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Review

Red blood cell-derived microparticles: An overview

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ABSTRACT

The red blood cell (RBC) is historically the original parent cell of microparticles (MPs). In this overview, we describe the discovery and the early history of red cell-derived microparticles (RMPs) and present an overview of the evolution of RMP. We report the formation, characteristics, effects of RMP and factors which may affect RMP evaluation. The review examines RMP derived from both normal and pathologic RBC. The pathologic RBC studies include sickle cell anemia (SCA), sickle cell trait (STr), thalassemia intermedia (TI), hereditary spherocytosis (HS), hereditary elliptocytosis (HE), hereditary stomatocytosis (HSt) and glucose-6-phosphate dehydrogenase deficiency (G6PD).

Contents

1. Early history

The earliest descriptions of MP were obtained from normal RBC (RMP) and from sickled RBC (SRMP). Microparticles derived from normal RBC were initially observed microscopically in the early 1900s. An example of the studies is shown by the classic work of Auer in 1933 in

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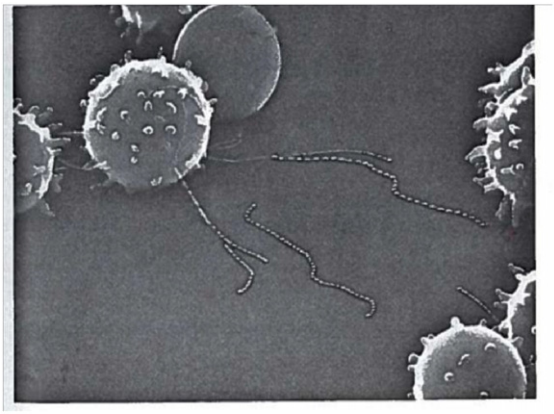
which he observed beaded and unbeaded 'filaments' shed from normal RBC which eventually formed single RMP [1]. Similar studies by other researchers were done during that period, showing similar findings [2,3]. A study by Bessis [4], using electron microscopy, described the transformation of mature RBC to budded RBC and microparticles (Fig. 1) as well as showing extracellular beaded strings and single RMPs (Fig. 2). Jensen, in 1973, using a laser beam for microincision of spiculated sickled RBC showed the development of beaded strings and 'round bodies' [5]. RMP production was later observed cinematographically while studying sickle RBC during deoxygenated and exposure to the sickle/unsickle cycle [6]. The earliest definitive studies of RMP were done in 1975 and 1976 by Allan and Michell in which RMP, were spectrin-free (skeletal-free), and were derived from normal human RBC. The investigators isolated, described and characterized the RMP. Isolation was obtained using the Ca^{2+} ionophore A23187 [7,8,9]. From their work, studies of cell-derived microparticles were begun. Lutz in1977 described the release and characterization of spectrin-free vesicles during ATP depletion [10].



Source: Bessis M, Mandon P. La microspherulation et les forms myelinques des globules rouges. Nouvelle Revue Francaise d'Hematologie. 1972;12: 443-454 [4].

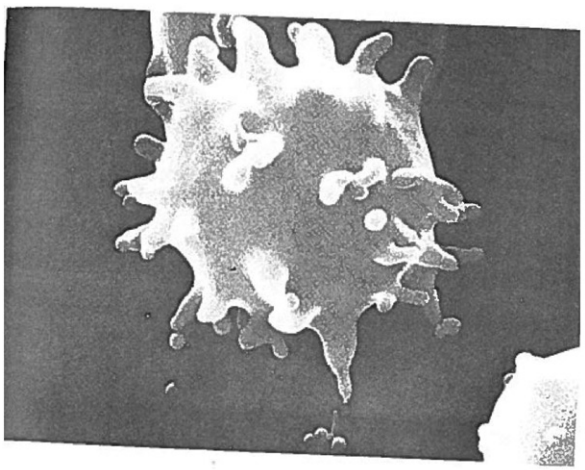
Fig. 2. Myelin forms as seen by electron microscopy.

2. Microparticles shed from normal RBC

2.1. Formation and release

Formation and release of RMP occur with activation or eryptosis of RBC. The process enables the clearance of senescent RBC. Normally, the RMPs are released as budded fragments from the RBC membrane undergoing stimulation or eryptosis. The process may be considered a primitive response to cell stress [11,12] and is thought to reflect a balance between cell stimulation and death. Specific stimuli known to induce RMP formation include activation of cells by oxidative injury, endotoxin, cytokines, complement, and high shear stress [13].

Several studies have described RBC changes which may contribute to the process leading to the formation and release of RMP. A study by Bosnan et al. [14] suggests that the RBC aging process harbors a specific band 3-centered mechanism for RMP generation which also allows for recognition and fast removal of the RMP by the immune system. Data by Salzer [15] has suggested that the calcium-induced specific enrichment of stomatin, synexin and sorcin in RMP indicates that these proteins are essential for the vesiculation process. Observation by Wagner [16] considers that the mechanism of vesiculation may be related to physical distortion of the RBC membrane by factors which affect cell membrane phospholipid organization [17].



Source: Bessis M, Mandon P. La microspherulation et les forms myelinques des globules rouges. Nouvelle Revue Francaise d'Hematologie. 1972;12: 443-454 [4].

Fig. 1. Budded RBC and microparticles.

2.2. Characteristics

The morphology, size, phenotype and concentration of RMP have been described by Arroud [18]. The study showed that morphologically RMP may take three shapes; spherical, tubular and large fragments. Approximately 95% of the RMPs are spheroid (buds) with diameter sizes between 100 nm and 300 nm. The measured diameter of RMP depends on the method of measurement. Measurement by a laser particle sizer is 300 nm in length [16].

2.3. Composition

RMP composition consists of residual hemoglobin, lipid and protein that contribute to the various biologic effects of the RMP. The amount of hemoglobin retained in RMP is approximately 20% of the amount of hemoglobin observed in the circulating RBC [19]. The loss of hemoglobin from the parent cells occurs during the total lifespan of the RBC [20].

The lipids in the RMP are in the form of stomatin-specific lipid rafts. The rafts are enriched in glycosylphosphatidylinositol-linked (GPI) proteins and are depleted of the major transmembrane proteins. The RMPs also contain the proteins synexin and sorcin and are highly enriched in stomatin, which is the major protein of the microvesicular rafts [15].

RMPs contain half the amount of membrane protein found in intact RBC membranes, when referred to phospholipid phosphorous content, due to the absence of spectrin. The content of glycophorin and cholesterol remains the same as in intact RBC membranes. The major integral proteins are present in RMP in similar quantities. Acetylcholinesterase activity is enriched twofold [10].

Phospholipid composition of the RMP is similar to that observed in the intact RBC membrane except for phosphatidic acid which is increased (10-fold) and for phosphatidylethanolamine (PE), which is slightly lower than in intact RBC membranes [10]. The exposure of phosphatidylserine (PS) and PE results in a loss of membrane asymmetry [21]. Although PS and PE exposure can result in a general loss of membrane asymmetry, there may also be other mechanisms of PS and PE exposure on RMPs that vary by cellular source [22].

The composition of RMPs induced by ATP depletion resembles the composition of RMPs which were induced, in vitro, by treatment with the Ca^{2+} ionophore, A23187 [7]. The main constituents of the RMP

membrane structure, as shown by electron microscopy, consist of two bimolecular leaflets covered with globin [23,24,25].

2.4. Clearance

Clearance of RMP occurs by several mechanisms. The RMP may be cleared by binding to the macrophage's scavenger receptors of the organ in which they originated, or by senescent neoantigen-specific autoantibodies, and eliminated through the mononuclear phagocyte system [20]. The Kupffer cells of the liver are the rapid clearance site in which removal occurs within 5 min [26]. Clearance of RMP has been observed during cardiac surgery using a cell-saver device [27].

2.5. Coagulation effects

RMPs trigger generation of thrombin via factor XIIa [28] and enhance thrombus formation, in vivo, in a tissue factor dependent manner [29]. RMPs support the anticoagulant reactions of the protein C system [30] and have broad hemostatic activity enhancing both primary (platelet) and secondary (coagulation) hemostasis [31]. Circulating RMPs in normal humans support low grade thrombin generation [32]. Elderly individuals in stable conditions also have decreased RMP levels associated with low procoagulant activity with a different response to sepsis in procoagulant activity [33].

2.6. Cell–cell communication

Findings in malaria have shown that the deployment of RMP by *Plasmodium* sp. has a major impact on disease outcomes and serves as an integral part in controlling stage switching in its life cycle. Clinical studies have shown that elevated levels of RMPs exist for the cell-to-cell communication required in patients with severe malaria. Recent discoveries indicate that *Plasmodium* sp. 'highjack' the RBC vesiculation system in order to cross-communicate. RMPs have been suggested as key mechanisms in both the transfer of DNA and parasite density regulation in malaria biology [34].

In paroxysmal nocturnal hemoglobinuria, cell-to-cell transfer of 2 GPI-anchored proteins, CD55 and CD59, by RMP has been demonstrated, in vitro, with retention of their function. Since RBC units stored for transfusion contain many RMPs, transfused blood could potentially serve as a source of CD55 and CD59 [35].

RMP, released during RBC lysis, may transfer phosphatidylserine (PS) to the surface of nucleated cells and "mark" them falsely positive as eryptotic cells [36].

2.7. Variables and factors which may affect analysis

Pre-analytic variables such as blood collection, poor venesection, shaking of samples, centrifugation protocol, handling and storage practices may affect the levels and analyses of RMP. Similarly, analytical/technical factors, which include flow cytometry, size gating, fluorescence gating, RMP enumeration (beads, "Swarm Effect") may affect RMP levels and analyses [37].

2.8. Factors which may affect formation

Study of the effects of RBC aging suggests that vesiculation is a mechanism for the removal of RBC membrane patches, which contain removal molecules, thereby postponing the untimely elimination of otherwise healthy erythrocytes [20]. Young RBCs shed more RMPs than old cells [38].

Amphiphilic drugs such as chlorpromazine, primaquine and tetracycline inhibit RMP formation [39]. These same drugs induce stomatocytosis. Chlorpromazine inhibits the budding shape of RBC and subsequent vesiculation of erythrocytes [40].

The Scott syndrome, which is a rare congenital bleeding disorder, contains a genetic defect in which RMP formation does not occur. The bleeding may consist of epistaxis, trauma related hematoma, bleeding after tooth extractions, and have other sources. This is due to an inability of RBC and platelets to vesiculate [41]. The defect is caused by an inability of the RBC and platelets to transpose the procoagulant, phosphatidylserine (PS), from the inner lipid portion of the RBC plasma membrane to the membrane surface [42]. RBCs share many of the membrane abnormalities reported for Scott syndrome platelets and suggest that the Scott syndrome defect is common to both cell lines [41]. The lack of vesiculation associated with the bleeding syndrome further suggests the important contribution vesiculation makes to coagulation.

At elevated pH levels (pH 11) RBCs are composed of spherical parent cells and large spherical daughter RMPs. The RMPs are free or are connected to the parent cells by a narrow neck. The shapes of RBCs at pH 11 correspond to some of the calculated shapes of a closed lipid bilayer, having an extreme area difference between the outer and the inner monolayers [43]. It is suggested that the observed shapes of the red blood cells at pH 11 are a consequence of the abolishment of skeleton–bilayer interactions.

At low pH levels, aggregation of the skeleton may occur and cause RBC shape transformation from a stomatocytic shape to a spherical parent RBC with the bilayer completely underlayed with the skeleton. The spherical daughter RMPs are skeletal-free [44]. The low pH, in human microvasculature, may be a contributing factor in the vesiculation process.

Human RBCs release RMPs when heated to temperatures close to the thermal transition for spectrin. Vesiculation increases as the temperature increases. Large (0–1.0 nm) cytoskeleton-free RMPs are obtained by release of RBC buds from fresh human and rabbit erythrocytes incubated at 45 °C and titrated with EDTA and $CaCl_2$. Usually only one bud per cell will be present. The immediate parent cells are initially spherical and followed by budding of the RBC membrane. Microscopic evaluation of RMP at elevated temperatures estimates that 80% of all RMPs are smaller than 0.4 μm and suggested that 86% of the lost erythrocyte material was not lost by vesiculation [45].

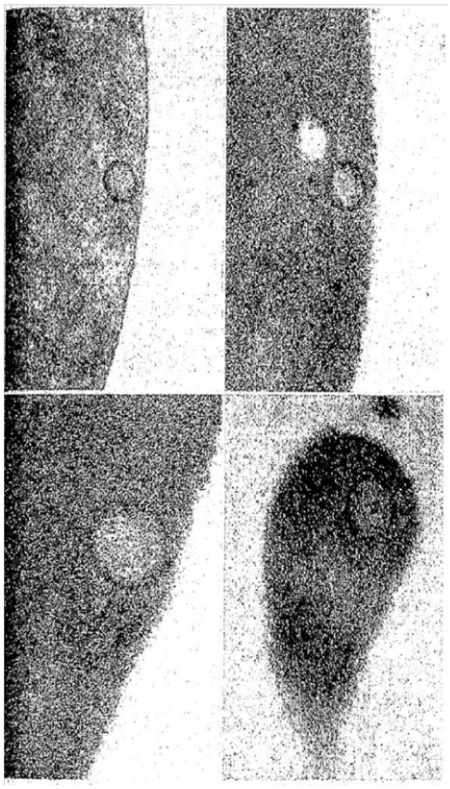
3. Microparticles shed from pathologic RBC

Hemolytic anemias with pathologic RBC, due to membrane defects, enzyme defects, or hemoglobin abnormalities, have decreased RBC survival times [46]. The cells may have defects in fragility and deformability and have increased susceptibility to shear stress and oxidative damage. The anemias with pathologic RBC are associated with vesiculation.

3.1. Sickle cell anemia

Historically, in 1982, sickle RBCs (SRBC) were the first pathologic cells to be described as a parent source of MPs [47]. The findings were confirmed in studies describing uncoupling of the membrane skeleton from the lipid bilayer [48,49]. The sickle microparticles (SMPs), which are spectrin-free, otherwise contain elements similar to the plasma membrane of the parent RBC. The contained elements are band 3, glycophorin A, band 4.1, enriched diglycerol with increased levels of intracellular Ca^{2+}. Phospholipid classes (sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine) are present [17,49]. Cholesterol concentration and acetylcholinesterase activity in the SMP were similar to the levels observed in the membrane of the parent cells [9].

Sickle MPs are of considerable interest. SMP may appear as "inward" or internal MPs (Fig. 3) [4,50,51,52]. The internal SMPs have several properties including the ability to eliminate residual organelles and the ability to reduce the surface area and volume of the parent cell [52]. The membranes of the internal SMPs also contain a Ca^{2+} pump [51] which allows Ca^{2+} to be moved from the parent cytoplasmic



Source: Bessis M, Mandon P. La microspherulation et les forms myelinques des globules rouges. Nouvelle Revue Francaise d'Hematologie. 1972;12: 443-454 [4].

Fig. 3. Intra-erythrocyte microspheres.

SRBC to the internal SMPs. When the host SRBCs are depleted of ATP, Ca^{2+} is lost. The internal SMPs subsequently undergo exocytosis [52].

These findings suggest that the derivation of SMP may vary. SMP may occur due to autophagy and exocytosis, as cited above [52], due to breakage of the spicules of deoxygenated SRBC [5,6] or due to released buds from pathologic non-spiculated RBC.

SMP and SRBC have phase changes which occur in the membrane lipids [53]. The changes may have clinical importance since phase changes in the membranes may contribute to the generation of anti-phospholipid antibodies, which are thrombophilic, and would consequently add to the coagulatory effect of sickle RMPs.

SMPs are scavengers of nitric oxide (NO) induced by the chronic hemolysis present in SCA. The finding inhibits NO bioavailability and impairs endothelial-dependent vasodilatation [54].

SMP levels are linked to the levels of hemolysis in patients with sickle cell anemia (SCA), and may occur concomitantly with the formation of irreversibly sickled cells [55].

SMP, via cell–cell transfer, transfers heme to endothelial cells which incites vasooclusion in animal models [56].

SMP detection may be affected by the presence of insoluble circulating protein complexes. The complexes overlap in biophysical properties (size, light scattering, and sedimentation) with SMP [57].

Sickle microparticles have important effects on coagulation activity. SMPs increase coagulation activity [58]. A later study has shown that measures of thrombin generation are significantly increased by SMP [59]. Further, a study has described acceleration of the propagation phase of thrombin in SCA [60].

Clinical findings of SMP in SCA are several including a 6-fold increase in SMP levels [61]. In children with SCA, HbF and hydroxyurea (HU) modulate both plasma concentration of HbF and SMP [62]. The clinical improvement associated with HU treatment in patients with SCA may be related to the depressed expression of phosphatidylserine (PS) which occurs in subpopulations of SRBC. The subpopulations are the source of PS^+ SMP [31].

3.2. Sickle cell trait (STr)

Individuals with sickle cell trait, which is ordinarily a benign disorder, have increased levels of coagulation activity which is less than that observed in homozygous sickle cell anemia and Hb SC disease [63]. Clinical studies show that the increase in coagulation activity is associated with a 2-fold increased risk of pulmonary embolism [64].

Individuals with STr may also have increased MP formation. A study of a set of monozygotic twins with STr showed that both twins had increased, but different levels of SMP [65]. Of interest was the finding that one of the twins was prothrombotic in contrast to her twin who was not prothrombotic. Both twins had normal RBC deformability, normal blood viscosity, and normal RBC aggregation [65]. The findings in this limited study suggest that individuals with STr may have a prothrombotic subgroup of individuals who have an increased risk for serious complications. The finding would contribute to the observation that individuals with STr have an increased incidence of exercise related death [66].

3.3. Thalassemia

Patients with thalassemia intermedia (TI) generate MP and have other significant laboratory and clinical findings associated with their generation [61]. The level of circulating thalassemic MP (TIMP) is 4 times greater than the normal level. TIMPs have a close relationship to intravascular hemolysis, as indicated by the relationship between the levels of TIMP and plasma hemoglobin. The TIMP and TIRBC–PS also have high levels of phosphatidylserine (PS) which are significantly different from normal levels.

TIMPs are related to coagulation as demonstrated by the significant relationship between TIMPs, which are PS(+), and markers of thrombin generation. These changes facilitate the thromboembolic complications seen in thalassemic patients.

Other significant changes occur in TI which are related to the TIMP. Oxidative damage in platelets and RBC in TI potentially induces production of TIMP with an altered proteome [67]. Thalassemic RBCs also release TIMP loaded with hemichromes by redox activation of p72sSyk kinase [68]. The effect of P72Syk kinase inhibitors on release of TIMP may indicate new perspectives for controlling the release of TIMP in hemolytic anemias.

Nitric oxide (NO) bioavailability is decreased in TI. The relationship between TMP levels and plasma hemoglobin, which occurs in thalassemia, suggests a mechanism linking the presence of vesiculation to hemolysis and consequently to decreased NO bioavailability [54].

Splenectomy in TI patients is associated with higher levels of TMPs, including PS^+ TIMPs and PS^+ TIRBCs, as well as increased coagulation activity [61]. The markers of thrombin generation, PF1.2, TAT and D-dimers are higher in splenectomized patients with the increase in TAT being significant. The non-significant, but higher levels of the other measures of coagulation, were possibly due to the small sample sizes. RBC counts in the splenectomized patients were lower (mean ± SEM) $2.88 \pm 0.32 \times 10^{12}$ than the counts observed in the unsplenectomized patients 3.8 ± 10^{12} ($p = 0.09$).

3.4. Hereditary spherocytosis (HS)

Hereditary spherocytosis is the most common inherited hemolytic anemia due to a RBC membrane defect. Most cases of HS are caused by deficiencies in the RBC membrane cytoskeleton proteins, ankarin, band 3, protein 4.2, beta-spectrin or alpha-spectrin [69]. The proteins are vital for vertical tethering of the RBC cytoskeleton network with the lipid bilayer. Protein loss results in reduced mechanical strength and loss of membrane surface area through vesiculation. Deficiency of

one or more of the cytoskeleton components may also create an area of weakness in the membrane facilitating RBC vesiculation. Alternatively, loss of one of the integral proteins, for instance band 3, may affect membrane integrity. Thus, the reason for the increase in released microparticles in HS may differ from the reasons for released RMP in other hemolytic anemias, which are due to deficiencies in other cytoskeleton proteins [46].

3.5. Hereditary elliptocytosis

Hereditary elliptocytosis (HE) is an anemia caused by an RBC membrane defect that is characterized by elliptical RBC. The basis for the problem is a defect in maintaining the lateral linkages of the RBC cytoskeleton by the membrane proteins. The defects include quantitative and/or qualitative alterations in the proteins, alpha-spectrin, beta-spectrin and protein 4.1. The RBCs are susceptible to shear stress-induced fragmentation associated with membrane loss and transformations of RBCs from the biconcave disk to an elliptical or oval shape. Membrane loss may potentially occur through shear-induced vesiculation. The exact relationship in HE and the increase in vesiculation is not clear [70].

3.6. Hereditary stomatocytosis (HSt) (xerocytosis)

Hereditary stomatocytosis (HSt) is a disorder that comprises a heterogenous group of syndromes in which the cell membrane is leaky to monovalent cations Na^+ and K^+. Abnormalities in actin–somatin may affect the vertical association between the lipid membrane and the cytoskeleton and thus regulate vesiculation [71]. A study of hereditary xerocytosis by Snyder [72] showed that the degree of increased vesiculation in the anemia was related to the level of the RBC life span. Xerocytosis, with mild anemia (^{51}Cr half-life survival of 15.5 days) had the highest amount of vesiculation as compared to patients with short RBC survival times, who had the smallest amount of vesiculation.

3.7. Glucose-6-phosphate dehydrogenase deficiency

In patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency, the effect of an increase in oxidation and phosphorelation of band 3 proteins may result in an increase in hemolysis as well as an increase in the release of microparticle-containing hemichromes [73,74]. G6PD enzyme activity is significantly correlated with MP concentrations. MPs in G6PD deficient patients are largely derived from RBC (RBC 45%, Platelets 30%). The MPs with PS positivity may activate the coagulation cascade, however the clinical effect is not known [75].

4. Conclusion

This overview describes the history of the origin of microparticles as a concept. The evolving knowledge about MP formation, characteristics, effects and factors which may affect formation and function of MP derived from normal and abnormal RBC is then described. Accumulated knowledge, where the RBC serves as the paradigm, shows that microparticle formation is a fundamental biologic process of cell aging, cell survival and cell clearance. Underlying disorders of Hb synthesis, RBC membranes or red cell enzymes, increase micro-vesiculation, thereby increasing procoagulant effects, and decreased NO bioavailability, which contribute to the clinical phenotype of each syndrome where microparticle formation is increased.

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