Microparticles in stored red blood cells: submicron clotting bombs?

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Introduction

More and more knowledge on blood microparticles (MPs) has been accumulated this last decade. However the role(s) of those small phospholipid vesicles still remain unclear. MPs, also named microvesicles or ectosomes, range from 0.1 to 1 μm in size, and are released in blood flow by various types of cells[1]. They are found in healthy individuals as well as in patients suffering from different diseases. The release of MPs is a highly controlled process prompted by various stimuli such as shear stress, complement attack, agonist (or pro-apoptotic) stimulation or damage[1,2]. If MPs were first described as cellular debris without any biological function[3], nowadays they are known as cellular effectors involved in numerous physiological processes such as haemostasis and inflammation[4], transfer of surface proteins[5], angiogenesis[6] or apoptosis[7]. Moreover, there is a link between MPs and several diseases such as thrombocytopenic disorder[8], thrombosis[1], cardiovascular diseases[9], diabetes[10], sickle cell disease[11], haemolytic anaemia[12] or sepsis[13], where an increase of the number of MPs in plasma has been demonstrated. Interestingly, a decrease in MPs numbers in plasma of patients presenting with the Scott syndrome, a rare bleeding disorder, has been reported[14].

MPs are also present, and accumulate, in blood products such as erythrocyte and platelet concentrates during storage[15-17]. Because the surface of MPs is made of negatively charged phospholipids, they may play a role in the coagulation cascade. Therefore, the purpose of this paper is to review the potential procoagulant effect of erythrocyte MPs and thus, their potential adverse effect after blood transfusion.

Microparticles and coagulation

Blood coagulation is an essential mechanism that prevents bleeding; this highly controlled process is carefully regulated in order to maintain blood circulation in case of injury while preventing vessels obstruction by clot formation. Coagulation factors, calcium ions and procoagulant membrane surfaces are the primary components involved in coagulation activation. In eukaryotic cells, including blood cells, it is established that neutral phospholipids (such as phosphatidylcholine) are mainly present on the outer membrane, whereas negative phospholipids (such as phosphatidylserine) are at the inner side of the membrane. Concerning blood cells various translocases are activated upon stimulation and phospholipid asymmetry is disrupted. Phosphatidylserine (PhSer) is externalized, modifying the neutral membrane charge into negative[18]. Activated blood cells and their MPs expose negative membrane and are thus thought to be procoagulant. The presence of negatively charged phospholipid membranes and calcium ions is required for the assembly of coagulation complexes such as tenase or prothrombinase. The role of tenase complex is to convert factor X (FX) into activated factor X (FXa), in both extrinsic and intrinsic pathways. The extrinsic tenase complex is composed of tissue factor (TF) and FVIIa[18] whereas the intrinsic tenase is composed of co-FVIIIa and factor FIXa[19,20]. The function of prothrombinase, constituted of co-FVa and FXa, is to convert prothrombin into thrombin. FIXa and FXa can convert their substrate without their respective cofactors, however negatively charged membranes catalyses the reaction, for example $3x10^5$ fold faster in the case of prothrombinase[21,22]. Figure 1 presents the possible site of actions of MPs on the coagulation cascade. The importance of negative phospholipids is to facilitate complex association, speeding up their association by around 1,000 fold for efficient haemostasis[23]. Because MPs express PhSer on their membrane, they could contribute to coagulation by providing catalyzing negatively charged membranes.

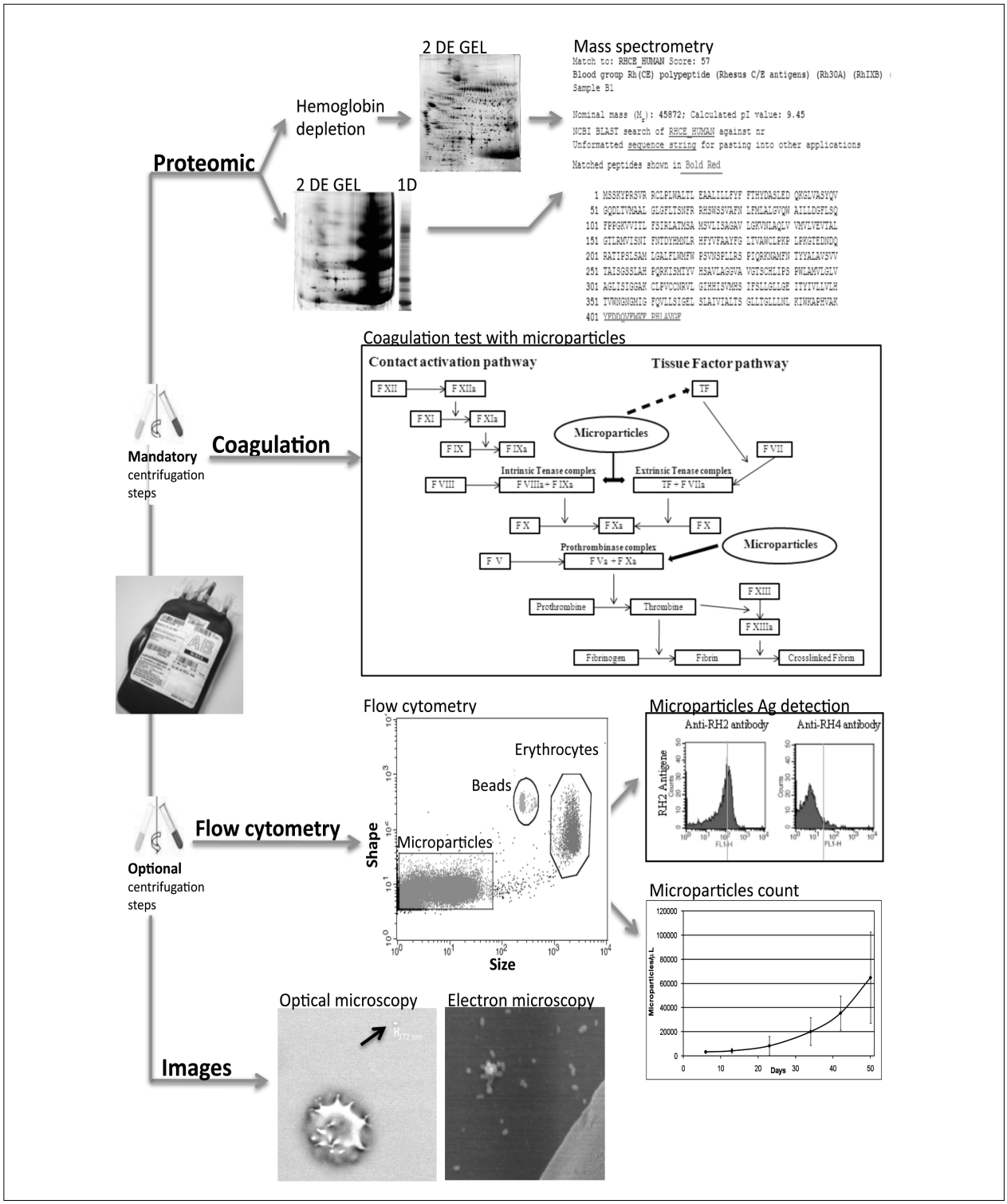

Figure 1 - General scheme of experiments on blood microparticles.
Proteomics, coagulation tests, flow cytometry and imaging by microscopy are the most common approaches to investigate microparticles. It is possible to study microparticles from various origins (whole blood, plasma as well as platelet or red blood cells concentrates). Of note, agonists such as calcium ionophore or protaglandine E2 are frequently used to induce cells microparticles release. Various centrifugation steps may be used to further isolate microparticles depending on the experiment condition. Indeed, in proteomics or coagulation tests, it is important to work on "purified" microparticles. *(continued)*

(*continued*) **Proteomics approach** - The characterisation of microparticle proteins is aimed to gain insights on the various biological processes involved in their production and function. Generally, after isolation of microparticles, they are directly solubilised in one-dimensional gel electrophoresis buffer. After migration and staining, protein bands are excised, digested by a proteolytic enzyme (usually trypsin), and identified by various mass spectrometry techniques. One-dimensional gel electrophoresis allows separating membrane as well as soluble proteins with a good compatibility to mass spectrometry analysis. Two-dimensional gel electrophoresis allows a better protein sorting, but this technique is poorly amenable to analysis of hydrophobic protein. In all technique, elimination of very abundant proteins, such as haemoglobin should be performed to detect the less abundant ones. This could be done by chromatography, "off gel electrophoresis" or by using "proteominer" techniques. As for one-dimensional gel electrophoresis, it is possible to excise spots of interest for identification by mass spectrometry. By using approaches described by Rabilloud *et al* [58] it is possible to improve the migration of hydrophobic proteins after two-dimensional gel electrophoresis, but the drawback of such approach is its lower compatibility with mass spectrometry. Therefore, most microparticle protein lists published were established using one-dimensional gel electrophoresis techniques followed by mass spectrometry. By this approach, we identified the presence of RHCE proteins in erythrocyte microparticle membranes, as showed on the mass spectrometry results presented in the scheme (a peptide corresponding to amino acids 401-417 from the RHCE protein was identified).

Coagulation approach - There are more and more evidences that microparticles are involved in coagulation. Therefore, different assays could be done on microparticles. Standard clinical coagulation tests could be done such as determination of the thrombin time, the activated partial thromboplastin time or new available tests allowing determination of FXa. These approaches will allow determining at what level microparticles are involved in clotting.

Flow cytometry approach - This is one of the most frequently used techniques to analyse microparticles. Indeed, it allows analyzing thousands of microparticles in one sample with determination of different markers and enables semi-quantitative analysis at the same time. For the semi-quantitative analysis, a precise number of fluorescent beads are used to determine the number of microparticles in a sample. Using the same number of beads, it is thus possible to compare the number of microparticles among different samples. Fluorescent cell-specific antibodies or annexin V (a phosphatidylserine marker) are used to tag microparticles and to differentiate them from the rest of the events. Using the antibody of choice and according to the type of microparticles, it is to detect and highlight surface antigens such as CD-235a, CD41, or even RH2 or RH4 (as presented on the scheme).

Imaging approach - Observation of microparticles by microscopy give information about their size and shape. Though numerous steps of preparation, scanning electron microscopy allows observing if microparticles are homogeneous in size or not. By confocal differential interference contrast microscopy, it is possible to observe forming microparticles from echinocyte.

It has been demonstrated that platelets-derived MPs have from 50- up to 100-times more procoagulant activity than platelets[24]. MPs membranes support more efficiently thrombin formation than platelet membrane when corrected for unit of surface, at least *in vitro*[4,25]. Not only platelet MPs are involved in this process, but erythrocyte MPs and other MPs have also a procoagulant activity through their negatively charged membrane as well[1,11,26].

Although controversial, some authors claim that MPs support clotting not only through their negatively charged membranes, but also by expressing an inactive form of tissue factor (TF) on their surfaces. The origin and mechanism of action of blood-born TF is still subject to debate, nevertheless authors have suggested that MPs may be a reservoir of blood-born TF[27]. Furie et al delineated a "*microparticles accumulation pathway*" as part of the coagulation cascade[25]. According to this model, some monocyte-derived MPs expressing TF in an "inactive" form bind to a forming clot in vessels and display active TF that helps to amplify coagulation. In an other hand, Connor et al proposed an activated factor X assay to measure clotting time of various samples containing MPs and their results show that there is a correlation between the number of MPs and clotting time; interestingly enough, the assay was insensitive to the presence or the absence of TF[28]. Other studies mention the presence of TF on endothelial-derived MPs[29], platelet-derived MPs[30,31], or even on erythrocyte-derived MPs[32], while some others did not detect any MPs-exposing TF[11]. All these pieces of evidence are difficult to explain, in particular because results often depend on the study design, methods of MPs isolation, and various pre-analytical factors; therefore one cannot exclude that discrepancies might result from different experimental methodologies[33].

Microparticles and hypercoagulability

Hypercoagulability state is associated with an increased risk of fibrin deposition in blood vessels, which could result in thrombus formation[34]. Hypercoagulable state has been often observed in case of chronic haemolytic anaemia's (sickle cell disease,

thalassemia, paroxysmal nocturnal haemoglobinuria, autoimmune anaemia)[35]. Frequently, there are erythrocyte membrane alterations with accumulation of negative procoagulant membranes in blood flow, platelet activation, and a decreased level of anticoagulant proteins, amplifying the risk of thrombosis[35]. In thalassemic patients, the main determinant of the thrombotic risk seems to be related to altered red blood cell membranes as well as to platelet activation, but not to plasma protein abnormalities[36]. Erythrocyte membrane alterations leads to the exposure of PhSer on the outer layer, cytoskeleton cleavage, membrane budding and release of MPs[8]. An elevated number of MPs in blood flow has been reported in patients suffering from different vascular diseases[1]. Consequently, MPs could have an essential causal role in hypercoagulable states, as reported by Van Beers et al in sickle cell disease[11]. The authors showed that, while the number of platelet-derived MPs was stable, there was an increase in erythrocyte-derived MPs during crisis compared to steady state.

Microparticles and red blood cells storage

Modification, alteration or even degradation of erythrocyte concentrates (ECs) occurs during storage (a phenomenon called the storage lesions)[7,37], and accumulating MPs have been considered as being one of the expression of storage lesions[15]. Red blood cells' ageing in blood bank conditions clearly differs from physiological *in vivo* ageing. Undeniably, conditions to which red blood cells are exposed during storage such as temperature and nature of the medium are dramatically different from *in vivo* conditions. Erythrocytes degrade and loose efficiency during storage. Indeed, erythrocytes change shape; their membranes become more rigid and there is a disruption of phospholipids asymmetry and release of fragments and MPs[37]. In addition, during the conservation of erythrocyte in plastic bags, not only physiological alterations occur, but also biochemical changes in the storage medium principally due to red cells metabolism. These modifications, physiological and biochemical are part of the storage lesions. There is an increase in the concentration of lipids, free haemoglobin, potassium, lactate and in contrast a reduction of pH, glucose, 2,3-diphosphoglycerate, sodium or adenosine triphosphate[38]. For example diminution of glucose and increase of lactate concentration reflects red blood cells glycolysis.

In blood flow, MPs are rapidly removed in the liver by Kupffer cells[39]. Release of MPs occurs throughout erythrocyte lifespan[39] and continues during storage, therefore they accumulate during storage[17]. Precise reasons of erythrocyte vesiculation are not elucidated, however MPs is a possible mechanism preventing red blood cell removal. MPs may help to clear away the C5-9 complement attack complex, band 3 neoantigen, IgG or other potential harmful agent from the membrane, when the erythrocyte is still viable[40-42]. In addition, MPs formation could be a mechanism involved to clear out methaemoglobin which is no more functional[37].

The precise consequences of storage, including storage lesions and MPs release, on transfusion efficiency are not clearly understood, and relatively little is known about their molecular bases and even less about their possible biomedical consequences on recipients. There is accumulating evidence that receiving "old" blood is not as advantageous as receiving fresher blood. In 2008, Koch et al showed a link between the age of the transfused ECs and post-transfusion complications. They claimed that life expectancy at 5 years was better for patients who received fresher blood (ECs stored for less than 14 days) as compared to older blood (stored for more than 14 days) in the course of cardiac surgery[43]. A recent study by Spinella et al concluded that ECs stored for more than 28 days was associated with an increased incidence of deep vein thrombosis and death from multi-organ failure[44]. Other studies on post-transfusion complications or mortality and storage time were also published and controversial[45-47]. However, although these studies raise important questions about transfusion safety and storage lesions, they are subjected to many discussions[48-51].

Due to the fact that transfused people are sick and polytransfused recipients are often in poor condition, it is difficult to attribute clearly an event to transfusion rather than on the clinical situation of the recipient. In a recent review, Zimrin and Hess analyzed several papers on clinical studies examining the effect of storage of red blood cells in transfusion and concluded that well designed epidemiologic studies are needed to demonstrate that a clear clinical effect may be due to ECs storage lesions. Despite the lack of conclusive

evidence that storing blood longer is worse, it is current practice to transfuse fresher blood to patient considered at high risk (such as in neonatology for example or cardiac surgery).

As stated before, MPs may have procoagulant activities; therefore, we may reasonably suggest that transfusion of "older" ECs containing a high number of MPs could increase the risk of adverse reactions, by inducing a hypercoagulable state leading to thromboembolic complications. Inversely, in many situations requiring blood transfusion, a hypercoagulable state may be useful to diminish or even helping to stop the bleeding.

Proteomics and microparticles analysis

Recent advances in proteomics provide new tools allowing studying protein content of MPs in order to elucidate their exact role. Several publications are focused on proteomics of MPs derived from various type of cells (see Table I). Results are different according to the MP cell types and the methodological aspects. To the best of our knowledge, most studies have adopted the same workflow for proteomic analysis of MPs as shown in the "proteomic" part of figure 1 (excepting for the initial sample that is not ECs in all cases). Briefly, once isolated by centrifugation, MPs are directly separated by one-dimensional gel electrophoresis (1D-GE) and finally protein bands are excised, and further digested by a proteolytic enzyme (usually trypsin) and identified by various mass spectrometry techniques. The main advantage of this technique is the compatibility with the analysis of hydrophobic proteins in contrast to two dimensional electrophoresis gel (2D-GE) approaches. Among different proteomic studies on MPs, various lists of MP proteins have been established. Mapping of MP proteins involved in coagulation is still lacking. In the study of platelet MPs proteome by Garcia et al[52] when the list of proteins is submitted to a QuickGO search with the GO reference "blood coagulation", 12 proteins out of 578 were found to be involved in blood coagulation. Recently, Bosman et al[53] established a reference proteome of red blood cells MPs. When the protein

Table I - Overview of studies on proteins of microparticles reported in the literature

Type of MPs	Theme	Technique	Main Results	Ref.
Red blood cells	Red blood cell membranes and MPs proteome	Centrifugation, LC-MS/MS	Storage-dependent changes in the RBC membrane proteins. MPs display similar change in their protein composition during storage.	53
Red blood cells	MPs during storage of EC	Centrifugation, Flow cytometry, 1 D SDS PAGE, Western Blot, MALDI-MS	Number of MPs increase during storage. Proteomic analysis revealed changes of protein expression comparing MPs to erythrocyte membranes.	17
Plasma	Proteome of Plasma MPs	Centrifugation, Flow cytometry, 2 D-GE, MALDI-MS	Comparison between plasma and MPs 2D-GE and LC-MS/MS to list MPs proteins. From the spotted sample, 83 different proteins and their respective isoforms were identified in which 13 have never been reported in human plasma.	55
Platelet	Proteome of platelet MPs	Centrifugation, 1D SDS-PAGE, LC-MS/MS	The identification of 578 proteins of platelet MPs was accomplished among which 380 were not identified previously in platelet proteome	52
Endothelial	Proteome of endothelial procoagulant MPs	Centrifugation, 1D SDS-PAGE, LC-MS/MS	This study describes the protein composition of endothelial cell MPs. Among proteins, presence of metabolic enzymes and bioactive effectors has been evidenced.	56
Malignant lymphocyte	Characterization the T-lymphocytes MPs proteome	Centrifugation, 1D SDS-PAGE, LC-MS/MS, MALDI-MS	In total, 390 proteins were identified, 34% of those proteins were from plasma membrane among which proteins involved in hematopoietic clusters of differentiation.	57

list was submitted to a QuickGO search with the same parameters as mentioned above, 5 out of 308 proteins were involved in coagulation, namely phospholipid scramblase 1, plasminogen precursor, fibrinogen beta chain precursor, complement component C9 precursor and beta-2-glycoprotein 1. Except for phospholipid sramblase 1, proteins were neither identified in red blood cells membrane proteome established by Bosman et al[53] nor by Pasini et al[54]. As procoagulant proteins of MPs were not found on red blood cells, we could hypothesize that those proteins were either of plasmatic origin with a non-specific binding to MPs or were proteins present on red blood cell membranes, but enriched on MPs microdomains.

Discussion

The important issue raised by the presence of MPs in blood products is to determine their exact involvement for the recipient. It has been demonstrated that MPs are present in blood products, and that they number increases during storage. Series of articles and reviews were published on the association between red cell storage duration and complications after transfusion. However few of them mentioned MPs as potential cause of these complications. Therefore, there is an urgent need of large clinical studies with the aim to determine precise effects (if any) of MPs in blood recipients. Furthermore, it is important to determine how MPs are involved in the coagulation cascade, by either passively acting by providing additional negatively charged membrane surface, or rather by allowing the expression of TF or other proteins implicated in the coagulation process. Furthermore, studies of MPs from platelets, endothelial cells or monocytes and comparison with EC-derived MPs are needed. Proteomic analysis, as well as quantitative proteomic evaluation of they protein content will open new avenues in haematology, because it will help to decipher the complexity of the hypercoagulable states and the effect of EC transfusion in routine clinical practice. Combining various approaches that are haemostatic investigations, MPs characterisation either by cytometry or by proteomics, clinical studies will certainly help to secure transfusion medicine.

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