Characterizing blood microparticles: Technical aspects and challenges

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Abstract: Although long considered to be cellular debris, microparticles (MPs) are more recently considered reflective of cellular stimulation, activation, and degeneration/apoptosis. MPs that arise from the cellular components of blood and the endothelial lining of blood vessels are referred to as blood MPs and by general consensus are small (≤1.5 μm), expose the anionic phospholipid (PL) phosphatidylserine (PS) on the outer leaflet of their membrane, and bear surface membrane antigens reflecting their cellular origin. This brief review summarizes the different approaches used by several groups to study blood MPs. The aim of this article is to review the technical aspects of characterizing the morphological and functional properties of blood MPs with emphasis on the preanalytical and analytical variables involved in these studies.

Keywords: microparticles, flow cytometry, techniques

Introduction

Cellular microparticles (MPs) are fragments of the plasma membrane that are shed by virtually all cell when subject to a number of stress conditions, including cellular activation and apoptosis (Beaudoin and Grondin 1991; Zwaal and Schroit 1997; Freyssinet et al 1999; Hugel et al 2005). Since the description of "platelet dust" by Wolf (1967), numerous studies have reported the presence of subcellular vesicles in centrifuged plasma. Although long considered to be cellular debris, blood MPs are more recently considered reflective of cellular stimulation, activation, and degeneration/apoptosis (Freyssinet 2003; Morel et al 2005; Boulanger et al 2006). By general consensus, MPs are small in size (≤1.5 μm), expose the anionic phospholipid (PL) phosphatidylserine (PS) on the outer leaflet of their membrane, and bear surface membrane antigens reflecting their cell of origin (Jy et al 2004). MPs that arise from the cellular components of blood and the endothelial lining of blood vessels are referred to as blood MPs. Apart from blood MPs, plasma contains smaller membrane vesicles (40–100 nm) termed exosomes and larger vesicles (>1.5 μm) termed apoptotic bodies that are derived from blood and vascular cells (Heijnen et al 1999; Freyssinet 2003). The aim of this article is to review the technical aspects of characterizing the morphological and functional properties of blood MPs with emphasis on both preanalytical and analytical variables.

Membrane phospholipid asymmetry and microparticle formation

All cell type's spontaneously shed blebs from their plasma membranes in an active process that appears to be important for membrane remodeling and antigenic turnover. In resting cells, the negatively charged phospholipids, including PS, are almost exclusively present in the inner leaflet of the cell membrane (Zwaal and Schroit 1997). Among the mechanisms proposed to account for this asymmetry are unidirectional enzymatic movement by an aminophospholipid translocase and/or association between

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the negatively charged phospholipids and elements in the cytoplasm, including cytoskeletal elements and their accompanying proteins (Bevers et al 1998). MP release is an integral part of the cell membrane remodeling process whereby the normal asymmetric distribution of phospholipids between the two leaflets of the plasma membrane is lost. Calcium entry, activation of calpain and scramblase activity are thought to be important steps in the loss of lipid asymmetry and microparticle formation, but the exact sequence of events is not completely understood (Fox et al 1990; Yano et al 1994; Dachary-Prigent et al 1995, 1997; Pasquet et al 1996, 1998; Bucki et al 1998, 2006; Kunzelmann-Marche et al 2001). The possible pathways leading to membrane budding and MP release have been recently reviewed (Freyssinet 2003; Boulanger et al 2006).

Membrane shedding by blood cells

Erythrocytes

Shedding processes are found in a variety of blood cells (Beaudoin and Grondin 1991). Human erythrocytes treated with ionophore A23187 in the presence of calcium, respond by budding to release MPs. Normal erythrocytes also release MPs following ATP depletion, heat treatment (49 °C), incubation at pH 5.4 and incubation with diamide (5 mMol/L) (Wagner et al 1986). Erythrocyte MPs are also found to be elevated in several pathological conditions associated with hemolysis (Hugel et al 1999) but are notably elevated in sickle cell anemia (Allan et al 1982). Erythrocyte-derived MPs are usually identified by the binding of antibodies to glycophorin A (CD235a), a protein uniquely expressed on the erythrocyte membrane.

White blood cells

These cells have a unique physiological role in inflammation, immune regulation, and thrombosis. Following exposure to various stimuli including lipopolysaccharide and phorbol myrisitate, monocytes are observed to vesiculate and release MPs (Satta et al 1994; Mesri and Altieri 1999). Monocytes express CD14, the lipopolysaccharide receptor strongly on their surface while granulocytes express CD14 weakly. Thus, monocyte-derived MPs have been identified by the surface expression of CD14. MPs derived from neutrophils have been identified in the blood of normal subjects and in patients with meningoccocal sepsis (Nieuwland et al 2000). Typically, polymorphonuclear neutrophilic leukocyte-derived MPs are identified using the leukocyte-specific markers lactoferrin or CD66b. Lymphocyte-derived MP have been identified using lymphocyte-specific markers CD4 and CD8 (Armstrong et al 1988; Aupeix et al 1997; Diamant et al 2002; Martin et al 2004).

Platelets

Microparticle formation in platelets occurs as a result of activation by various stimuli (epinephrine, adenosine diphosphate, collagen, thrombin, A23187) (Horstman and Ahn 1999) and during storage (Bode et al 1991). Increased platelet MP formation has been documented in a variety of clinical conditions that are associated with platelet activation (Solum 1999). Platelet-derived MPs that express surface glycoproteins and antibodies to CD41, CD42, and CD61 have been used to detect platelet-derived MPs (Lee et al 1996; Combes et al 1997; Diamant et al 2002). CD61 is less specific as a platelet marker since this integrin is also expressed on blood monocytes and antibodies that recognize on the CD61 epitope will identify both platelet- and monocyte-derived MPs.

Endothelial cells

Endothelial cells undergo injury and vesiculation following *in vitro* exposure to complement, thrombin and a variety of agents such as ionophore, lipopolysaccharide, autoantibodies, and cytokines (interleukin-1 and tumor necrosis factor-α) (Hamilton et al 1990; Combes et al 1999). Elevated endothelial MPs have been documented in several pathological situations including atherosclerosis, sepsis, and diabetes mellitus. Several endothelial specific antibodies have been used to detect MPs in these situations including CD31, CD51, CD105, CD144, and CD146 (Sabatier et al 2002). Of this extensive list, CD144 and CD146 are endothelial-specific markers as they have not yet been found expressed on any other blood cell in humans.

Assays to characterize microparticles

In general, most assays have focused on characterizing the antigenic composition of cellular microparticles using antibody capture-based ELISA (Aupeix et al 1997; Freyssinet et al 1999; Mallat et al 1999) or flow cytometry (Combes et al 1997, 1999). Most investigators have elected to characterize blood MPs using flow cytometry and the technical protocols of some have been reviewed in a recent forum (Jy et al 2004). In addition to analyzing MP antigens, several investigators have assessed the coagulant function of blood microparticles using functional assays (Westerman et al 1984; Mallat et al 1999; Berckmans et al 2001; Joop et al 2001; Shet et al 2003; Aras et al 2004). These assays

either measure acceleration of plasmatic coagulation by MP-derived phospholipids or triggering of plasmatic coagulation by MP-associated tissue factor. The PF3 assay, in which exposure of platelet poor plasma to Russell's viper venom in the presence of calcium causes coagulation, but only in the presence of certain phospholipids (supplied by blood MPs), has also been used as a functional assay (Hardisty and Hutton 1965).

Preanalytical variables

The measurement of blood MPs requires careful attention to collection and processing of blood samples. While there have been some general guidelines regarding preanalytical variables that may be important, these have been more consensus-driven statements rather than guidelines based on scientific evidence. Furthermore, several aspects of blood collection such as venepuncture needle size, use of a tourniquet during blood collection, phlebotomy using a vacutainer vs a syringe, and the type of anticoagulant used (sodium citrate vs ethylenediaminetetraacetic acid) could have an impact on the measurement of blood MPs. Additionally, sample processing following blood collection is extremely variable between investigators and could be partially responsible for the discrepancies in blood MPs levels reported in the literature. While separating the cellular elements of blood from the plasma containing MPs, careful attention must be paid to centrifugation speed. In our experience, a 2-step centrifugation using 1500 × g for 10 minutes and then 13,000 × g for 10 minutes resulted in platelet-free plasma (when assessed by flow cytometry and light microscopy) (Shet et al 2003). The second centrifugation step is particularly efficient at rendering plasma relatively "platelet free" by eliminating small platelets but it is unclear if large bloods MPs are also depleted in this process. There are several difficulties in separating platelets, microparticles, exosomes, and cellular debris as individual components. We have attempted to isolate blood MPs from platelet-poor plasma by using a high-speed centrifugation step. However, the sample that we work with contains exosomes. Isolating blood MPs from exosomes by centrifugation is technically challenging and the need for such separation is probably dependent on the nature of the study being performed.

Analytical variables

Flow cytometry

Flow cytometric analysis of blood MPs appears to be the most favored method to characterize blood MPs (Jy et al 2004). Typically, MPs are identified as particles with a forward angle light scatter (FALS) smaller than an internal standard consisting of 1–1.5 μm sized latex particles (Shet et al 2003). Most investigators do not use a lower size limit but some have arbitrarily chosen 100 nm below which particles that are recognized by the instrument are not considered MPs (Boulanger et al 2006). Others have used platelets as an internal standard and gated particles with a FALS value below that of the smallest platelet (Nieuwland et al 2000). While the identification of MPs on the basis of size using FALS tests the limit of sensitivity of flow cytometry, some have overcome this problem by adjusting the parameters of the instrument to detect fluorescent intensity (Horstman et al 1994). Flow cytometry can also be used to enumerate blood MPs by adding a known number of fluorescent or nonfluorescent latex particles to the sample prior to performing analysis (Combes et al 1997, 1999; Shet et al 2003). The number of MPs present in the sample is derived by dividing the number of MP events counted by the volume of the sample analyzed, ie, (beads counted/beads added) × sample volume, and adjusting for the final dilution of the original blood sample. Alternatively, if the flow cytometry instrument delivers the sample to the optics cell by screw-driven syringe at a known rate, then the sample MP count can theoretically be calculated. This method of enumeration is less desirable since it makes assumptions regarding the instruments fluidics and has unacceptable day-to-day variability (Reverter et al 1996).

Antibody capture assays

ELISA-based MP capture assays have been devised and tested to simplify and semi-automate the characterization of blood MPs. These assays have utilized annexin V as a means of capturing phospholipids (Aupeix et al 1997; Mallat et al 1999) or antibodies to specific blood MP membrane antigens, eg, αIIbβ3 (Mallat et al 2000), or antibodies to nonspecific blood MP membrane antigens that are shared by several cell types eg, antibody 1B5 (Aras et al 2004) and have been reviewed elsewhere (Freyssinet et al 1999). Such assays have reduced the challenges of analyzing large sample numbers.

Other assays

In addition to the above techniques, the antigenic characteristics and the membrane composition of blood MPs have been studied using electron microscopy (Heijnen et al 1999; Aras et al 2004) (Figure 1 and Figure 2), confocal microscopy (Combes et al 1999), high performance liquid chromatography (Weerheim et al 2002), capillary

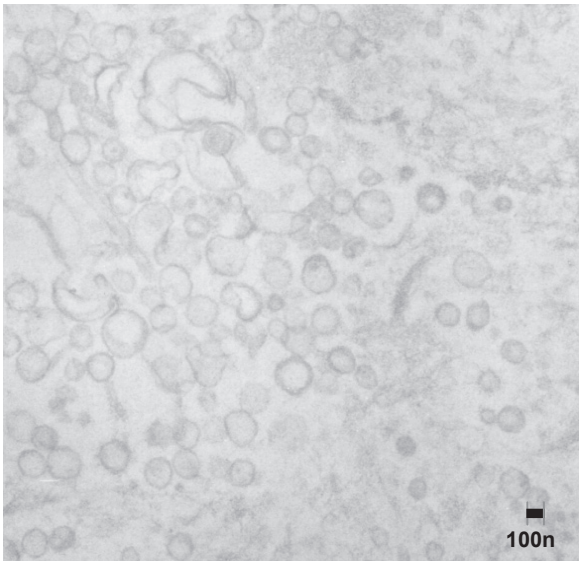

Figure 1 Ultrastructure of blood MPs from a patient with sickle cell anemia using electron microscopy.

electrophoresis (Xiong et al 2003), and mass spectrometry (Jin et al 2005; Miguet et al 2006) with varying degrees of success. Assays that measure the pro/anticoagulant functions of blood MPs are somewhat under-utilized perhaps in part due to the technical challenges in developing standardized and reproducible assays.

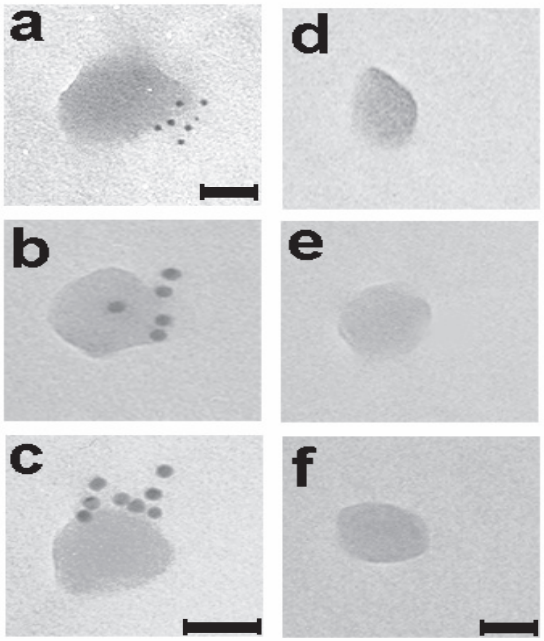

Figure 2 Immunogold labeling of blood MPs. Blood MPs obtained by ultracentrifugation were incubated with rabbit polyclonal anti-tissue factor antibody (**a**; 6 nm gold), anti-CD144 antibody (**b**; 12 nm gold), and anti-CD14 antibody (**c**; 12 nm gold). No labeling was observed with control antibodies (**d**, **e**, and **f**).
Note: Bars = 100 nm.

Limitations

Flow cytometry provides useful information about blood MPs but there are some limitations associated with this technique that are worth considering. The definition of a blood MP using flow cytometry is still an area of great debate. In our studies, we used somewhat rigid criteria to define blood MPs in order to preserve specificity while sacrificing sensitivity to a certain extent. Blood MPs were defined as particles that were 1. Removable from platelet-free plasma by ultracentrifugation; 2. Were small in size ($\leq$1μm); and 3. Labeled with annexin V. While such criteria may be possible in certain situations, they are not practical or possible in all conditions. For instance, most would agree that blood MPs demonstrate surface PS as evidenced by annexin V labeling, but clearly not all plasma particles $\leq$1 μm label with annexin V (Shet et al 2003; Boulanger et al 2006). Furthermore, a substantial portion of plasma particles $\leq$1 μm label with antibodies to cell surface markers, but not annexin V, which suggests that there are PS (–) blood MPs (Shet et al 2003). An alternative explanation could be that the exposure of PS on the outer aspect of some blood MPs is incomplete resulting in absent/reduced annexin V labeling or that some MPs contain scramblase activity. Finally, the contribution of exosomes and apoptotic bodies to the flow cytometric signal obtained cannot be completely ignored. Thus, while a consensus flow cytometric definition of blood MPs would have utility, one should be aware of the limitations of such definitions when studying biological processes. Modern flow cytometers are equipped with less powerful lasers and have a threshold for particle size detection ~500 nm below which sensitivity is unpredictable according to instrument manufacturers (Becton Dickenson, San Jose, CA; pers comm). Detecting MPs using fluorescence as the primary detection parameter has overcome this problem to a certain extent, but most investigators do not clearly indicate instrument setting in their description of the methods. Finally, the choice of well-characterized antibodies directed against a cell membrane antigen that not shared by other cell lineages present in blood is an important prerequisite to characterizing the cellular origin of blood MPs. Such antibodies should be primarily labeled with a flourophore, as the use of secondary antibodies is not recommended.

Microparticles in health and disease

The general consensus is that plasma levels of blood MPs reflect equilibrium between their release and their clearance by monocytes-macrophage system. Since clearance of cells undergoing apoptosis or injury is extremely efficient, the

presence of blood MPs is considered an objective *in vitro* parameter that serves as evidence of deleterious cellular alterations *in vivo*. However, very little is published about the clearance and fate of blood MPs in any specific disease state leaving one to speculate that their presence reflects either a defect in recognition of these particles or an overwhelming of the clearance mechanism by their sheer number (Willekens et al 2005). Experimental evidence both *in vitro* and *in vivo* has demonstrated that blood MPs are capable of influencing diverse biological function (Zwaal and Schroit 1997; Barry and FitzGerald 1999; Horstman and Ahn 1999; Mesri and Altieri 1999; Freyssinet 2003; Hugel et al 2005; Boulanger et al 2006). Blood MPs have been associated with several disease processes and while they seem to be useful markers there is insufficient evidence to demonstrate that this association is causal. Clearly, elevated blood MPs in several disease processes result from inflammation, cellular activation and apoptosis. Such MPs, through their diverse biological effects, unequivocally contribute to vascular disease and comorbidity and are therefore likely to be very important in the disease process.

Conclusion

Several studies clearly demonstrate that blood MPs could be mediators of disease in some cases, markers of disease in other cases, and have prognostic potential, but the pathophysiological relevance of blood MPs is still in its infancy. Many of the studies mentioned in this review demonstrate that detection, phenotypic characterization, and quantification of blood MPs are interesting and potentially valuable in patients that are at risk for vascular events. However, conversion of this potential into a reality will necessitate a standardized approach to the detection and characterization of blood MP. In addition, attention to the preanalytical and analytical variables and refinement in the techniques used to assess the cellular origin of blood MPs could help shed light on the mechanisms of MP release, function, and clearance.

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