receptors for these subendothelial molecules (collagen: glycoprotein (GP) Ia/IIa and GP VI; VWF: GP Ib/IX/V) act in concert: binding of VWF to platelets is enhanced by their binding to collagen. Platelet adhesion initiates the reactions of shape change, secretion and activation of GP IIb/IIIa (the fibrinogen receptor) ligand binding sites. These reactions result in the formation of platelet-platelet aggregation. In conclusion, platelet aggregate formation requires the synergistic interaction of both fibrinogen and VWF/collagen and their platelet membrane receptors (5).

Alternatively, activation of platelets (as appears from shape change, degranulation, secretion and conformational changes in the fibrinogen receptor) can also be achieved by a number of (circulating) soluble agonists, such as serotonin (6), thromboxane A_2 (7), ADP (8), adrenalin (9), vasopressin (10) and thrombin (11). All of these have their own receptor(s) and intracellular signalling pathways. Apart from these stimulatory substances, there are also a number of circulating platelet inhibitory molecules, e.g. prostacyclin, prostaglandin and nitric oxide. Therefore, and in view of the fact that platelet activation is *not* an all-or-nothing phenomenon, it is possible that

activated platelets circulate. This may contribute to platelet ageing, or, more precise, platelet heterogeneity (12).

There is increasing evidence that *circulating* (i.e. not adhering) platelets play a role in the capture, rolling and adhesion process of monocytes (13). In this role, platelets bridge the distance between monocytes and endothelial cells. The same holds true for platelet-derived microparticles. The main ligand/receptor combination in this bridging process is P-selectin glycoprotein ligand (PSGL)-1/P-selectin. P-selectin is a membrane-bound protein of blood platelets. Degranulation of alpha granules in blood platelets leads to increased expression of this membrane receptor (14). Hence activated blood platelets could – more than resting platelets – be an intermediate in one of the primary stages of atherothrombosis, i.e. the adhesion and migration of monocytes.

It is clear that the above-mentioned, not mutually exclusive mechanisms of platelet activation lead to a variety of changes of the organellar and molecular structure of blood platelets: activation and rearrangement of the cytoskeleton leads, among others by redistribution of two types of intracytoplasmatic membrane systems (the open canalicular system and the dense tubular system), to a rapid shape change with the formation of pseudopodia and the redistribution of several transmembrane receptors; degranulation leads to an altered intracytoplasmic granule pattern, to the expression of granule membrane-bound proteins to the outer surface and to the release of their contents; the lipid distribution of the membrane changes (more negatively charged phospholipids migrate to the outer leaflet of the lipid bilayer), and microparticles are shed (15). Many of these changes can be detected by fluorescent flow cytometry.

Flow cytometry measured function of platelets in whole blood

The way in which platelet activation in whole blood can be evaluated by fluorescence cytometry is by measuring the surface expression of activation-dependent epitopes, either by evaluating the percentage of circulating platelets labelled ‘positive’, or by measuring the fluorescence intensity of the whole circulating blood platelet population. Thus, several manifestations of platelet activation in whole blood can evaluated by labelling platelets with monoclonal antibodies directed against different (activation-dependent) epitopes. More specifically, a labelling panel consisting of antibodies directed against CD42b, CD62P and CD63 and PAC-1 (see below) evaluates, respectively, altered expression of the VWF receptor, alpha granule degranulation, lysosome degranulation and a conformational change of the fibrinogen receptor.

CD41, also known as platelet glycoprotein IIb or integrin $alpha_{IIb}$, is part of the platelet glycoprotein IIb/IIIa (gpIIb/IIIa or integrin $alpha_{IIb}beta_3$) complex that serves as a fibrinogen receptor. In whole blood, its expression is specific to blood platelets. CD41 positivity can therefore be used to discriminate blood platelets from other cellular constituents.

CD42b, also known as GPIba, GPIbalpha or glycocalicin, is part of the CD42a-d complex. This is a transmembrane protein complex serving as a receptor for VWF and thrombin. The actual binding site for VWF and

thrombin lies on CD42b. Alteration of the expression of CD42b on the outer surface of a blood platelet is associated with blood platelet activity.

CD62P, also known as P-selectin, GMP-140 or PADGEM, is an integral membrane protein of 140kDa found in the membrane of alpha granules of resting platelets and in the Weibel-Palade bodies of endothelial cells. Activation of platelets causes a rapid translocation of CD62 to the cell surface, where it mediates adhesion, principally to neutrophils and monocytes.

CD63, also known as LIMP, LAMP-3, gp55, or melanoma-associated antigen (ME491), is a 53 kD lysosomal glycoprotein, and is thought to function as a transmembrane adaptor protein. Activation of platelets causes lysosomal degranulation, which is accompanied by a translocation of CD63.

PAC-1, a monoclonal antibody, recognizes an epitope on the glycoprotein IIb/IIIa (gpIIb/IIIa or integrin alpha$_{IIb}$beta$_3$) complex of activated platelets at or near the platelet fibrinogen binding location on the glycoprotein IIIa (gpIIIa or beta$_3$) subunit. This epitope has not yet been assigned a CD number. Platelet activation induces a calcium-dependent conformational change in gpIIb/IIIa that exposes a ligand binding site. PAC-1 binds only to activated platelets and inhibits fibrinogen- mediated platelet aggregation.

Flow-cytometric measurement of platelet activity in unfixed whole blood is a sensitive approach that has several advantages over other methods of platelet function measurement (4). Firstly, it enables simultaneous analysis of multiple aspects of platelet physiology to be conducted on large numbers of single platelets in a relatively short time. Secondly, analysis can be carried out in whole blood which reduces in vitro handling artifacts. Thirdly, it enables 100% of the platelet population to be studied, including giant platelets, platelet-derived microparticles and platelet leukocyte-aggregates. Fourthly, analysis of unfixed samples allows investigation of the platelet response to agonist stimulation. Fifthly, whole blood analysis means that platelets are studied in autologous plasma and in the presence of other cells, which can contribute to the overall platelet response through the release of soluble mediators.

There are some disadvantages to flow-cytometric analysis of platelet function. Firstly, flow cytometers are expensive instruments and monoclonal antibodies are expensive reagents. Secondly, sample preparation is often quite complicated, depending on the type of platelet function is measured. Thirdly, blood samples should be processed within 30 minutes of drawing when unfixated blood is used and within 24 hours when immediate fixation is applied.

Applicability and concluding remarks

Whole blood flow-cytometric studies have demonstrated circulating activated platelets in patients with coronary artery disease, peripheral artery disease and ischaemic cerebrovascular disease (16-21). Flow-cytometric analysis of platelet activation markers may predict an increased risk of cardiovascular disease. Flow cytometry can also be used to study several manifestations of platelet activity in whole blood in patients with (treatment directed at) atherothrombosis-related conditions, such as patients with hypertension (22), dyslipidaemia (23) or renal failure (this thesis), women with pregnancy-induced hypertension or pre-eclampsia (24;25), and women treated with postmenopausal hormone replacement therapy (this thesis). It may provide insight as to whether, to what extent and by which activation pathways, platelets play a role in the pathogenesis of diseases related to these conditions.

References

(1) Michelson AD. Methods for the measurement of platelet function. Am.J.Cardiol. 2009; 103(3 Suppl):20A-26A.

(2) Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ et al. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. Thromb.Haemost. 1998; 79:885-896.

(3) Michelson AD, Shattil SJ. The use of flow cytometry to study platelet activation. In: Watson SP, Authi KS, editors. Platelets, a practical approach. Oxford: IRL Press, 1996.

(4) Goodall AH, Appleby J. Flow-cytometric analysis of platelet-membrane glycoprotein expression and platelet activation. In: Gibbins JM, Mahout-Smith MP, editors. Platelets and megakaryocytes, volume 1: functional assays. Totowa, New Jersey: Humana Press, 2004: 225-253.

(5) Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. Blood 1999; 94:172-178.

(6) Julius D, Huang KN, Livelli TJ, Axel R, Jessell TM. The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. Proc.Natl.Acad.Sci.U.S.A. 1990; 87:928-932.

(7) Hirata M, Hayashi Y, Ushikubi F, Yokota Y, Kageyama R, Nakanishi S et al. Cloning and expression of cDNA for a human thromboxane A2 receptor. Nature 1991; 349:617-620.

(8) Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. Blood 2002; 99:193-198.

(9) Regan JW, Nakata H, DeMarinis RM, Caron MG, Lefkowitz RJ. Purification and characterization of the human platelet alpha 2-adrenergic receptor. J.Biol.Chem. 1986; 261:3894-3900.

(10) Inaba K, Umeda Y, Yamane Y, Urakami M, Inada M. Characterization of human platelet vasopressin receptor and the relation between vasopressin-induced platelet aggregation and vasopressin binding to platelets. Clin.Endocrinol.(Oxf.) 1988; 29:377-386.

(11) Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991; 64:1057-1068.

(12) Mathur A, Martin JF. Platelet heterogeneity: physiology and pathological consequences. In: Gresele P, Page CP, Fuster V, Vermylen J, editors. Platelets in thrombotic and non-thrombotic disorders. Cambridge: Cambridge University Press, 2002: 70-79.

(13) Theilmeier G, Lenaerts T, Remacle C, Collen D, Vermylen J, Hoylaerts MF. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. Blood 1999; 94:2725-2734.

(14) Lim YC, Snapp K, Kansas GS, Camphausen R, Ding H, Luscinskas FW. Important contributions of P-selectin glycoprotein ligand-1-mediated secondary capture to human monocyte adhesion to P-selectin, E-selectin, and TNF-alpha-activated endothelium under flow in vitro. J.Immunol. 1998; 161:2501-2508.

(15) Hartwig JH. The platelet cytoskeleton. In: Michelson AD, editor. Platelets. Burlington: Academic Press, 2007: 75-97.

(16) Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J.Am.Coll.Cardiol. 1998; 31:352-358.

(17) Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. Circulation 2001; 104:1533-1537.

(18) Yip HK, Chen SS, Liu JS, Chang HW, Kao YF, Lan MY et al. Serial changes in platelet activation in patients after ischemic stroke: role of pharmacodynamic modulation. Stroke 2004; 35:1683-1687.

(19) Klinkhardt U, Bauersachs R, Adams J, Graff J, Lindhoff-Last E, Harder S. Clopidogrel but not aspirin reduces P-selectin expression and formation of platelet-leukocyte aggregates in patients with atherosclerotic vascular disease. Clin.Pharmacol.Ther. 2003; 73:232-241.

(20) Robless PA, Okonko D, Lintott P, Mansfield AO, Mikhailidis DP, Stansby GP. Increased platelet aggregation and activation in peripheral arterial disease. Eur.J.Vasc.Endovasc.Surg. 2003; 25:16-22.

(21) Riba R, Nicolaou A, Troxler M, Homer-Vaniasinkam S, Naseem KM. Altered platelet reactivity in peripheral vascular disease complicated with elevated plasma homocysteine levels. Atherosclerosis 2004; 175:69-75.

(22) Labios M, Martinez M, Gabriel F, Gomez-Biedma S, Guiral V, Vivo M et al. Flow cytometric analysis of platelet activation in hypertensive patients. Effect of doxazosin. Thromb.Res. 2003; 110:203-208.

(23) Labios M, Martinez M, Gabriel F, Guiral V, Martinez E, Aznar J. Effect of atorvastatin upon platelet activation in hypercholesterolemia, evaluated by flow cytometry. Thromb.Res. 2005; 115:263-270.

(24) Konijnenberg A, van der Post JA, Mol BW, Schaap MC, Lazarov R, Bleker OP et al. Can flow cytometric detection of platelet activation early in pregnancy predict the occurrence of preeclampsia? A prospective study. Am.J.Obstet.Gynecol. 1997; 177:434-442.

(25) Konijnenberg A, Stokkers EW, van der Post JA, Schaap MC, Boer K, Bleker OP et al. Extensive platelet activation in preeclampsia compared with normal pregnancy: enhanced expression of cell adhesion molecules. Am.J.Obstet.Gynecol. 1997; 176:461-469.