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Microparticles in sickle cell disease

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Abstract

Several pathophysiological pathways in sickle cell disease (SCD), the most prevalent hemoglobinopathy worldwide, result in activation of circulating blood cells and the release of submicron vesicles, so-called microparticles (MPs). MPs are candidate biomarkers in vascular disease that exhibit functional biological properties. Compared to healthy individuals, higher level of plasma MPs, mostly derived from platelets and red blood cells (RBC), has been repeatedly observed in SCD patients in their steady-state condition. In contrast, conflicting results have been obtained on the impact of SCD complications and hydroxyurea treatment on circulating MP concentrations, largely due to non-standardized pre- and analytical procedures. Several factors responsible for the increased release of MPs by RBC have been identified in SCD such as sickling/unsickling, oxidative stress and abnormal activity of RBC acid sphingomyelinase. Besides their well-known pro-coagulant effect, sickle RBC-derived MPs produced *ex vivo* can induce ROS production by endothelial cells and promote a pro-inflammatory and pro-adhesive phenotype that may lead to renal occlusion in SCD mice. However, the functional properties of circulating MPs in human sickle cell disease remain to be studied and fully characterized.

Introduction

Sickle cell disease (SCD) is a group of genetic disorders that have in common the production of the abnormal hemoglobin S (HbS) instead of hemoglobin A. HbS occurs as a result of a single base mutation in exon 1 of the β-globin gene that causes the substitution of valine for glutamic acid at the sixth position of the β-globin molecule. Sickle cell anemia (SCA) refers to the disorder produced by homozygous HbS inheritance, whereas co-inheritance of HbS and hemoglobin C or β-thalassemia are at the origin of the two other sickle cell syndromes most commonly encountered, *i.e.* hemoglobin SC (HbSC) disease and hemoglobin S/β-thalassemia (HbS/β-thal) [1].

In deoxygenated conditions and after a delay period, HbS molecules polymerize inducing the sickling of affected red blood cells (RBC) and leading to decreased deformability and increased fragility. Sickle RBC, which exhibit abnormal adhesive properties to endothelial cells [2], do not easily traverse the microcirculation, causing frequent vaso-occlusive episodes [3]. Vaso-occlusion is not only due to sickling of RBC, but also to endothelial-leukocyte-RBC interactions, and is enhanced by inflammatory processes and endothelial-leukocyte-RBC interactions [4,5]. Vascular obstruction and ischemia are followed by a restoration of blood flow, which further provokes reperfusion injury. These cycles of hypoxia-reperfusion cause oxidant and inflammatory stress, which increase the expression of several endothelial cell adhesion molecules [5]. Furthermore, sickle RBC exhibit a reduced lifespan associated with intravascular hemolysis, leading to the release of free Hb and heme into the blood. These bioactive molecules are responsible for the decrease in nitric oxide (NO) bioavailability, which may be involved in several sickle complications such as priapism, leg ulcer, pulmonary hypertension and stroke [6,7]. Currently, hydroxyurea (HU) is the only drug known to reduce

the occurrence of some complications such as painful vaso-occlusive crisis (VOC), acute chest syndrome (ACS) and the need for transfusion [8,9]. Originally prescribed to SCA patients because of its ability to induce expression of fetal hemoglobin (which is known to alleviate HbS polymerization), it has subsequently been shown to possess pleotropic effect that in aggregate contribute to its therapeutic efficacy [10].

Several of the pathophysiological pathways known to be associated with SCD, such as oxidative and inflammation stress, may activate circulating blood cells and lead to the release of sub-micron vesicles, named microparticles (MP) [11]. During the last two decades, a large number of studies in the SCD field have been performed to better describe the quantitative and qualitative profile of MPs in SCD patients and their involvement in SCD pathophysiological processes. In this review, we will focus on the characteristics and functional properties of plasma MPs and present evidence that MPs could be both biomarkers and bio-effectors in SCD.

Microparticles: definition and genesis

MPs fall under the umbrella term of extracellular vesicles (EV), which is used to designate all types of vesicles released from cells, the best characterized being exosomes, apoptotic vesicles (also called apoptotic bodies), and MPs [12]. These various types of EV differ in regards to several characteristics such as size, density, morphology, composition (protein, lipid and nucleic acid) as well as sub-cellular origin [12]. It should be noted that the overlap of some of these parameters between EV sub-types has been associated with technical purification difficulties and thus the ability to attribute particular functions to each subtype [12]. Nevertheless, there is accumulating evidence that EV carry diverse cargoes including

proteins, RNA species such as mRNA and miRNA and lipids that can be transported and exchanged between cells, strongly suggesting that EV play key roles in cell-cell communication at both paracrine and systemic levels [13,14].

MPs are defined as phospholipid microvesicles with a diameter ranging from 100 to 1,000 nm that are derived from the cytoplasmic membrane of cells submitted to stress conditions that result in apoptosis or activation. These conditions lead to local cytoskeletal rearrangements and membrane budding [15,16]. Indeed, the increase of intracellular Ca^{2+} induced by these conditions affects the function of three enzymes, namely floppase, scrambase and flippase that are involved in the asymmetry of the cellular lipid bilayers, and leads to the externalization of phosphatidylserine (PS) [17,18]. PS exposure is believed to be a key event in MP formation. Indeed, Scott syndrome, characterized by impaired ability to externalize PS and linked to genetic defects of several enzymes involved in cell PS exposure, has been associated with a decrease of MP shedding and a bleeding disorder [19,20,21]. Moreover, the rise of intracellular Ca^{2+} activates proteases that cleave the cytoskeleton, weaken its interaction with the cytoplasmic membrane, thereby allowing the release of MPs [22]. Accordingly, MPs are usually described as exhibiting PS externalization, although MPs without externalized PS have also been described [23].

The protein composition of MPs reflects that of the cell from which they are derived, including cell-type specific antigens which allow the identification of their cellular origin. Moreover, MPs also contain functional molecules that could have been induced on the parental cell by the factor responsible for triggering MP release [24,25]. Thus, both cell origin and nature of trigger(s) impact the phenotype of MPs and their functional properties. It has been shown for example that the level of externalized PS and the type of membrane receptors

exhibited by endothelial cell-derived MP vary according not only to the triggering factor, namely apoptotic stimuli or tumor necrosis factor, but also to the vascular origin of the endothelial cells [25].

MPs have been detected in multiple biological fluids including urine, broncho-alveolar lavage fluid, sputum, synovial fluid, ascites, saliva and plasma [26]. In physiological conditions, it has been shown that plasma samples contain platelet-, erythrocyte-, endothelial cell- and leukocyte-derived MPs [22].

Functional properties of MPs

A large number of functional properties have been associated with MPs. The first and probably best described property is the ability to promote coagulation [27,28]. Two physical characteristics of MPs have been linked to their procoagulant activity. The externalization of PS results in a negatively charged surface, allowing assembly of coagulation factors and thrombin generation [29]. MPs released from endothelial cells and monocytes may also display tissue factor (TF) on their surface and thus support coagulation via the factor VII (FVII)/TF-dependent pathway [24,30]. Furthermore, it has been shown that MPs from leukocytes can transfer TF to platelets and contribute to the recruitment of cells and the accumulation of TF at sites of vascular injury [31]. Furthermore, tumor-derived MPs are known to exhibit procoagulant properties [32]. In agreement with their described procoagulant properties, high MP concentrations have been detected in various clinical conditions associated with increased incidence of thrombosis including paroxysmal nocturnal hemoglobinuria [33], cardiovascular disease [34] and deep venous thrombosis [35].

Various blood cell-derived MPs have also been shown to regulate the production of reactive oxygen species (ROS) and thus oxidative stress level. MPs shed by endothelial cells [36], monocytes [37] and lymphocytes [38] promote endothelial O_2^- and H_2O_2 production in cultured endothelial cells through processes involving different enzymatic systems, and thus may induce apoptosis [39].

Pro-inflammatory stimuli provoke the release of MPs, which in turn may directly contribute to the inflammatory response. For instance, MPs derived from polymorphonuclear leukocytes or from monocytes promote the production of IL-6/MCP in cultured endothelial cells [40] and IL-8/MCP in airway epithelial cells [41], respectively. The effects of MPs seem to be cell type-dependent since it has been shown that macrophages are inhibited upon incubation with MPs released from polymorphonuclear leukocytes [42,43] suggesting that some MPs may exhibit both pro- and anti-inflammatory properties.

MPs from various blood cell types may also induce a pro-adhesive phenotype to endothelial cells. In this respect, treatment of endothelial cells with platelet- and endothelial cell-derived MPs are associated with increased expression of cell adhesion molecules and monocyte-endothelial cell interactions [36,44].

In addition to previously cited biological pathways, MPs have been implicated in the regulation of angiogenesis, vascular function and apoptosis [45,46].

However, it should be noted that most of the previously cited studies addressing these mechanisms have been performed with MPs generated *in vitro*. Since the biological content

and the properties are related to the triggers leading to their release, the functional properties of circulating MPs produced *in vivo* need to be confirmed.

Mechanisms of MP-mediated biological effects

In contrast to coagulation, the molecular mechanisms involved in the other biological effects mediated by MPs remain elusive and alternative putative processes have been proposed.

Several studies have documented direct physical interactions of MPs with their target cells [36,47,48]. Triggering of signaling pathways may result from binding of MP surface antigens to their specific counter-receptor. For example, the induction of monocytic cells adhesive receptor expression by endothelial-derived MPs could be inhibited by treatment with antibodies directed against ICAM-1, an adhesive receptor expressed by MP-derived endothelial cells, and its counter receptor, beta2 integrin expressed by monocytes [49]. Complementary experiments strongly suggested that the induced pro-adhesive phenotype of monocytic cells did not involve membrane fusion, but rather receptor binding [49]. Alternatively, the binding of MPs to target cells may change the panel of exposed antigens and thus modify functional properties of these cells. For instance, such a mechanism has been proposed for the increase of leukocyte-leukocyte interactions mediated by platelet-derived MPs [50]. The fusion of MPs with cells and the subsequent transfer of their content has also been proposed to be involved in the cellular relocation of functional membrane receptors [51] and bioactive substances such as lipids [44,52] shown to mediate several MP functional properties.

MPs may contain enzymes with reactive oxygen species (ROS)-generating capacity [53,54]. Thus, they have the potential to directly produce ROS known to be involved in several pathways mediated by MPs such as apoptosis, inflammation and endothelial dysfunction [55].

However, the *de novo* production of ROS by MPs remains to be proven. In contrast, it has been unambiguously shown that MPs derived from erythrocytes may scavenge nitric oxide (NO) almost as efficiently as plasma hemoglobin because of their hemoglobin high content [56,57].

In aggregate, these studies strongly suggest that the diverse biological effects induced by MPs are mediated by several distinct mechanisms.

Characterization of MPs

A large number of approaches have been used for MP characterization and detection including flow cytometry, solid-phase methods followed by enzyme-linked immunosorbent assay or functional assay, electron microscopy, atomic force microscopy, nanoparticle tracking analysis, dynamic light scattering and tunable resistive pulse sensing [26,58,59]. Each of these approaches has inherent advantages and limitations; however, since this topic was recently reviewed, it will not be discussed further in the present report [60,61,62]. Up to now, the most widespread technique used to analyze MPs is flow cytometry. This approach allows rapid determination of cellular origins and enumeration of MPs, and is available at most research facilities. However, flow cytometry has its own limitations, in particular for the detection of small vesicles [58], although recent generation flow cytometers are capable of detecting MPs as small as approximately 0.2 μm in diameter [63]. In addition to differences of flow cytometer characteristics, it has been clearly established that analytical and pre-analytical procedures such as sample preparation are important sources of variability [61]. Indeed, the lack of consensus in these variables contributes to the difficulty in drawing firm conclusions about MP characteristics and roles in clinical conditions such as SCD [11].

Quantitative and qualitative microparticles pattern in sickle cell disease

Compared to healthy individuals, a 3- to 4-fold increase of plasma MPs has been repeatedly reported in SCD patients at steady-state condition, i.e. remote from acute complications, by multiple groups [11] using either flow cytometry [64 - 71] or solid-phase methods [72]. In these studies, MPs were found to derive mainly from platelets and RBCs; those originating from other blood-cell types such as endothelial cells, monocytes or granulocytes were either detected at variable levels or were undetectable [65]. Surprisingly, one group reported lower concentrations of MPs in SCD patients compared to controls [73].

Multiple reasons could account for these discordant results. Since several of these studies were performed in SCD patients with a variety of sickle cell syndromes, i.e. SCA, HbSC disease or S/β-thal [64 – 66,71,73], it is tempting to speculate that these discrepancies could be related to the proportion of patients with each syndrome. However, a recent study performed on 180 SCD children -- 84 with HbSC disease and 96 with SCA -- showed that although the former group exhibited lower blood MP concentrations, resulting mainly from a decrease of MPs originating from RBC and to a lesser extent from platelets, no significant difference in the numbers of MPs derived from other blood cells between the two groups was detected [74]. To our knowledge, no study dedicated to patients with S/β-thal has been conducted. Therefore, the variability of the cellular origins of MPs in SCD patients reported in the literature is most likely the consequence of non-standardized pre-analytical and analytical procedures, known to be critical factors impacting MP pattern, both quantitatively and qualitatively [11].

The clinical course of the disease may also affect MP concentration. So far, conflicting results have been obtained on the impact of SCD complications on circulating MP concentrations.

Six studies described increased numbers of MPs during sickle cell crises compared to steady-state disease [64,66,71,72,73,75], while van Beers EJ *et al* detected similar MP concentrations in both conditions [65]. In addition, the cellular origins of MPs for which an increased concentration was observed varied between studies. Several important parameters differed between these studies, including cross-sectional or longitudinal design, clinical definition of sickle crisis or delay between blood sampling and hospital admission, among others. The largest longitudinal survey published so far, with 32 SCA patients, showed a 2-fold increase in the concentration of RBC-derived MPs during painful vaso-occlusive crisis [75]. Such an increase in MPs originating from RBC has also been reported by three other groups [66,71,73]. However, further studies are warranted to better document the quantitative and qualitative changes in MPs during SCD-related complications.

High MP concentrations were reported in SCD patients exhibiting a severe vaso-occlusive phenotype [66,69] and a past history of complications such as acute chest syndrome, pulmonary hypertension [66] or osteonecrosis of the femoral head [76]. Nevertheless, such associations with previous vaso-occlusive complications, acute chest syndrome or pulmonary hypertension have not been reproduced in other studies [74], while the usefulness of MPs as biomarkers of osteonecrosis need to be confirmed.

Hydroxyurea (HU) effect on MP profiles

The effect of HU therapy on circulating MPs remains unclear. Indeed, HU treatment has been associated with either reduced plasma concentration of MPs, mainly those originated from RBC and platelets [66,69,77], increased levels of MPs [73,78] or no change in MP concentration [71]. Since MP concentrations exhibit high inter-individual variability, the cross-sectional design of the previously cited studies may account partly for these inconsistent

results. Currently, only one longitudinal study of patients treated with HU has been performed demonstrating similar MP concentrations before and after 2 years of treatment [79]. Interestingly, modification of two quantitative flow cytometry parameters of MPs shed by RBC, namely forward scatter (FS) and mean fluorescence intensity (MFI) of Annexin V (related to MP size and density of phosphatidylserine at the membrane surface, respectively), were detected in this study. HU-treated patients exhibited significantly higher FS indices and lower MFI values compared to values before treatment. Moreover, the relationship between these two parameters and HU dose strengthens the hypothesis of an HU-mediated effect. Further studies are needed to confirm these observations and, more importantly, to analyze their potential functional consequences.

Triggering factors of MP release in sickle cell disease

Several factors responsible for the increased release of MPs have been identified in SCD. The best characterized are those targeting RBCs and platelets, while those affecting the other blood cell types have been less studied.

Sickle RBCs were the first pathologic cell type to be identified as a source of MPs [80]. Indeed, it has been established that repeated RBC sickling/unsickling induce the shedding of MPs due to uncoupling between the membrane skeleton and the lipid bilayer, resulting partly from RBC membrane protein oxidation [81 - 83]. These oxidative processes, as well as sickling of RBC, are the main driving forces of intravascular hemolysis. Therefore, it is not surprising that several studies have detected relationships between RBC-derived MP concentration and Hb, as well as expression of hemolytic parameters [65,69,77,84].

Another mechanism induced by alteration in the RBC membrane is involved in the generation of sickle RBC-derived MPs. Mechanical stress in RBC, exacerbated in SCD, activates

sphingomyelinase, an enzyme implicated in membrane stability, vesiculation and MP formation [85,86]. A recent study showed that the abnormal activation of acid sphingomyelinase in sickle RBCs enhanced the shedding of MPs by these cells [87].

In addition, it has been shown that thrombospondin-1, a major platelet protein detected at high level in sickle plasma [88], triggers *in vitro* erythrocyte conversion into spicule-covered echinocytes and induces RBC-derived MP shedding [89].

Several mechanisms responsible for enhanced MP release by platelets in sickle patients have been characterized. Free hemoglobin, detected at high levels in SCD patient blood as a consequence of intravascular hemolysis, is clearly a major factor contributing to platelet activation in SCD by limiting the bioavailability of NO, a molecule known to inhibit platelet activation [90,91]. More recently, it has been shown that free HbS may directly participate in platelet activation. Indeed, its binding to GP1bα on the platelet surface induced activation via the Lyn, PI3K, Akt and ERK pathway with associated shedding of MPs [92]. Lastly, the release of intra-erythrocyte ADP by intravascular hemolysis may also lead to platelet activation [93].

MP formation from other blood cell types is presumably the consequence of several pathophysiological processes such as hypoxia/reperfusion, inflammation, and oxidative stress, among others.

Microparticles in sickle pathophysiology

The involvement of MPs in pathophysiological processes of SCD including coagulation, inflammation and abnormal cellular adherence has been proposed in several studies.

Different cell type-specific MPs may trigger coagulation through TF-independent or TF-dependent mechanisms. The former mechanism may involve MPs originating from RBCs and platelets, the two most abundant circulating MPs in SCD that do not express TF but exhibit docking sites for activated clotting factors; indeed, an accelerating effect on thrombin generation was observed with RBC-derived MPs in *in vitro* assays [67,94]. Accordingly, positive correlations between RBC-derived MP concentrations and expression of coagulation markers such as prothrombin fragment F1+2 and D-dimer as well as acceleration in the propagation phase of thrombin generation have been observed [65,67]. Interestingly, a recent study presented evidence that the density of externalized PS, assessed by the mean fluorescent intensity of Annexin V, is higher for RBC-derived MPs compared to platelet-derived MPs [74]. On the other hand, initiation of coagulation by TF-bearing MPs was supported by the inhibition of clotting activity of sickle MPs in normal plasma using a neutralizing antibody to TF, as well as the correlations between TF positive MP numbers and levels of circulating coagulation markers [64]. Despite these discrepancies, these data strongly suggest that MPs of various cellular origins in SCD are bio-effectors involved in the hypercoagulation state and thrombosis, both considered a leading cause of death and significant contributors to vessel occlusion in SCD patients [95 - 97].

Other functional properties for sickle RBC-derived MPs have been documented. Injection of RBC-derived MPs, produced *in vitro* from sickle RBCs, in SCD mice induced renal vaso-occlusion [89]. *In vitro*, these MPs induced ROS production by endothelial cells, endothelial apoptosis and a pro-adhesive phenotype. A subsequent study by the same group demonstrated heme docked by externalized PS in MPs could be the dominant driving factor of these biological effects, which also included compromised microvascular dilation [98]. In addition, these MPs could be internalized by monocytes, where they promoted the production and

secretion of some proinflammatory cytokines and enhanced monocyte adhesion to endothelial cells [87]. Although these data strongly suggest functional links between RBC-derived MPs with pathologic processes in SCD, it is worthwhile to note that these studies have been performed with MPs generated *in vitro* and thus which may not fully recapitulate the properties of circulating MPs.

Conclusion

This overview describes the current state of knowledge regarding MP formation, structural characteristics, biological impacts and factors which affect genesis and function of MPs derived from blood cells in SCD. Despite a significant volume of studies, uncertainties remain including the precise quantitative and qualitative pattern of circulating MPs in SCD, at steady state, during acute SCD complications and in SCD patients treated with HU. These unsolved points illustrate the need for standardized procedures and improved protocols for MP purification and analysis. Carefully designed studies based on large and well-characterized SCD cohorts are currently needed to clarify the biomarker status of MPs in SCD. Furthermore, the functional properties of circulating MPs, in addition to those generated *in vitro*, remain to be resolved.

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