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Microparticle Analysis in Disorders of Hemostasis and Thrombosis

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Abstract

Microparticles (MPs) are submicron vesicles released from the plasma membrane of eukaryotic cells in response to activation or apoptosis. MPs are known to be involved in numerous biologic processes, including inflammation, the immune response, cancer metastasis, and angiogenesis. Their earliest recognized and most widely accepted role, however, is the ability to promote and support the process of blood coagulation. Consequently, there is ongoing interest in studying MPs in disorders of hemostasis and thrombosis. Both phosphatidylserine (PS) exposure and the presence of tissue factor (TF) in the MP membrane may account for their procoagulant properties, and elevated numbers of MPs in plasma have been reported in numerous prothrombotic conditions. To date, however, there are few data on true causality linking MPs to the genesis of thrombosis. A variety of methodologies have been employed to characterize and quantify MPs, although detection is challenging due to their submicron size. Flow cytometry (FCM) remains the most frequently utilized strategy for MP detection; however, it is associated with significant technological limitations. Additionally, pre-analytical and analytical variables can influence the detection of MPs by FCM, rendering data interpretation difficult. Lack of methodologic standardization in MP analysis by FCM confounds the issue further, although efforts are currently underway to address this limitation. Moving forward, it will be important to address these technical challenges as a scientific community if we are to better understand the role that MPs play in disorders of hemostasis and thrombosis.

Keywords

microparticles; microvesicles; coagulation; thrombosis; hemostasis; flow cytometry

Introduction

Historical Perspective

Microparticles (MPs) were first described by Chargaff and West [1] in the mid-20th century as a “precipitable factor” present in plasma that could promote coagulation processes. Wolf [2] in 1967 described “platelet dust” that was formed as a result of platelet shedding, which

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also exhibited procoagulant activity and was detectable in the 0.1 to 0.3 μm size range by transmission electron microscopy in the precipitate of ultracentrifuged plasma. Now understood to be platelet MPs (PMPs), this observation has led to an exponential growth in the study of MPs derived from platelets and other cell types, and with it a greater understanding of their overall biologic relevance. Although the study of MPs has now expanded beyond the realm of coagulation and into other areas of (patho)physiology, significant research effort remains focused on this aspect of MP function. As depicted in Figure 1, total MP publications have increased steadily over the past decade, with coagulation-related MP publications showing a similar increase and continuing to represent a significant portion of the total MP publications.

Definition

MPs are defined as heterogeneous, submicron (0.1 to 1 μm) vesicles released from cell membranes in response to specific stimuli or apoptosis. They have an intact phospholipid membrane and express membrane antigens specific to their cell of origin [3]. The working definition of a MP generally includes both the size discrimination, as well as the presence of externalized phosphatidylserine (PS) on the membrane [4, 5]. Newer evidence, however, supports the notion that not all MPs expose PS on their surface [6–10], and that PS content may vary depending on the cell of origin and stimulus or mechanism by which they are formed [11]. Whether this is due to a true lack of PS exposure, or whether PS expression is below the detection threshold of conventional techniques, particularly on smaller MP subsets, is unclear [12]. To complicate matters, it has also been theorized that the presence of a cell-specific antigen on the surface of a MP does not necessarily identify its cell of origin. Soluble antigens from other cell types may adhere to MPs, or fusion may occur between MPs from one cell type with the cellular membrane of a different cell, thereby allowing the detection of a MP expressing an “adopted” antigen [13, 14].

MPs must also be distinguished from two other bioactive vesicles released from cells. Exosomes are preformed vesicles < 100 nm that are generated in endocytic multivesicular bodies and released via exocytosis. They are more homogeneous in size than MPs, carry different membrane antigens, and play an important role in the immune response [15–19]. Conversely, apoptotic bodies (AptB) are produced during the latter stages of cell apoptosis [20]. They are typically larger than MPs (1–3 μm), although a few may be smaller (0.5 μm) [21]. Similar to MPs, they express PS on their surface; however, in contrast to MPs, AptB carry DNA and histones, which is one of their hallmarks [21, 22]. It should be pointed out that the term “extracellular vesicles” is increasingly being used in the scientific literature and is a term that encompasses MPs exosomes, and AptB [23]. Additionally, the term microvesicle is frequently encountered and in general is synonymous and interchangeable with the term MP [24].

Cellular Sources and Formation

Circulating MPs are most commonly derived from blood and endothelial cells, although other sources, such as tumor cells [25], are capable of producing MPs that appear in blood. In healthy individuals, PMPs have generally been accepted to be the most abundant MP subtype [26, 27]. More recent data, however, suggest that a significant portion of PMPs may

actually be derived from megakaryocytes in bone marrow [28]. To identify specific MP subsets by flow cytometry (FCM) according to their cell of origin, antibodies to common antigens of the parent cell are typically used (Table 1 and Figure 2), often in combination with Annexin V or another marker for PS, such as lactadherin.

The formation and release of MPs from cells typically occurs upon stimulation or induction of apoptosis. It is considered a broad primitive response to stress shared by all eukaryotic cells [29] and is thought to reflect a dynamic balance between cell proliferation, stimulation and death [30]. Specific stimuli known to induce MP formation include activation by substances such as endotoxin or cytokines, and partial or complete lysis such as by complement, oxidative injury, and high shear stress [31, 32]. Mechanistically, evidence continues to emerge regarding the cellular processes that lead to formation and release of MPs. In brief, loss of cellular membrane phospholipid asymmetry with resultant PS exposure appears to be a critical component of MP formation [33]. This process is governed by several phospholipid transporters (“flippase”, “floppase” and “scramblase”), which under basal conditions preserve the normal phospholipid asymmetry of the cellular membrane, with the negatively charged PS confined primarily to the inner leaflet. Calcium influx also appears to be a necessary prerequisite for MP formation, as it contributes to both PS externalization, as well as membrane cytoskeleton remodeling through activation of calpains and caspases necessary for cleavage of cytoskeletal proteins [34]. Upon stimulation, the loss of phospholipid asymmetry along with cytoskeletal disruption eventually leads to membrane blebbing and MP formation and release.

Biological Functions of Microparticles

Microparticles and Coagulation – Mechanistic Insight

This section of the review will briefly summarize what is known about MPs and their contribution to coagulation processes, as this information provides a backdrop for better understanding their potential relevance in disorders of hemostasis and thrombosis. This topic has also been extensively reviewed recently [35]. In basic terms, coagulation refers to the processes that regulate blood clot formation, whether it be under physiologic conditions to prevent hemorrhage (hemostasis) or under pathologic conditions (thrombosis). Additionally, coagulation processes can be further divided into those that promote blood clotting (procoagulant) and those that counterbalance or regulate blood clotting (anticoagulant and fibrinolytic).

The potential procoagulant function of MPs may be related to the presence of PS on the outer membrane, as well as the possible presence of tissue factor (TF). MP-associated PS provides a catalytic surface for the assembly of enzymatic coagulation complexes that initiate and maintain coagulation [36]. This function may underlie the contribution of MPs to both the physiologic process of hemostasis as well as the pathologic process of thrombosis [37]. Interestingly, it has been estimated that a PMP generated *ex vivo* has 50- to 100-fold higher procoagulant activity than the same area on an activated platelet [38], which may help account for the potential thrombogenicity of certain MPs. TF is the principal physiological initiator of coagulation *in vivo* through its interactions with the coagulation protease Factor VII/VIIa and is constitutively expressed by most vessel wall component

cells other than endothelium [39]. It is often therefore described as a “hemostatic envelope” that surrounds the vasculature, preventing excessive hemorrhage upon injury. Circulating TF in the blood may, however, be present at very low concentration, with monocytes believed to be the primary source [40]. The presence of TF on some monocyte-derived MPs (MMPs) and tumor-derived MPs is well established; however, whether PMPs or endothelial MPs (EMPs) express biologically active TF remains a matter of debate [41, 42]. Although likely only a small fraction of total TF in the blood (most of which is likely to be cell-bound), MP-borne TF is thought to be functionally active and may thus contribute to the procoagulant nature of MPs.

More recent data also point to a role for MPs supporting coagulation independent of TF and the extrinsic pathway of coagulation. PMPs and red cell MPs (RMPs) generated *ex-vivo* have been shown to initiate and support thrombin generation through the intrinsic pathway in a Factor XII-dependent manner [43], meaning that the procoagulant properties of MPs are abolished when Factor XII is inhibited. Similarly, RMPs in sickle cell disease [44] and in banked units for transfusion [45] have also been shown to promote coagulation through the intrinsic pathway in a Factor XI-dependent manner, again through abolished MP procoagulant properties when Factor XI is inhibited. These findings shed new light on the procoagulant repertoire of MPs and their possible impact through alternative mechanisms in coagulation initiation, although further studies are needed for verification as well as to elucidate the mechanism by which this occurs. With the renewed interest in the possible role of the intrinsic pathway in thrombosis [46, 47], additional studies are also needed to define the role MPs might play in this context.

In addition to the procoagulant functions of MPs, evidence exists regarding their ability to regulate coagulation through anticoagulant or fibrinolytic mechanisms. MPs have been demonstrated to harbor functionally active tissue factor pathway inhibitor (TFPI) on their membrane [48, 49], and support activated protein C and protein S mediated regulation of coagulation [50–52], both of which are normal anticoagulant pathways in the blood. Newer evidence also establishes that MPs support plasmin generation [53, 54], another regulatory mechanism by which fibrin clots are degraded. These more recent discoveries point to a more complex role of MPs in coagulation, where it is likely that the balance between pro- and anticoagulant properties ultimately determines their net effect in hemostasis and thrombosis (Figure 3).

Role of Microparticles in Thrombosis

As a corollary to studies that have investigated mechanisms by which MPs may contribute to the process of coagulation, the role of MP participation directly in the process of pathologic thrombosis *in vivo* has also received attention. Utilizing a mouse model of arteriolar thrombosis, it has been demonstrated that TF^+ MPs (presumably MMPs) accumulate at the site of thrombus formation and participate in clot propagation [55]. Other studies utilizing mouse models of venous thrombosis have additionally implicated MPs [56, 57]. Although these data are important, they have limitations due to the imperfect nature of murine models of thrombosis [58], which either use vessel injury or ligation to initiate

thrombus formation. Additionally, exogenous MPs are often infused in these studies, which may also limit the applicability of these findings to human thrombotic disorders.

Thus, there is ample evidence to assert that MPs play a biologically plausible role in coagulation disorders, and in the remainder of this review we will focus on the literature addressing MP analysis by FCM in disorders of hemostasis and thrombosis, as well as the technical challenges and limitations encountered when using this approach.

Phenotypic vs Functional Assays for Microparticle Analysis

FCM remains the most commonly utilized approach for the detection and analysis of MPs [59]. This platform is advantageous in that it provides not only quantitative information but also qualitative information by immunophenotyping particles and thereby identifying their cellular origin. Numerous other modalities have also been used for MP analysis, including immunoassays, atomic force microscopy, electron microscopy, dynamic light scattering, and impedance-based FCM [60]. These methodologies will not be reviewed further; however, each has its own advantages and disadvantages. In general, though, these techniques are either not widely available, are low throughput, or do not provide both qualitative and quantitative data.

It is also worth briefly discussing functional assays that have been used in the study of MPs in coagulation disorders. Generally speaking, these assays assess MP pro- or anti-coagulant functions. MPs are isolated from plasma using either capture techniques or ultracentrifugation and are interrogated for their ability to support or inhibit coagulation using either clot-based or chromogenic endpoint assays. TF-dependent procoagulant activity can be assessed through the use of a specific blocking antibody to TF [61].

There are limited data on the correlation between functional assays and flow cytometric analysis of MPs; however, what data do exist are inconsistent. Several studies have shown a positive correlation between MPs detected by FCM and MP procoagulant activity [62, 63], while others have failed to demonstrate a correlation [13, 64, 65]. The lack of a positive correlation between FCM and functional assays is not surprising, since more sensitive assays (such as atomic force microscopy) detect upwards of 1000-fold more MPs than conventional FCM [66]. Additionally, dynamic light scattering has shown the median size of MPs to be under 300 nm, with the fraction of MPs < 200 nm in plasma accounting for at least 50% of the thrombin generating capacity [67]. Since FCM does not detect these smaller MPs, it is understandable why functional assays and FCM may fail to correlate. It also appears that the plasma centrifugation protocol may influence assay correlations, as increased numbers of contaminating platelets prior to freezing will erroneously lead to an improved correlation [68]. This is explained by the presence of a greater number of larger PMPs from fractured platelets that are then detectable by FCM. More investigation in comparing the two types of assays is warranted, but functional assays remain a useful tool in supplementing FCM when evaluating MPs in disorders of coagulation.

Microparticle Analysis in Disorders of Hemostasis

One might assume that a lack or decrease in circulating MPs could contribute to a clinically relevant bleeding phenotype, and in fact, this has been shown to be the case. Scott Syndrome is a very rare genetic disorder characterized by impaired outward transmembrane migration of PS on cell surfaces, including platelets. Individuals with this condition have a moderate bleeding tendency due to impaired ability to carry out enzymatic coagulation processes on the platelet and/or MP surface [69]. Flow cytometric analysis has demonstrated markedly decreased levels of circulating MPs in affected patients [70], which is intuitive given that PS externalization is an important step in MP formation. To what degree the lack of MPs in these patients directly contributes to their bleeding symptoms is not known, but this hypothesis seems likely given the proposed importance of PMPs in hemostasis [71].

Several studies have also examined a potential role of MPs in hemostasis in congenital bleeding disorders. Hemophilia A is a rare inherited bleeding disorder characterized by deficiency of coagulation Factor VIII (FVIII), which in its severe form results in spontaneous hemorrhage [72]. Current treatment involves replacement of the deficient coagulation factor using infusions of recombinant or plasma derived FVIII concentrates [73]. One study evaluated MPs by FCM in hemophilia A patients before and after receiving FVIII infusion for a documented clinical bleeding event. The authors observed a significant decrease in total MPs, PMPs and EMPs after treatment [74]. It was suggested that MP incorporation into a developing hemostatic plug at the site of injury explained their decreasing numbers after treatment.

Von Willebrand disease (VWD) is another congenital bleeding disorder characterized by either a qualitative or quantitative deficiency of von Willebrand factor (VWF). It is characterized primarily by mucocutaneous bleeding [75]. Therapy typically consists of infusing plasma derived VWF containing concentrates or desmopressin (aka DDVAP) during acute bleeding episodes [76]. DDAVP promotes hemostasis by increasing endogenous levels of VWF and FVIII [77], and by increasing platelet activation [78]. The number of PMPs and VWF-bound MPs increases significantly after DDAVP administration [79], in conjunction with increased VWF functional activity in plasma. Furthermore, depletion of MPs from plasma significantly decreases the VWF functional activity observed after DDAVP administration. These data provide evidence that DDAVP administration increases MP numbers, including VWF-bound MPs, and that this MP-VWF contributes to the increased VWF functional activity. Thus, MPs appear to contribute to the therapeutic efficacy of DDAVP in the treatment of VWD.

Microparticle Analysis in Thrombotic Disorders

To date, there has been much more interest in studying MPs in thrombotic disorders (as opposed to disorders of hemostasis), and increased circulating MPs have been reported in many inherently prothrombotic conditions (Table 2). Additionally, MP numbers are often correlated with markers of an activated coagulation system, and in some cases with the presence or absence of a historical thrombotic event. These studies are almost exclusively retrospective or cross-sectional, and therefore need to be interpreted with caution. While the existence of an association between elevated MP numbers and prothrombotic conditions has

been repeatedly reported, a causal relationship between increased circulating MPs and thrombotic events cannot necessarily be concluded.

A specific area that has received considerable attention is cancer-associated thrombosis. In general, increased MPs have been detected using numerous methodologies, including FCM in patients with a variety of tumors [80–85]. Several studies have also shown a relative increase in MPs in cancer patients with thrombosis compared to cancer patients without thrombosis [86–89]. These data pertain primarily to procoagulant functional analysis of MPs; however, at least one study has shown increased TF+ MPs by FCM [90].

There are some prospective data in cancer that have linked elevated levels of MPs with future occurrence of thrombosis [88, 91–93]. One study showed that TF+ MPs detected by FCM was predictive of thrombosis in brain tumor patients [94]. However, other studies failed to show increased MPs as predictive biomarkers of future thrombosis [82, 95, 96]. The reason for these discrepant results is not clear, but may be related to either variability in thrombotic risk with different malignancies or differences in methodologies and analytical variables. Although prospective in nature, these data have limitations due to lack of serial MP measurements over time. In that regard, probably the most convincing evidence linking MPs to cancer-associated thrombosis comes from a study that prospectively examined serial MP TF-dependent procoagulant activity (MP-TF activity) in pancreatic cancer patients [97]. Herein, there was a significant correlation between increasing levels over time and subsequent development of thrombosis; however, the study conclusions were limited due to its small size.

There have also been quite discrepant studies evaluating MPs in the setting of thrombosis without an underlying prothrombotic condition (ie. idiopathic thrombosis). Several cross sectional studies have reported increased MP procoagulant functional activity [98, 99], while others have shown increased MPs by FCM [90, 99–102]. Still other studies failed to demonstrate an increase in MPs, either using functional assays [103–105] or FCM [106, 107]. Again, these contradictory results are most likely attributable to differences in methodologies and techniques.

Challenges in Flow Cytometric Analysis of Microparticles

Despite FCM being the most frequently utilized methodology for the analysis of MPs, numerous challenges exist, in large part related to limitations in the ability to detect submicron particles. Additionally, differences in how samples are processed and analyzed can have significant impact on the results obtained. Thus, important factors related to the analysis of MPs can be divided into pre-analytical and analytical variables (Table 3).

Pre-Analytical Variables in Microparticle Analysis

Numerous pre-analytical variables, most of which pertain to the collection and handling of specimens, can directly impact MP analysis. Although these variables can theoretically affect results regardless of the detection methodology, the majority of information addressing pre-analytical variables has been identified in studies utilizing FCM. Major pre-

analytical variables include the method of blood collection including type of anticoagulant used, timeframe and method for processing samples, and method of sample storage.

(i) Blood Collection—Several issues regarding the method of blood collection can contribute to an artifactual elevation in MP numbers. Use of a tourniquet, traumatic venipuncture, small-diameter needles, and use of vacuum-filled containers may cause hemolysis, platelet activation or endothelial damage, all of which can falsely increase the number of MPs detected. It is therefore standard practice to discard the first several milliliters of blood to minimize these variables [11]. The type of anticoagulant used for blood collection may also have effects on MP analysis. Specifically, heparin has been shown to cause increased MP numbers compared to other anticoagulants [108]. Additionally, PMP levels can increase due to *ex-vivo* vesiculation of platelets in citrate, with no such increase observed in samples obtained in citrate-theophylline-adenosine-dipyridamole [109] or acid-citrate dextrose [110], although sample agitation makes this much more pronounced [111]. In general, however, citrate is most commonly used due to its wide availability and acceptable results, provided that sample agitation and delays in processing are avoided.

(ii) Sample Processing—MP analysis is generally performed on plasma samples, although can also be done in whole blood [112] or on isolated MPs [107]. Although data directly comparing MP numbers assessed in each sample type from the same individual are lacking, it is likely that the results would be variable across techniques. Particularly when MPs isolated from plasma by ultracentrifugation are re-suspended for analysis, aggregation of MPs is highly likely to occur using such high centrifugation speeds, thus changing their size profile and altering the number of detectable MPs within the appropriate size-based gate. As such, it is recommended that MP analysis be performed in plasma. Delays in plasma preparation should also be avoided to help prevent the *ex-vivo* generation of MPs from blood cells.

For plasma MP analysis, the centrifugation protocol is highly important. Numerous centrifugation protocols have been employed [113], resulting in either platelet-poor or platelet-free plasma. Depending on the centrifugation speed, there is either the potential for loss of MPs in the sediment or the supernatant, as well as the risk of contamination of the sample with residual platelets. During a freeze/thaw cycle, these residual platelets can be fractured, leading to artificially increased PMP numbers [111, 114]. When MP analysis is performed on fresh samples, there is probably minimal effect on the results unless the centrifugation speed is high enough to pellet a portion of the MPs with the cellular fraction. Overall, the goal is to avoid loss of MPs, thus maintaining sensitivity, without sacrificing specificity through the contamination of samples with residual platelets and resultant false increase in MP numbers.

Another potential pitfall is the possibility for “micro-clot” formation to occur in the plasma prior to analysis. This typically occurs with Annexin V staining, which requires the addition of calcium to plasma samples anticoagulated with calcium chelators (such as citrate). The addition of calcium also enables the enzymatic processes of coagulation to occur, which can lead to formation of a fibrin clot [115]. These “micro-clots” can then bind MPs, creating large aggregates that fall outside the MP size gate and thus artificially reduce the number of

MPs detected. The use of heparin for blood collection has been proposed as a solution to this problem, since it inhibits coagulation and prevents "micro-clot" formation [116]. Alternatively, an anticoagulant such as Hirudin, a direct thrombin inhibitor, can be incorporated into the calcium-containing Annexin V buffer to prevent this artifact, which also avoids the known increase in MPs that occurs when blood samples are collected in heparin. This latter method is currently being utilized in the most recent standardization workshop for flow cytometric evaluation of MPs sponsored by the International Society on Thrombosis and Haemostasis (ISTH).

(iii) Handling and Storage Practices—The method of transportation of samples and potential agitation during transport are thought to influence MP numbers, particularly in blood samples anticoagulated with citrate, due to *ex-vivo* generation of MPs from blood cells. More widely recognized, however, is the impact of storage methods on MP analysis. Several studies have shown an increase in MP numbers when comparing frozen to fresh samples [117, 118], although this increase appears to be minimal over a 12 month period [111]. There is also the theoretical risk of fracturing MPs, causing an artificial increase in overall MP numbers [106]. Ideally, fresh plasma samples should be analyzed immediately to avoid this artifact. However, in addition to being impractical, labor-intensive and inefficient, this restriction would severely limit the ability for collaborative studies.

Analytical Variables in Microparticle Analysis

Several analytical variables can also affect the detection and enumeration of MPs by FCM. The most obvious of these is the type of flow cytometer used for MP evaluation and its intrinsic ability to discriminate submicron particles. Newer generation flow cytometers have improved detection capabilities in the 200–300 nm range and should be the preferred option when studying MPs [119], although the use of older generation flow cytometers with suboptimal resolution is still common practice. Apart from the type of flow cytometer used, other analytical variables include gating strategies for both size and fluorescence and the use of counting beads for enumeration.

(i) Size Gating—Initial gating for MP analysis is typically based upon size through the use of calibration beads. Generally, a bead that approximates 1 μm is used to set the upper size limit for MP detection, and all events smaller in size are interrogated for PS and/or cellular antigens using fluorescently labeled antibodies. Plastic beads, however, remain an imperfect model for size calibration since factors other than size can influence FSC, including relative refractive indices of both particles and suspension medium, presence of surface absorptive material, particle shape, and surface roughness [120, 121], which are not equivalent between biological entities and beads. This has led to controversy over their appropriate application and use [122–125]. A proposed solution to this problem is the use of biological entities, such as bacteria or viruses, for size calibration, although this strategy would also need standardization [123, 124].

(ii) Fluorescence Gating—Another challenging analytical variable is discriminating a positive fluorescent signal from background when using fluorescently labeled antibodies. Most laboratories use isotype controls (ITCs) to aid in setting gates for positive *vs.* negative

events. This technique has classically been used for cellular phenotyping, but in recent years its use even in this field has been challenged [126–128]. Although intended to account for background fluorescence and nonspecific binding, different ITCs can manifest various levels of background staining depending on their concentration, degree of aggregation, and fluorophore:antibody ratio [129]. Furthermore, the degree of nonspecific binding of an ITC may or may not reflect an equivalent degree of nonspecific binding as that of the antibody of interest. Therefore, the indiscriminant use of ITCs can significantly affect the number of positive events detected [130]. Adding to the complexity is the fact that many of the antigens used for fluorescent labeling, such as those on leukocyte and endothelial MPs, are weakly expressed and do not provide a clear separation in fluorescence between positive and negative events, rendering the use of ITCs even more troublesome. As such, titration of ITCs against the specific antibody of choice at its intended concentration should always be done in an antigen free sample to match background fluorescence of the ITC to that of the specific antibody. Options for antigen free samples include MP free plasma obtained through the use of detergents to lyse MPs [131] or via ultracentrifugation. Additionally, titration of specific antibodies should also be done to ensure optimal concentration and to help limit the effects of nonspecific antibody binding [132, 133]. Lastly, all antibodies and ITCs should undergo ultracentrifugation prior to use to pellet free fluorochrome aggregates, which can be detected in the MP size gate and interpreted as a false positive fluorescent signal [134].

(iii) Counting Beads and Enumeration—Counting beads are commonly used for the enumeration of MPs by FCM. These calibrated bead solutions have a known concentration and typically fluoresce brightly in a wide range of excitation and emission wavelengths. A known volume of beads is added to a sample, and by comparing the ratio of bead events to MP events, absolute numbers of MPs can be calculated. Although a useful tool, caution is needed when using counting beads for enumeration of submicron particles. Designed primarily for cell counting, the available beads are typically in the 5–10 micron size range. Therefore, when analyzing MPs, particularly if pushing the detection threshold to its lower limits, the flow cytometer may not have the dynamic range necessary to accurately detect and count the beads. To help avoid this potential pitfall, counting beads should always be assessed using thresholds and settings for both MP analysis and large particle (ie. cellular) analysis to ensure that similar numbers of beads are counted with each set of parameters. Additionally, counting beads on the smaller end of the size spectrum should be chosen for MP analysis to help lessen the chance for error.

Accurate enumeration of MPs can also be complicated by “swarm effect” as recently described by van der Pol and colleagues [135]. Swarm effect is encountered when multiple small particles, which alone are below the detection threshold of the cytometer, are simultaneously present in the laser beam and thus generate a single event signal. In this situation, the flow cytometer-determined concentration of particles underestimates the true concentration, and the relationship between count rate and prepared concentration is non-linear. This detection of coincident events can be controlled for by optimization of flow rates and dilutions [136], and therefore it is important to use low flow rates and optimal sample dilutions in order to avoid or minimize this complication. The advent of imaging flow

cytometry appears to enable accurate counting of individual MPs regardless of sample concentration [137], however this modality is still in its infancy.

The Need for Standardization

Due to the inherent difficulties associated with MP detection by FCM, as well as the numerous pre-analytical and analytical variables discussed above, it is clear that standardization of practices in flow cytometric analysis of MPs is urgently needed. To that end, an attempt at standardization of PMP enumeration was recently undertaken by the ISTH. Using a mixture of fluorescent beads of known sizes (Megamix™ – Biocytex, Marseille, France) and gating strategies to set both an upper and lower size limit (~500 nm) for MP detection, it was shown that a window of MP analysis could be reproducibly set on different cytometers of the same model to allow consistent PMP enumeration over time [138]. This protocol was then adopted as part of an ISTH workshop in an attempt to validate its use, wherein it was shown to facilitate the reproducible enumeration of PMPs across different flow cytometers and in different labs, though modifications of the protocol were required for certain cytometers [139]. As a next step, a second ISTH workshop has attempted to standardize PMP enumeration down to ~300 nm utilizing a similar bead-based gating strategy. The results of this exercise are expected in late 2014/early 2015.

In addition to standardization attempts focused on analytical variables, the Vascular Biology Scientific sub-Committee of the ISTH also organized a workshop aimed at standardizing pre-analytical variables that can critically impact MP measurements and remain a major source of variability [140]. Herein it was shown that a standardized pre-analytical protocol could reduce the inter-laboratory variability of flow cytometric evaluation of PMPs, although variability was not completely eliminated. Together, the results of these workshops are promising, although much work is still needed, particularly to help standardize the evaluation of MP subsets other than PMPs that are more challenging to detect, such as endothelial and leukocyte MPs. Hopefully, however, these efforts will serve as a first step towards continued exercises aimed at standardizing methodologies for MP analysis to allow better comparison of results across studies and promote the development of collaborative studies across centers.

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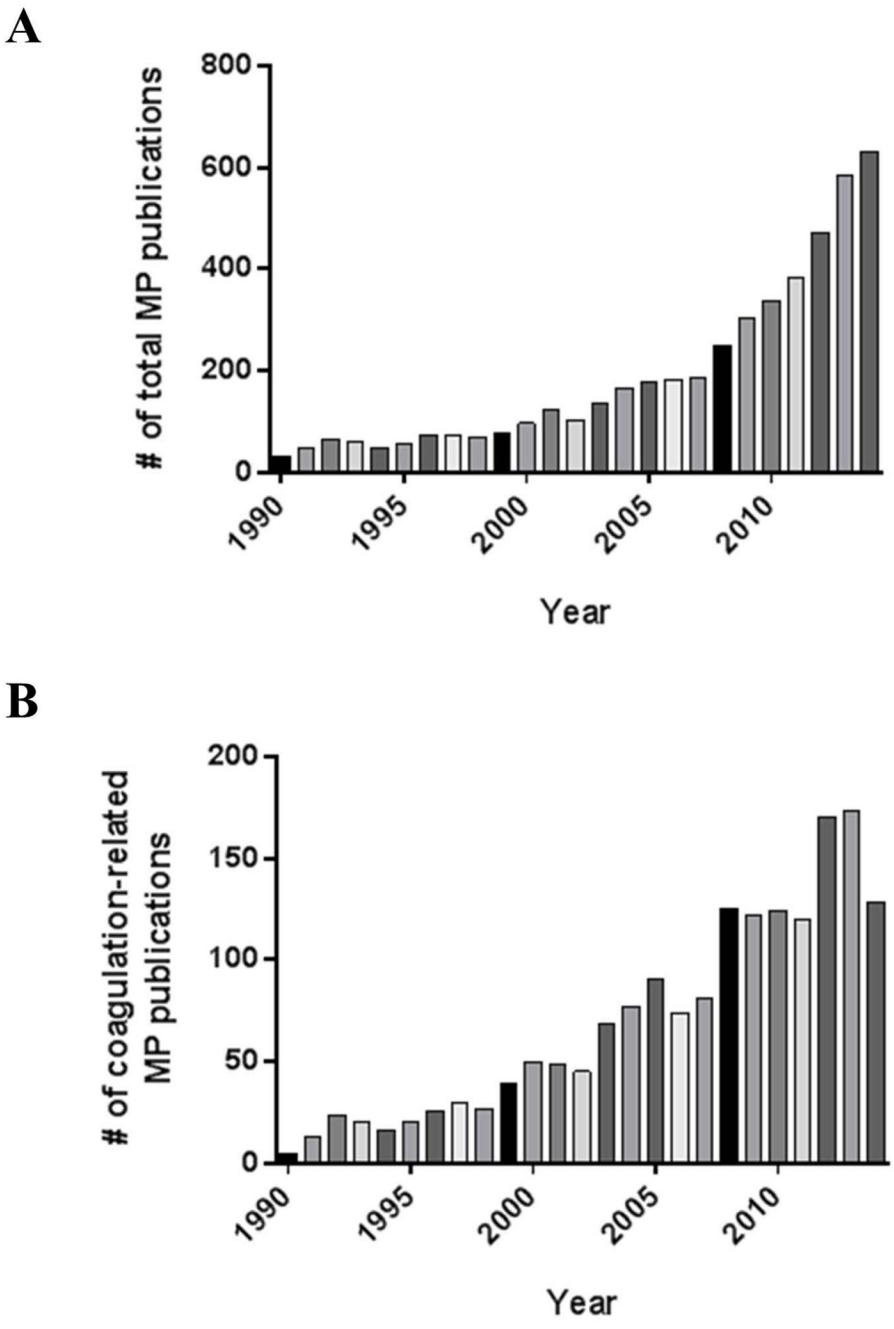
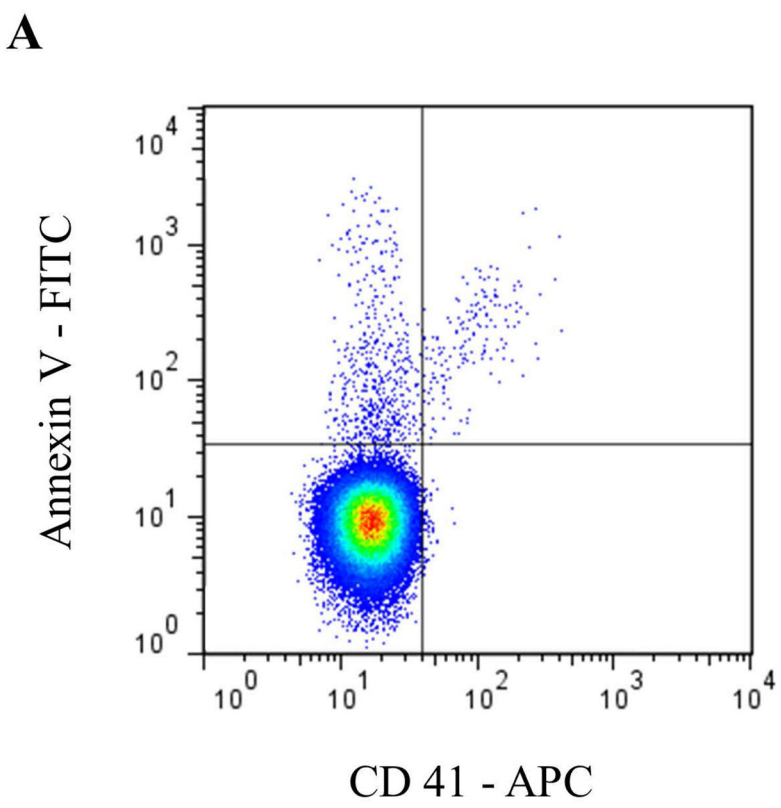


Figure 1.

Number of (A) total MP publications, and (B) specific coagulation-related MP publications by year since 1990. Total MP publication numbers were acquired utilizing a PubMed search for articles from 1990–2014 with keywords “microparticles or microvesicles”, while excluding studies related to pharmacology, drug delivery and non-biological entities. Coagulation-related MP publication numbers were acquired utilizing a PubMed search for articles from 1990–2014 with keywords “microparticles or microvesicles” in conjunction with coagulation specific terms such as “thrombosis” and “hemostasis”.



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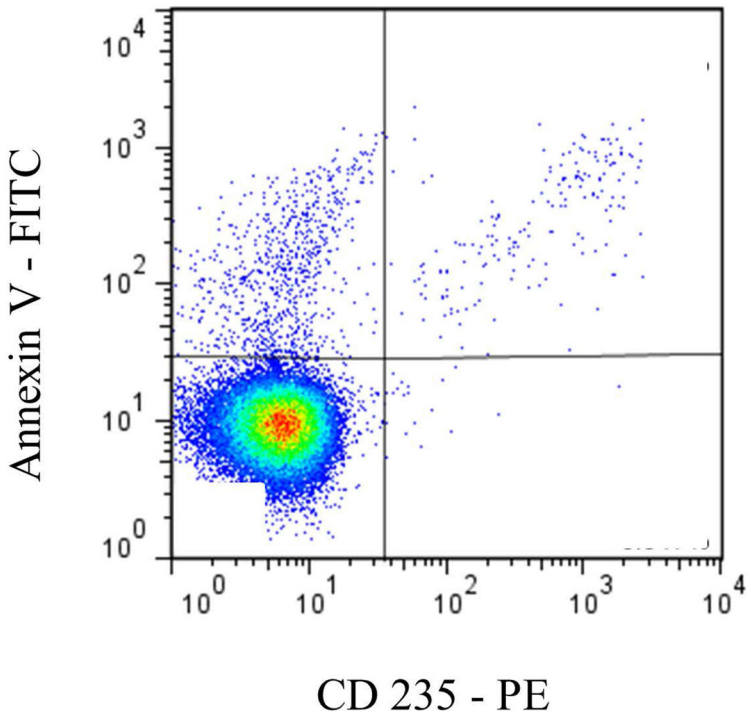

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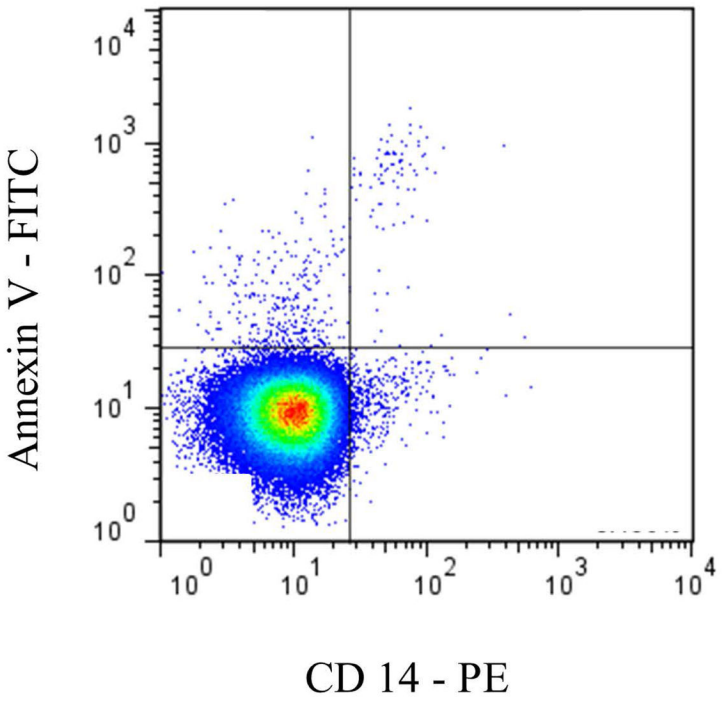

Figure 2.

Representative staining and gating strategies for A) platelet microparticles (PMP), B) red blood cell microparticles (RMP), and C) monocyte microparticles (MMP) analyzed in platelet free plasma on a Stratedigm S1000Ex flow cytometer. Fluorescent gating was performed within the MP size gate of 200–900 μm, which was initially set utilizing polystyrene beads (data not shown). PMP = dual positive Annexin V/CD41 events. RMP = dual positive Annexin V/CD235 events. MMP = dual positive Annexin V/CD14 events.

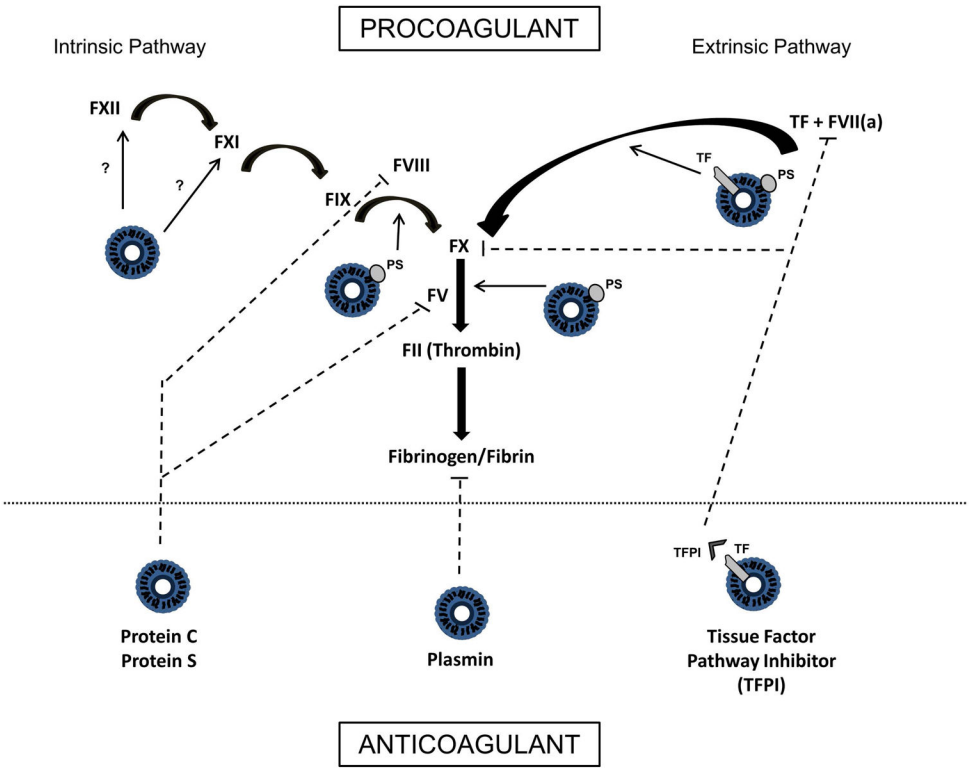

Figure 3. Multifaceted role of MPs in coagulation processes

Simplified schemata of the coagulation cascade showing the different potential contributions of MPs. MPs support coagulation through exposure of phosphatidylserine (PS), which provides a catalytic surface for assembly of the coagulation complexes. Tissue factor (TF) bearing MPs can activate coagulation through the extrinsic pathway. MPs may also support coagulation through the intrinsic pathway, although the mechanism by which this occurs is not fully known. Anticoagulant properties of MPs include the ability to support Protein C/ Protein S mediated regulation of coagulation, as well as tissue factor pathway inhibitor (TFPI) mediated inhibition of TF/VIIa activity and FX. MPs can also support plasmin generation, an enzyme that solubilizes and degrades clots. (Bolded arrows indicate activation steps [ie FXII activates FXI]. Dashed lines indicate inhibitory effects. Unbolded arrows emanating from MPs indicate areas of MP participation in coagulation activation processes.)

Table 1

Common antigens used to stain and identify specific MP subsets according to cell of origin

MP subtype	Antigen	Alternative Name (if applicable)
Platelet microparticles (PMP)	CD41 CD42a CD42b CD61* CD62P*	GPIIb GPIX

Table 2

Prothrombotic conditions with reported increased microparticles.

Prothrombotic condition	References
Sickle cell disease	[141–143]
Malignancy	[80, 82, 83, 86, 88–90, 93, 94, 97, 144]
Thrombotic thrombocytopenic purpura(TTP)	[145, 146]
Antiphospholipid antibody syndrome	[147–149]
Sepsis	[150–153]
Myeloproliferative disorders	[154–157]
Inflammatory bowel disease	[158–160]
Nephrotic syndrome	[161]
Paroxysmal nocturnal hemoglobinuria	[142, 162]
Systemic vasculitis	[163, 164]
Pregnancy/Preeclampsia	[165–168]
Systemic lupus erythematosus	[169–171]
Thrombophilia	[172, 173]
Trauma	[174–176]

Table 3

Overview of major challenges related to flow cytometric analysis of MPs.

Important Issues in MP Analysis by Flow Cytometry	
Pre-Analytical	**Analytical/Technical**
• Method of blood collection – Tourniquet use – Needle diameter – Type of anticoagulant • Sample processing – Sample type (whole blood, plasma, isolated MPs) – Time to sample preparation – Centrifugation protocol – “Micro-clot” formation • Sample handling and storage – Sample transportation/agitation – Fresh vs freeze/thaw	• Flow cytometer – Intrinsic resolution capabilities • Size gating – Beads vs biologicals • Fluorescence gating – Proper use of isotype controls (ITCs) – Detection of dimly expressed antigens – Titration of antibodies and ITCs – Fluorochrome aggregates • MP enumeration – Use of counting beads – “Swarm effect”