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Phosphatidylserine Shedding from RBCs – A Mechanism of Membrane Modulation and Damage Control

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1. Introduction

Normally, phospholipids (PLs) are distributed across the membrane of all cells, including the RBCs, asymmetrically [1]: aminophospholipids such as phosphatidylserine (PS) are mainly localized in the cytoplasmic leaflet of the membrane, whereas lipids with a choline head (e.g., phosphatidylcholine) are mainly localized in the outer leaflet [2]. The PS distribution across the cell membrane is in a dynamic equilibrium; while the enzyme aminophospholipid translocase inserts it inward, the scramblase causes its externalization. Some of this external PS is shed into the extracellular medium either as membrane-bound vesicles [3] or as membrane-free PS [4]. In RBCs, exposed PS is one of the signals of senescence, mediating the removal of old or damaged RBCs from the circulation [5]. Shedding of PS may reduce this signal and thus function to moderate RBC removal [6]. PS externalization and shedding are also associated with development of RBCs in the bone marrow, fulfilling various structural and functional purposes [7]. In the present review I will summarize our studies on changes in PS distribution and shedding during maturation and ageing of erythroid cells.

2. Methodologies

For these studies we have employed two analytical methodologies, Nuclear Magnetic Resonance (NMR) spectroscopy [4] and flow cytometry [8]. Using ^1H - and ^{31}P -NMR procedures, we measured absolute concentrations of metabolites in aqueous and organic extracts of the cells [9-12].

Flow cytometry was employed to measure various parameters of cellular oxidative stress: generation of reactive oxygen species (ROS) and membrane lipid peroxides, the intracellular content of reduced glutathione [13], as well as the contents of the labile iron [14] and calcium (Ca). These measurements are based on changes in the fluorescence intensities of specific probes.

To measure the cellular distribution of PS and its shedding from erythroid cells, we developed a two-step fluorescence inhibition assay [8]. PS is usually estimated by staining cells with annexin V which specifically binds to PS. Fluorochrome-conjugated annexin V is

used to determine the percentage of PS-carrying cells by flow cytometry [15, 16]. This method is applicable to populations containing a significant fraction of positive cells, for example, following exposure to an apoptosis-inducing agent. However, *in vivo*, at a given time, only a small fraction of any cell population is apoptotic, making their determination statistically unreliable. In addition, this procedure does not yield information regarding the inner PS, which is not exposed on the outer surface of the cells, nor on the PS shed into the surrounding medium. Moreover, the method refers usually only to strongly positive cells, giving the impression that the process occurs in an "all or none" fashion, neglecting cells with less than maximal amount of bound annexin V. Most importantly, the procedure provides relative comparison rather than absolute quantitative values.

To overcome these limitations, we developed a novel flow cytometry methodology that provides a quantitative measurement of the external PS as well as the intracellular and shed PS. The procedure entails two steps: In the first step, the outer PS of intact cells or the total PS of cell lysates and supernatants, or human serum is bound to excess amount of fluorescent-annexin V. In the second step, the residual, non-bound fluorescent-annexin V is quantified by binding to PS exposed on apoptotic cells (e.g., 6-day old HL-60 cells) which serve as an indicator reagent. The fluorescence of these indicator cells is reciprocally proportional to the amount of PS on the measured cells in the first step [8].

3. PS exposure and shedding during the lifespan of RBCs in the circulation

During their life in the circulation, RBCs are exposed to several stress situations, which are (i) physical, occurring when they squeeze through small capillaries, (ii) hyperosmotic, when they travel through the kidney medulla, and (iii) oxidative, when they pass the oxygenated lung. These stress conditions affect the RBC composition and properties, leading to their senescence and eventually to their elimination from the circulation.

One of the signals of senescence is PS externalization. PS-carrying RBCs undergo phagocytosis (erythrophagocytosis) by macrophages in the reticulo-endothelial system (extravascular hemolysis) [5]. Under normal conditions this occurs in humans after 120 days, but under pathological conditions this process is accelerated, thereby shortening the life-span of RBCs, causing hemolytic anemia. These hemolytic anemias are hereditary, such as the hemoglobinopathies, thalassemia and sickle cell disease, or acquired, such as the myelodysplastic syndromes.

Using the methodologies described above, we studied the externalization and shedding of PS in RBCs during their senescence and compared RBCs derived from the peripheral blood of normal donors and patients with hemolytic anemias. ^{31}P -NMR analysis indicated that compared to normal RBCs, thalassemic RBCs have lower concentrations of total cellular PS which was associated with increased PS shedding [4]. Flow cytometry measurements, using fluorescent annexin V as a probe, confirmed these results and further demonstrated that despite of the decreased total cellular PS, in thalassemic RBCs, the PS exposed on their outer membrane was significantly increased. This was reflected not only by moderate increase in the percentage of annexin V positive cells as measured by the direct method, but also by a significant increase in exposed PS on the entire population as measured by the indirect method. The increased PS exposure reflected the balance between the decrease in the inner membrane PS and the increase in the shedding of PS into the extracellular milieu. The increased PS shedding by thalassemic RBCs was also reflected in the higher PS concentration in sera of thalassemic patients compared with normal donors. It should be mentioned that

while shedding is often described in the context of microparticles, i.e., membrane-bound vesicles [3], we have shown that the majority of the shed PS is membrane-free [8].

PS shedding has a profound effect on the membrane composition and functionality. The PLs and cholesterol are the major lipid membrane components. Using a $^1\text{H-NMR}$, we determined their ratio in normal and thalassemic RBC membranes and in supernatants following *in vitro* incubation [6]. The results indicated a significant decrease in PLs in the membranes and an increase in the supernatants, while cholesterol was only slightly decreased in the membrane and was minimal in the supernatants. These changes resulted in an increased cholesterol/PL ratio in the RBC membranes. Thalassemic RBCs demonstrated a higher basal cholesterol/PL ratio than normal RBCs. These findings suggest that shedding is a selective process involving mainly PLs and leading to relative accumulation of cholesterol in the membrane.

PS shedding and the consequential changes in the membrane composition and properties affect its functionality. It increased its osmotic resistance and the susceptibility of RBCs to undergo erythrophagocytosis. Using cultured macrophages, we have shown that while PS externalization increased phagocytosis, the shed PS prevented it, probably by competitive binding to PS receptors on the macrophages [6].

PS shedding may play a role in the functioning and fate of mature RBCs in the circulation:

- a. Shedding of PS-enriched membranes [3, 4] might cause size reduction which characterizes RBC senescence as well as microcytic anemias. Shedding might serve mainly to rejuvenate the plasma membrane of the RBCs by removing its damaged components [17].
- b. The cholesterol content of the RBC plasma membrane was reported to affect its mechanical properties (fluidity) [18, 19]. During physiological aging, senescent RBCs showed an increased cholesterol/PL ratio followed by greater membrane strength [20]. We have shown that in RBCs PS shedding and relative accumulation of cholesterol are associated with a greater osmotic resistance [6].
- c. PS externalization has been suggested as one of the mechanisms of senescent RBC clearance from the circulation by PS receptor carrying reticuloendothelial system macrophages [5]. We have shown that whereas PS externalization increases phagocytosis, PS shedding decreases it [4]. The latter effect may be attributed to a decrease in the exposed PS, as well as competition by the shed PS for the macrophage PS receptors. Thus, the balance between PS externalization and shedding may play a role in controlling the fate/lifespan of the RBCs in the circulation under both physiological and pathological conditions, e.g., in thalassemia where RBCs were shown to have increased PS shedding [4, 8].
- d. Finally, it is worth mentioning that PS has procoagulant properties [21]. Exposed and shed PS could be involved in normal and pathological homeostasis [1]. Thus, thalassemic patients with increased exposed and shed PS are prone to thromboembolic complications [22, 23].

4. PS shedding during development of erythroid cells

RBCs are produced in the bone marrow by a well regulated process (erythropoiesis) that involves proliferation and maturation. PS externalization and shedding have important roles in this process [7]. We studied normal human bone marrow cells as well as two *in vitro* models of erythropoiesis, primary cultures of human erythroid precursors and a

murine erythroleukemia cell line. The human erythroid precursors are derived from progenitors present in the peripheral blood of normal donors. They are stimulated by the physiological inducer erythropoietin to proliferate and mature into hemoglobin-containing nucleated orthochromatophilic normoblasts. This system provides a reliable *in vitro* model that recapitulates many aspects of erythroid maturation [24]. The murine cells, derived originally from the spleen of viral induced leukemia, were stimulated to undergo erythroid maturation by hexamethylene bis acetamide [25] [26]. In all these systems, both PS exposure and shedding were found to be high in early precursors, and to be reduced during maturation.

Several suggestions might be raised regarding the role of PS shedding in the maturation of erythroid cells:

- a. Size reduction characterizes not only RBC senescence in the circulation, but also erythroid maturation in the bone marrow. During their maturation erythroid precursors undergo a gradual and continuous, but a significant, reduction in size [27, 28]. This is an important functional adaptation generating mature RBCs small enough to pass through narrow capillaries. It also generates a high surface to volume ratio that promotes gas exchange between the RBCs and tissue cells during this passage. Shedding of PS-enriched membranes [3, 4] during maturation might be the cause or the outcome of the size reduction process. We have shown that inhibition of PS externalization/shedding prevented size reduction in differentiating erythroid cells [7], favoring the first possibility.
- b. Apoptosis of nuclear cells involves PS externalization [29]. RBC production is regulated by apoptosis of erythroid precursors, which is controlled by erythropoietin, serving as an anti-apoptotic agent [30]. We found that depletion of erythropoietin during maturation of cultured erythroid precursors results in PS externalization, suggesting that this process is involved in the apoptosis of erythroid precursors as part of normal or pathological (ineffective) erythropoiesis (e.g., in the myelodysplastic syndrome or thalassemia), while PS shedding may have an opposite effect.
- c. During their early development in the bone marrow, erythroid precursors are found in erythroblast islands, where they surround a central macrophage [31]. A diverse array of adhesion proteins expressed on the erythroblast surface mediate its interaction with both stromal cells and the extracellular matrix [32, 33]. It is possible that the outer PS on these precursors may assist in their attachment to macrophages carrying PS receptors, thus forming the erythroblast islands. Outer PS shedding (in addition to PS internalization) may lessen this adhesion and facilitate the release of erythroid precursors from the island as they mature. This possibility awaits experimental confirmation.
- d. During maturation, erythroid precursors expel their cellular organelles, including the nucleus, mitochondria and ribosomes, by exocytosis through membrane-bound vesicles [34, 35]. Recent results indicated that enucleation is caused by the coalescence of vesicles at the nuclear-cytoplasmic junction, whereas, mitochondria are eliminated through selective autophagy [36]. Plasma membrane remodeling by PS redistribution might also be part of this process. Yoshida et al. have shown that "the nuclei are engulfed by macrophages only after they are disconnected from reticulocytes, and that phosphatidylserine, which is often used as an 'eat me' signal for apoptotic cells, is also used for the engulfment of nuclei expelled from erythroblasts" [37].

5. Mechanisms involved in PS shedding

We studied several mechanisms in relation to the above described changes in PS distribution: the oxidative status of the cells, changes in Ca-flux and microtubule (MT) polymerization.

6. Oxidative stress

The oxidative status of cells depends on the balance between oxidants (such as ROS) and antioxidants. Under pathological conditions, the balance leans towards generation of excess oxidants, which is accompanied by reduced content of antioxidants, resulting in oxidative stress. Although free radicals have important roles in normal physiology, such as in signal transduction, in excess they interact with and damage various components of the cells (e.g., proteins, lipids and nucleic acids). Many diseases are associated with oxidative stress, including hemolytic anemias. Although these anemias vary as to their etiology, in all cases the damage to erythroid cells is mediated by oxidative stress [38]. Using flow cytometry, we have demonstrated oxidative stress in normal mature RBCs treated with various oxidants: increased generation of ROS and membrane lipid peroxides and decreased content of reduced glutathione - the main cellular antioxidant. Similar results were obtained in RBCs derived from patients with thalassemia, sickle cell disease, myelodysplastic syndromes (MDS), paroxysmal nocturnal hemoglobinuria, spherocytosis and other hemolytic anemias. ¹H-NMR analysis demonstrated oxidative stress in such RBCs by a high lactate/pyruvate ratio [4].

Oxidative stress reduces the activity of the enzyme translocase [39], causing the equilibrium that exists between the PS on the inner and the outer membrane leaflets to lean towards externalization. We have found that oxidatively stressed RBCs (old vs. young RBCs, thalassemic vs. normal RBCs, oxidant treated vs. non treated normal RBCs) have less total cellular PS but more exposed and shed PS [6]. Ameliorating the oxidative stress, e.g., by treating thalassemic cells with an antioxidant (e.g., vitamin C or N-acetyl cysteine) resulted in opposite results [8].

As mentioned above, in RBCs, exposed PS is one of the signals of senescence [5] and that it induces erythrophagocytosis. This process is accelerated in hemolytic anemias resulting in short survival of RBCs in the circulation. Although in patients with hemolytic anemia the proliferation of erythroid precursors in the bone marrow is increased (by stimulating the production of erythropoietin in the kidneys), when the condition is chronic the production of mature RBCs is futile due to increased premature death (by apoptosis) and lack of maturation of the erythroid precursors (ineffective erythropoiesis) [40]. The supply of mature, functional, RBCs is thus insufficient.

The main cause of oxidative stress in hemolytic anemias is iron overload due to increased iron absorption and repeated blood transfusions. When the iron content in the serum exceeds the binding capacity of transferrin, the iron-transport protein, surplus iron appears as "non-transferrin bound iron", which is taken up by cells, including RBCs, by mechanisms that are transferrin receptor-independent [41]. The incoming iron accumulates intracellularly as "labile iron pool" [42, 43], which is of redox potential due to its participation in chemical (Haber-Weiss and Fenton) reactions that generate ROS.

The oxidative status affects PS externalization/shedding also in developing erythroid cells. It is modulated throughout maturation; from being very high in early erythroid precursors

it is reduced considerably as the cells mature. This change is most probably related to the decrease in the metabolic rate. Most of the ROS produced by cells are originated in the mitochondria in the process of oxidative energy production [38]. Erythroid maturation involves a loss of mitochondria and a decrease in energy production which results in lower generation of ROS and consequently, in lower PS externalization and shedding.

7. Ca-flux

Increase in the intracellular Ca concentration is a well-known mechanism of PS [44]. We studied the relationship between the oxidative state, changes in Ca-flux and PS shedding [6]. It was found that the oxidatively stressed thalassemic RBCs with their increased PS shedding have high Ca content which could be corrected by treatment with antioxidants. The low Ca content and PS-shedding of normal RBCs could be increased by treatment with oxidants. Modulating the Ca content of normal RBCs by treatment with the Ca ionophore A23187 or by varying the Ca concentration in the medium confirmed that increasing the inward Ca flux induced PS externalization and shedding.

8. Microtubule (MT) polymerization

Several lines of evidence suggest an interaction between the plasma membrane PLs and cytoskeleton components [45, 46], including the MTs [47,48] - the key components of the cytoskeleton [49]. MTs are made up of $\alpha\beta$ -tubulin heterodimers [49], and they readily polymerize and depolymerize in cells. MTs are involved in a variety of cellular processes such as cell division, maintenance of cell shape, cell signaling and migration, and cellular transport [50] as well as maturation and stress. During erythroid maturation, MTs undergo dramatic changes in distribution to become absent in mature mammalian RBCs [51]. It has been shown that at early stages of maturation of murine erythroid precursors MTs are radially arranged just under the plasma membrane. Addition of the MT depolymerization promoters, colchicine or vinblastine, caused MTs to disappear completely. This, however, did not affect enucleation [51]. Addition of paclitaxel (Taxol), which enhances MT polymerization and stabilization, to these cells caused the resulting pre-mature RBCs (reticulocytes) to contain abnormally high numbers of polymerized MTs [51]. Treatment of patients with Taxol caused PS externalization and short survival of their RBCs [52].

We investigated the effect of MT depolymerization in developing erythroid cells on their membrane PS distribution and shedding using cultured human and murine erythroid precursors. Cells were treated with the MT depolymerization enhancer - colchicine and inhibitor - Taxol. The effect of these modulators was studied on the constitutive shedding as well as shedding induced by the Ca-ionophore A23187 [53]. We found that treatment with colchicine and Taxol markedly increased both the constitutive and the induced PS externalization. PS shedding, however, was increased by colchicine, but was inhibited by Taxol.

As discussed above, PS shedding is one of the mechanisms of membrane remodeling [54], including changes in the membrane cholesterol/PL ratio [4, 8]. Using $^1\text{H-NMR}$, we showed that colchicine, by enhancing shedding, increased the cholesterol/PL ratio, whereas Taxol, by inhibiting shedding, decreased this ratio.

Many compounds that alter the polymerization dynamics of MTs block mitosis, and consequently, induce cell death by apoptosis [55]. One of these compounds, Taxol, a

promoter of MT polymerization and stabilization, is being used for treatment of patients with various malignancies such as breast cancer [56] or ovarian cancer [57]. A significant side effect of this treatment is severe anemia which was related to the effect of Taxol on PS externalization of mature RBC [52]. In line with the importance of PS shedding in erythroid development, the present finding that Taxol affects PS shedding may suggest that anemia in patients treated with Taxol might be also due to its effect on erythropoiesis in the bone marrow.

9. Summary

PS externalization and shedding undergo a bi-phasic modulation in erythroid cells: both are decreased during maturation but increased during aging. This redistribution of PS is the outcome of multiple factors and mechanisms, including changes in the cellular oxidative status, Ca concentration and MT polymerization which affect the inward and outward PS flow and PS shedding. These dynamic processes are ongoing continuously and simultaneously, and may have opposite effects. For example, PS exposure on thalassemic RBCs, induced by their high intracellular oxidative status and Ca concentration, is blunted by increased PS shedding. Only when PS externalization overcomes the ability to remove it by shedding are thalassemic RBCs removed by erythrophagocytosis. Further study on the roles played by PS shedding in the production and clearance of RBCs is crucial for understanding its effect on the pathological consequences of hemolytic anemias and for the planning of novel therapeutic modalities to overcome them.

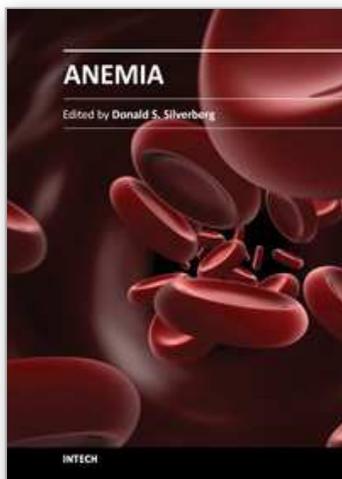
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This book provides an up- to- date summary of many advances in our understanding of anemia, including its causes and pathogenesis, methods of diagnosis, and the morbidity and mortality associated with it. Special attention is paid to the anemia of chronic disease. Nutritional causes of anemia, especially in developing countries, are discussed. Also presented are anemias related to pregnancy, the fetus and the newborn infant. Two common infections that cause anemia in developing countries, malaria and trypanosomiasis are discussed. The genetic diseases sickle cell disease and thalassemia are reviewed as are Paroxysmal Nocturnal Hemoglobinuria, Fanconi anemia and some anemias caused by toxins. Thus this book provides a wide coverage of anemia which should be useful to those involved in many fields of anemia from basic researchers to epidemiologists to clinical practitioners.

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