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Imaging flow cytometry in the assessment of leukocyte-platelet aggregates

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ABSTRACT

Platelets are subcellular blood elements with a well-established role in haemostasis. Upon activation platelets undergo granule exocytosis, resulting in α-granule P-Selectin being expressed on the cell membrane. This allows binding of activated platelets to P-Selectin glycoprotein ligand 1 (PSGL-1) expressing leukocytes, forming leukocyte-platelet aggregates (LPAs). Whole blood flow cytometry (FCM) has demonstrated that elevated circulating LPAs (especially monocyte LPAs) are linked to atherothrombosis in high risk patients, and that activated platelet binding influences monocytes towards a pro-adhesive and pro-atherogenic phenotype. However, a limitation of conventional FCM is the potential for coincident events to resemble LPAs despite no tethering. Imaging cytometry can be used to characterize LPA formation and distinguish circulating MPAs from coincidental events. Platelets and leukocyte subsets are identified by expression of surface markers (e.g. the lipopolysachharide receptor CD14 on monocytes, glycoprotein Ib CD42b on platelets). In conventional FCM, all events with both leukocyte and platelet characteristics are designated as LPAs. However, by using an 'internal' mask based on the brightfield image and the fluorescent platelet identifier, imaging flow cytometry is able to distinguish leukocytes with tethered platelets (genuine LPAs) from leukocyte with coincidental, untethered platelets nearby. Mechanisms (e.g. adhesion molecules) or consequences (e.g. signal transduction) can then be separately analysed in platelet tethered and untethered leukocytes. Imaging flow cytometry therefore provides a more accurate approach for both enumeration and analysis of LPAs than conventional FCM.

1. Introduction

1.1. Platelet physiology

Platelets are small a nuclear cells with high biological significance [1]. A complex cytoskeletal network allows platelets to quickly release granular proteins, up-regulate specific membrane receptors and interact with other cell types when platelets are activated. This, in turn, gives platelets the ability to rapidly alter phenotype in response to *in situ* agonists or stimuli and carry out their biological function in haemostasis: platelet activation, exocytosis, adhesion and aggregation.

Platelets contain three main intracellular granules: α-granules, dense bodies (δ-granules) and lysosomes [1]. Granules serve as storage and secretory vesicles which readily release their constituents (known as the platelet releasate) *in situ* at the site of vascular injury and platelet activation. Furthermore, granule molecules translocate to the surface of plasma membrane, allowing up- or down-regulation of surface receptor expression. One of the hallmarks of platelet exocytosis is the translocation of P-Selectin, a protein that is usually sequestered within granules, to the surface of platelets.

1.2. Leukocyte-platelet aggregates

Platelets interact with various circulating leukocytes, including monocytes, neutrophils, dendritic cells, and others [2]. Previous research has shown that activated platelets bind preferentially to monocytes [3]. Monocytes are involved in wound repair, immune

Abbreviations: AF488, Alexafluor 488; APC, allophycocyanin; BP, bandpass; BSA, bovine serum albumin; BV421, Brilliant Violet 421; FCM, flow cytometry; IFC, imaging flow cytometry; ISX, Amnis Imagestream^{X}; LPA, leukocyte-platelet aggregate; MFI, median fluorescence intensity; MPA, monocyte-platelet aggregate; PSGL-1, P-Selectin glycoprotein ligand 1; RMS, root mean square; SD, standard deviation; TRAP, thrombin receptor activating peptide (SFLLRN); WBC, white blood cell (leukocyte).

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surveillance and the inflammatory processes. Circulating monocyte-platelet aggregates (MPA) are a sensitive in vivo marker of platelet activation [2,3]. Increased levels of MPA are found in patients with patients with coronary artery disease, and are particularly elevated in unstable disease, such as acute coronary syndromes or recent myocardial infarction [4]. This suggests that MPAs may play an important role in mediating the disease pathology of unstable coronary artery disease [5]. Hence the mechanism of MPA has been an area of research interest because the interruption of the adhesive interaction may represent a new therapeutic approach to cardiovascular disease [6].

P-Selectin on the surface of activated platelets that have undergone granule exocytosis binds to P-Selectin glycoprotein ligand-1 (PSGL-1) on monocytes and other leukocytes in a Ca^{2+}-dependent fashion, initiating this interaction [1,7]. Flow cytometry analysis shows that increased platelet activation as measured by the expression of P-Selectin and PAC-1 binding on platelets correlates with the amount of MPA formed [8]. P-selectin/PSGL-1 mediated platelet binding causes monocyte NF-κβ activation/translocation, superoxide anion production, and promotion of MCP-1, TNF-α, IL-8, IL-1β and COX-2 [5,9]. This interaction is believed to be one of the key mechanisms for the initial accumulation of monocytes to damaged endothelium, and a key component in the early, inflammatory accumulation of monocytes in atherogenesis [5].

Abundant data from animal models support the concept of atherosclerosis as an inflammatory disease, and the potential role for MPAs in atherogenesis [5,10]. In hypercholesteremic rabbits and ApoE-deficient mice, intravital microscopy has shown that activated platelets preferentially adhere to the site of atherosclerotic lesions before the lesions are detectible [11,12]. Circulating activated platelets exacerbate atheroma formation when introduced to juvenile ApoE knockout mice [13], and treatment of these juvenile animals with anti-platelet drugs such as thienopyridines, or disruption of CD40-CD40L signaling reduces the size and improves the stability of the plaque [14].

Antibody inhibition studies in animal models consistently suggest potential other counter-receptors apart from PSGL-1 on monocytes that interact with P-Selectin on platelets [15,16]. In a recently published study, we demonstrated increased circulating MPAs occur in healthy children in the absence of P-selectin expression, overt circulating platelet activation or increased numbers of circulating degranulated platelets [17]. This suggests P-Selectin/PSGL-1 independent tethering of monocytes and platelets in circulation may be more significant in children [18]. Sensitive and specific tools to assess different types of platelet-monocyte interaction are necessary to elucidate the molecular mechanisms of interaction in health and disease.

1.3. Flow cytometry

The application of flow cytometry in platelet testing is one of the major advances in platelet research [8,19]. The basic principle of flow cytometry is to use fluorescently conjugated monoclonal antibodies against specific molecular targets on the cells of interest. The individual labelled cells pass through a series of focused laser beams at different excitation wavelengths. The emitted light is filtered and spectral overlap corrected for to determine the relative amount of each antibody bound per cell, revealing antigen density and characteristics of cells being measured. In this way, platelets are identified through characteristic laser scatter and expression of platelet-specific glycoprotein receptors (such as CD42b and CD61), and markers of platelet activation, (e.g. VASP dephosphorylation) and granule exocytosis (e.g. surface expression of P-selectin) can be measured.

Whole blood flow cytometry is a well-established technique for measuring circulating MPAs [19,20]. In lysed whole blood, monocyte events are gated by characteristic laser scatter and expression of CD14 (the lipopolysaccharide receptor). Monocyte events which are positive for a platelet specific epitope are identified as MPAs, and the percentage of monocytes with one or more adherent platelets is recorded. Abundant P-selectin dependent MPAs in the context of thrombo-inflammatory disease are readily identified in this manner, and the method is a sensitive and reliable indicator of platelet activation in the context of acute atherothrombosis [3,4,21].

However, measurement of this heterotypic cellular interaction by flow cytometry is potentially problematic, as the concentration of platelets in blood is orders of magnitude higher than that of monocytes. Non-adherent but coincident monocytes and platelets passing through the point of laser interrogation near each other may be indistinguishable from a genuine tethered MPA event. This is a problem particularly at high differential sheath and sample pressures (higher flow rates), and in certain flow cytometers or settings double positivity attributable only to coincidence may exceed 30% [22,23]. This may explain, in part, the large variability in reported levels of circulating MPAs in the literature. P-selectin negative monocyte-platelet events are often dismissed as a function of coincidence. However, our expanding understanding of P-selectin/PSGL-1 independent mechanisms of leukocyte-platelet interaction (particularly in paediatric populations) requires that we confidently differentiate between coincident events and genuine leukocyte-platelet tethering.

Epifluorescent and confocal microscopy have been used to image MPAs [24], but are not capable of providing the high-throughput quantitative data required to measure moderate, but clinically relevant, increases in MPAs. Locating and analysing sufficient MPAs by microscopy is extremely labour intensive. Furthermore, because P-Selectin positive platelets can bind monocytes even after fixation, attempts to concentrate the samples in order to help improve this have the potential for pre-analytical formation of artefact MPAs. For these reasons, epifluorescent microscopy has not been effectively used to discriminate genuine MPAs from coincident events.

1.4. Image cytometry

While previous attempts have been made to circumvent the issue of coincidence in determining monocyte-platelet aggregates by computer modelling of coincidence [22,23], recent developments in imaging cytometry present an opportunity to directly assess tethering.

Previous studies using stationary image cytometry with a combined bright-field and fluorescent monocyte marker recognition software algorithm to identify monocyte-platelet aggregates have shown promise [25]. While limited to scanning 80 stationary fields of view/sample, this approach showed good correlation with traditional flow cytometry ($r = 0.944$).

Imaging flow cytometry by Amnis Imagestream^{x} (ISX) is a versatile state-of-the-art platform that integrates fluorescence microscopy with flow cytometry (1000 cells/s). Equipped with up to five excitation lasers (405, 488, 561, 592 and 658 nm) and 12 detection channels this platform provides the potential to bridge qualitative and quantitative analysis of leukocyte-platelet aggregates. The use of imaging flow cytometry allows both identification and characterization of leukocyte-platelet aggregate events and distinct phenotypes with high-throughput analytical power. By using visualisation software and an algorithm mask, coincidental events can be identified and excluded from analysis. We have previously shown this