Membrane Lipid Alterations in Hemoglobinopathies

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The red blood cell (RBC) membrane is a complex mixture of lipids and proteins. Hundreds of phospholipid molecular species spontaneously arrange themselves in a lipid bilayer and move rapidly in the plane as well as across the bilayer in a dynamic but highly organized fashion. Areas enriched in certain lipids determine proper protein function. Phospholipids are asymmetrically distributed across the lipid bilayer with phosphatidylserine (PS) exclusively on the inside. Both the composition and organization of the RBC membrane is well maintained. Alterations lead to apoptosis during erythropoiesis or early demise of the cell in the circulation. The mechanisms that govern the maintenance of the lipid bilayer are only recently being unraveled at the individual protein level. Oxidized lipids are rapidly repaired using fatty acids taken up from plasma to maintain membrane integrity. Several isoforms of a RBC acyl-Coenzyme A (CoA) synthase have been reported, as well as the first member of a family of lysophospholipid acylCoA acyltransferases. Phospholipid asymmetry is maintained by the recently identified RBC amino-phospholipid translocase. These enzymes, essential in maintaining membrane lipid organization, are affected by oxidant stress or an increase in cytosolic calcium. Normal lipid composition and organization is lost in subpopulations of RBC in hemoglobinopathies such as sickle cell disease and thalassemia. Despite elaborate antioxidant systems, lipids and membrane proteins, including those that maintain lipid organization, are damaged in these cells. This in turn leads to improper repair of damaged RBC membranes and altered interactions of RBCs with other blood cells and plasma components that play a role in the pathology that defines these disorders. The altered lipid bilayer in RBCs in hemoglobinopathies leads to premature removal (anemia) and imbalance in hemostasis, and plays a role in vaso-occlusive crisis in sickle cell disease. Lipid breakdown products of PS-exposing cells result in vascular dysfunction, including acute chest syndrome in sickle cell disease. In summary, altered membrane lipids play an important role in the pathology of hemoglobinopathies and characterization of the proteins involved in lipid turnover will elucidate the pathways that maintain plasma membrane organization and cellular viability.
brane. The phospholipids differ with respect to their polar head group, the backbone to which the head group and apolar fatty acyl chains are bound (glycerol or sphingosine) as well as the length and level of unsaturation of the acyl groups, connected to the backbone through ester or ether bonds. The acyl chains of the phospholipids are 16 to 22 carbon atoms long with none to 6 double bonds. This large variety in chain length accommodates proper packing of lipids in the bilayer and governs protein-lipid interactions. These lipids form a highly dynamic lipid bilayer in which proteins are embedded and, together with the underlying membrane skeleton, provide a strong and flexible membrane for the red cell to perform its tasks in the circulation. Both the composition and organization of the lipids are well maintained during the life of the cell, and alterations will lead to a dysfunction of the membrane and a loss of cellular viability. The lipids move rapidly in the plane of the bilayer, but this movement is not a random process. Both lipid and protein organization will lead to domains in the membrane where certain lipids and proteins are enriched. These microdomains or “rafts” are involved in specific physiologic processes such as signal transduction as the components of these pathways aggregate to act in concert. These rafts are in general enriched in molecules such as sphingomyelin, saturated glycerol phospholipids and cholesterol. Lowering of the cholesterol content of the membrane tends to “dissolve” these rafts, leading to an altered function of the membrane, and changes in the cytosol such as an increase in calcium can lead to shedding of vesicles enriched in specific lipids and proteins. In addition to the heterogeneous distribution in the plane of the bilayer, phospholipids also transfer from the outer to the inner monolayer and back. This flip-flop across the membrane is not random and is orchestrated by proteins in the bilayer. The choline-containing phospholipids (PC and SM) are mainly found in the outer monolayer, while the amino phospholipids are predominantly (PE) or exclusively (PS) found in the inner monolayer. This highly asymmetric plasma membrane distribution is typical for most mammalian cells.

A loss of this asymmetry and the exposure of PS on the surface of the cell is an early event in apoptosis and leads to the recognition and removal of cells by macrophages.

**Oxidant stress**

High levels of oxygen, together with iron-containing hemoglobin, leads to the generation of reactive oxygen species (ROS) in RBCs. The high level of ROS in the RBC is counteracted by an elaborate antioxidant system, which includes proteins such as superoxide dismutase and catalase, thiol species such as glutathione and peroxiredoxin, and vitamin E. All of these mechanisms are aimed to neutralize the oxidant stress on the cell and avoid damage to proteins and lipids. Glycolysis plays an important role in regenerating these antioxidants to retain the proper reducing state in the cell. Despite these antioxidants, damage will occur, and repair of oxidized molecules is needed to maintain functional viability of the membrane. Since the RBC does not have the ability to replace its proteins and lacks de novo lipid synthesis, the onslaught of oxidant stress will ultimately take its toll on the RBC and is likely one of the significant factors that determines its lifespan in the circulation. Altered hemoglobin will lead to the increased oxidative stress in hemoglobinopathies. This requires an even more active antioxidant system and repair of oxidant damage to maintain plasma membrane viability. This increased oxidant stress plays an important role in apoptosis during RBC development (ineffective erythropoiesis) as well as the reduced lifespan in the circulation.

**Lipid Repair**

The double bonds in the phospholipid acyl chains are vulnerable to oxidative modification. The addition of oxygen in the apolar chains will alter the local packing of the bilayer and affect its functionality. Repair of this breach is accomplished by deacylation of oxidized phospholipid molecular species followed by rapid and selective reacylation of lyso-phospholipids using fatty acids from plasma (Figure 2; see Color Figures, page 516) via the so-called Lands pathway.

**Deacylation**

Due to the altered hydrophobicity of the oxidized acyl chain, phospholipases gain access to the exposed ester bond and will remove the oxidized fatty acid. The lyso-phospholipid that is generated by this process needs to be replaced. This is accomplished by an ATP-dependent process in which fatty acids are taken up from plasma, activated to acyl-Coenzyme A (CoA) and used to generate phospholipid. This is a highly selective process of which the details are only partly understood.

**Fatty Acid Activation**

Long-chain acyl-CoA synthetases (ACSL) are necessary for fatty acid degradation, phospholipid remodeling, and production of long acyl-CoA esters that regulate various physiological processes. These enzymes play a crucial role in plasma membrane phospholipid turnover in RBCs. In humans, five ACSL genes have been identified with as many as three different transcript variants for each. Several isoforms of acyl-CoA synthetase (ACSL6) that generate fatty acyl CoA (FA-CoA) from fatty acid in the RBC, the first step in the reacylation process, were recently identified. The detailed structure of mammalian ACSL has not been reported, but comparison to the crystal structure of a bacterial homolog of ACSL suggest that the different isoforms of ACSL6 differ in the proximity of the catalytic site of the enzyme where the fatty acid substrate enters the enzyme and seems involved in the formation of a pocket, in which the fatty acid is locked during the formation of the CoA ester bond. The depth and width of the pocket likely defines the substrate specificity of each of the isoforms. These proteins act in a dimeric form and are inhibited by the prod-
uct they generate, changing the ability to form dimers affects the activity of the protein. It is currently unknown whether homodimers, heterodimers or their interaction with other proteins modulates their activity.

Reacylation
Enzymatic studies show the presence of several forms of lyso-phospholipid acylCoA acyltransferases in the RBC, with specificity towards the different headgroups. The associated proteins of this activity, which incorporates FA-CoA into lyso-phospholipids, have been elusive. Recently, the PC reacylating enzyme in RBCs (LPCAT) was shown to be a product of the AYTL genes. Three LPCATs, products of the AYTL1, 2 and 3 genes, are members of a novel LPCAT family of which all three genes are expressed in a murine Friend erythroleukemic cell line. Ayt1 mRNA was detected in mouse reticulocytes, and the presence of the product of the human ortholog was confirmed in adult human RBCs. The LPCAT activity of both Ayt1 and 2, which exhibit a helix-turn-helix structural calcium-binding motif (EF-hand) at the C-terminus, was modulated by calcium and magnesium in an E. coli expression system. The incorporation of fatty acids in PC was altered by oxidative stress. Characterization of the product of the Ayt12 gene as the PC re-acylating enzyme in RBCs represents the first established biological function of a LPCAT and the first identified plasma membrane lyso-phospholipid acyltransferase.

Modulation
Both lysophospholipids and FA-CoA are molecules with detergent like characteristics, and their level in the membrane needs to be closely regulated. One of the proteins that plays a role in modulating this is an acylCoA-binding protein (ACBP) in the RBC cytosol. ACBP binds FA-CoA, acts as a buffer for the production of this molecule by ACSL6, and can deliver it as a substrate to LPCAT.

In summary, whereas the RBC does not have de novo lipid synthesis, phospholipids are rapidly turned over in deacylation and reacylation processes that involve several proteins that can be affected by cytosolic factors.

Phospholipid Asymmetry and Transbilayer Movement
The asymmetric distribution of the amino-containing phospholipids, PS and PE, across the two leaflets of biological membranes plays an essential role in eukaryotic cells (Figure 1; see Color Figures, page 516). PS exposure on the cell surface is a normal process in hemostasis as blood clotting factors organize on activated platelets that expose PS. PS-exposing cells are recognized by macrophages in early apoptosis, making this an essential step in tissue remodeling. When RBCs lose their ability to maintain phospholipid asymmetry, PS is exposed, leading to RBC removal. When these cells are not readily removed, they can induce pathophysiologic responses such as imbalanced hemostasis, and interactions with other blood cells and with endothelial cells of the vascular wall, as found in sickle cell disease and thalassemia.17-20

Flippase
 Plasma membrane phospholipid asymmetry was originally defined in RBCs. The choline-containing phospholipids, PC and SM, are preferentially present in the external leaflet. PS and PE are maintained in the RBC inner leaflet by an ATP-dependent transporter known as the flippase. Membrane-bound Mg2+ -ATPases, exclusively found in eukaryotes, seem to play a key role in the maintenance of the membrane lipid organization. This subfamily of P-type ATPases has been reported to actively translocate aminophospholipids across membranes, and several members of this family have been identified in the genome of mammals. The RBC flippase, a vanadate-sensitive ATPase of approximately 110 to 120 kDa, was recently identified as two isoforms of the type 4-ATPase 8A member 1 (ATPA8A). The structure and mode of action of these proteins in the plasma membrane needs to be established, and it is not known whether the two isoforms have different functions. The different forms of this protein may transport aminophospholipids at different rates for different molecular species.

Scramblase
In addition to active movement from outer to inner monolayer, several proteins have been identified that can transport lipids from the inner to outer monolayer by either a directional active (ATP-consuming) process or a bidirectional scrambling process.21

Sulphydryl modification will affect both the flippase activity as well as phospholipid scrambling. In addition, calcium plays a crucial role in the ability to maintain phospholipid asymmetry. An increase in cytosolic calcium lowers flippase activity and increases phospholipid scrambling, ultimately leading to PS exposure.

PS Exposure
Both the inward movement of PS and scrambling of the bilayer such that PS is exposed is highly controlled in the cell. Even when the scrambling is activated, as long as the flippase is active, PS exposure will be minimal. However, when both the flippase is deactivated and scrambling of the lipids across the bilayer is proceeding, PS will be exposed on the surface of the cell, with important physiologic consequences. During platelet activation, exposure of PS is essential, as this will form the docking site for hemostatic factors, such as the prothrombinase complex. The proteins in this complex assemble on the PS surface, and prothrombin is cleaved to thrombin, an essential step in blood coagulation. On the other hand, random, unwanted exposure of PS on plasma membranes would lead to a prothrombotic state and imbalance of the normal hemostatic processes. Exposure of PS is also an important trigger for recognition and removal of cells. Early in apoptosis or
programmed cell death, PS is exposed on the surface of the cell. Macrophages recognize this abnormal surface and will engulf the cell as it undergoes its apoptotic program, ensuring that the cell is processed before it will expel its potential noxious breakdown products in the environment. Therefore, PS exposure has become a powerful indicator of the onset of apoptosis, and the whole process of programmed cell death is obviously of high importance in tissue remodeling. Both calcium and oxidant stress are implicated in apoptosis as well as in PS exposure of RBCs. While the RBCs, without internal organelles or a nucleus, cannot undergo apoptosis as classically defined, the processes at the plasma membrane level that lead to PS exposure are likely to be similar.

PS exposure in RBCs in vitro can be initiated by inhibition of the flippase and loading the cell with calcium. Flippase inhibition can be accomplished by ATP depletion, sulfhydryl modification or specific inhibition of the ATPase activity with compounds such as vanadate. Scrambling seems to need an increase in cytosolic calcium. PS exposure in individual cells can be visualized by the use of fluorescently labeled annexin compounds in combination with fluorescent microscopy or flow cytometry. The biochemical pathways leading to PS exposure are only partly understood, but the loss of phospholipid asymmetry can be triggered by a number of conditions and seem to involve oxidant stress, calcium and protein kinase activity.

Membrane Lipids in Hemoglobinopathies
The phospholipid’s molecular species composition is highly maintained during the life of the RBC, with fatty acids rapidly taken up from plasma. This substrate pool changes depending on the intake of fatty acids from the diet. A rapid turnover with a changing substrate pool and a defined end result indicates a highly selective system of reacylation. Only a few members of the enzymes involved are identified to date, and little is known about the interactions between these proteins and between these proteins and their lipid environment that would clarify the mechanisms that govern this selectivity. This phospholipid turnover and repair can be expected to be higher in hemoglobinopathies due to the increased oxidant stress and damage to the lipids. Several reports indicate evidence of lipid oxidation in RBCs from sickle cell or thalassemia patients, suggesting that phospholipid repair is not efficient enough to maintain the proper molecular species composition in these cells. When the molecular species composition of density fractionated sickle RBCs are analyzed, a complex picture arises. While it seems that the more dense fractions are relatively lower in species with unsaturated fatty acids, the prime target for oxidant damage, significant differences are found in the density fractions from patient to patient. This points at a process in which the repair system is affected differently in individual cells or cell populations. All RBCs are exposed to the same pool of fatty acid substrates (plasma). Therefore, the difference seems to be related to the activity of the enzymes that use this pool for phospholipid repair. When the exchange of phospholipids between RBCs is facilitated by lipid exchange, the most dense fractions change their density. This suggests that a “normalization” of the lipid bilayer affects ion and therefore water transport across the membrane. The RBC is not able to replace proteins when they are irreversibly damaged by reactive oxygen species. When RBCs are challenged with oxidant stress in vitro, incorporation of fatty acids in RBC phospholipids is affected. Since several proteins and isoforms of these proteins are involved in the lipid repair process, damage of any of these may affect their proper function. Currently, little is known about the sensitivity of these proteins towards oxidant stress. In addition to direct oxidant damage of these proteins, alterations in the ionic balance of the cytosol may also play a role. The recently identified LPCAT activity, which exhibits an EF-hand motifs at the C-terminus, was modulated by calcium and magnesium. Since both cytosolic calcium and oxidant stress are altered in subpopulations of sickle RBCs, this suggests that LPCAT may act differently in subpopulations of sickle RBCs, leading to insufficient repair or “mis-repair.”

Together, oxidant stress and alterations in RBC cytosol in hemoglobinopathy RBCs will lead to the inability to maintain a proper lipid composition, which in turn leads to alterations in membrane function, including ion and water transport across the bilayer and altered red cell density distributions.

PS Exposure in Hemoglobinopathies
Exposure of PS on subpopulations of RBCs is important in the pathology of the disease. In PS-exposing RBCs the flippase is deactivated and phospholipid scrambling is increased. Both oxidant stress and elevated cytosolic calcium play a role in this process. Although the percentage of PS-exposing RBCs in hemoglobinopathies seems low (a few percent), the large number of RBCs in the circulation provides a significant surface of PS-exposing membrane. As PS exposure is a trigger for removal, their presence in the circulation suggests a high rate of generation of such cells that is not compensated by efficient removal, which may be related to lack of spleen function.

Consequences of PS Exposure
The presence of PS on the surface of RBCs has been reported in sickle cell disease and thalassemia, and is an important factor in the pathology of hemoglobinopathies. The altered membrane surface will lead to an alteration in the interaction with other cells in the circulation. As PS-exposing cells are recognized and removed from the circulation, the loss of phospholipid asymmetry is involved in the reduced RBC lifespan. Exposure of PS during erythropoiesis, as an early step in apoptosis, plays an important role in the ineffective erythropoiesis, particularly evident in thalassemia. Therefore, PS exposure is important factor in the anemia. The altered surface also leads to increased
interaction with endothelial cells and therefore is related to vaso-occlusive crisis. Recognition of PS-exposing cells is not limited to cell-cell interactions. Proteins and enzymes in plasma will interact with PS-exposing surfaces. Activated platelets expose PS on their surface as a normal step in hemostasis, and form the docking site for complexes such as the prothrombinase complex where factors Xa, Va and II bind to generate factor IIa. Given the number of RBCs and platelets in the circulation, even a low number of PS-exposing RBCs can lead to an imbalance in hemostasis, and the loss of RBC phospholipid asymmetry has been correlated with the prothrombotic state. PS-exposing cells also become targets for enzymatic breakdown by phospholipases. Secretory phospholipase A₂ (sPLA₂), an important lipid mediator in inflammation, does not break down normal RBCs but will hydrolyze lipids in PS-exposing RBCs. This in turn will generate lysophospholipids and free fatty acids, including arachidonic acid, important building blocks for thromboxanes and leukotrienes. Excessive amounts of both fatty acids and lysophospholipids can have profound effects on the vasculature. Interestingly, under conditions that lead to PS exposure, a phospholipase D is activated that generates phosphatidic acid from PC in RBC. In the presence of sPLA₂, PS-exposing cells generate lysophosphatidic acid (LPA), which affects vascular integrity. Measuring levels of sPLA₂ has shown to be predictive for the onset of acute chest syndrome (ACS) in sickle cell patients. Intervention based on this level has allowed the prevention of this devastating syndrome and the major cause of death in this patient population. Together these data suggest that the inflammatory increase of sPLA₂ may not only predict ACS but also play a role in the observed vascular damage. This is currently evaluated in a national Institutes of Health (NIH)—supported study using a sPLA₂ inhibitor as well as other interventions.

In summary, the mutations in the globin genes that underlie hemoglobinopathies have profound effects on RBC membranes. Normal lipid composition and organization is lost in subpopulations of these cells, which in turn leads to altered interactions with other blood cells and plasma components that play a role in the pathology that defines these red cell disorders.

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