

## Regulation of Sickle Cell Phospholipid Organization

Frans Kuypers, PhD Principal Investigator

### A. Specific Aims:

- 1) Assess the influence of the increased presence of inflammatory lipid mediators in SCD on the loss of phospholipid asymmetry in subpopulations of sickle cells.
- 2) Investigate the role of intracellular signaling pathways in the erythrocyte in the regulation of the flippase and the scramblase.
- 3) Investigate the role of altered erythrocyte lipids on cell-cell interaction and physiological processes in the circulation. To accomplish these goals, we will use a multidisciplinary approach using biochemistry and cell biology techniques to study the mechanisms that maintain normal phospholipid asymmetry and lead to the dysfunction of these mechanisms in SCD.

Red cell (RBC) membrane phospholipid composition and organization are essential for proper membrane function. The well-preserved asymmetric phospholipid distribution in the red cell (RBC) is lost in a sub-population of phosphatidylserine (PS) exposing RBC (PSRBC) in sickle cell disease (SCD). The presence of these cells is associated with an imbalance in hemostasis and vascular abnormalities. Density fractions of RBC in SCD patients show different phospholipid molecular species composition. Whereas RBC do not generate phospholipids *de novo*, they actively incorporate fatty acids from plasma into phospholipids via reacylation of lyso-phospholipids. Long-chain acyl-CoA synthetases (ACSL) and AcylCoA acyltransferase play crucial roles in this turnover. Our long-term goal is to understand the dynamic regulatory mechanisms that are involved in plasma membrane phospholipid composition and organization, and the physiological consequences that result from the presence of PS-exposing RBC in the circulation if these systems are altered.

### B. Studies and Results:

The proteins involved in the maintenance of RBC plasma membrane phospholipid composition have been elusive to date, hampering a better understanding of the molecular mechanisms that maintain normal composition and lead to dysfunction as observed in SCD. In the current year we have made significant progress in the definition of these proteins, that will allow us to define the alterations that result in the abnormal membrane function of SCD RBC. PS exposure in SCD RBC occurs when the aminophospholipid translocase or flippase is dysfunctional. We have been able to define the RBC flippase as a member of membrane-bound  $Mg^{2+}$ -ATPases, exclusively found in eukaryotes, that play a key role in the maintenance of the membrane lipid organization (1). To study relation between structure and function we have been able to express this protein in well defined yeast vesicles, and have begun to study different (mutated) forms of the protein to better understand how this transporter actually moves aminophospholipids from outer to inner monolayer. Importantly it will help us to define how calcium and thiol modification, factors known to lead to PS exposure in SCD, alters its function.

We have defined several isoforms of Acyl-CoA Synthetase Long-chain member 6 (ACSL6), and as such set the stage to define structure function relationships of this protein of which the activity is altered in SCD (2). We have been able to express this protein as in an active form in a bacterial expression system, and have begun to study different (mutated) forms of the protein to better understand how the first step in lipid molecular species turnover and repair after oxidant damage is regulated(2-4). ACSL6 is the first synthase defined to be located in a plasma membrane and we feel that our studies will be an important contribution to better understand phospholipid homeostasis in SCD RBC but also in plasma membranes in general. In PS exposing SCD RBC phospholipase D action generated phosphatidic acid. In the presence of sPLA2, an important factor in the inflammatory state of SCD and a keyplayer in acute chest syndrome, these cells are broken down to release lysophosphatidic acid or LPA. We have shown that LPA is elevated in SCD and in a vascular model system we show that LPA can lead to vascular leak (5). Lysophospholipids are re-acylated in a normal functional RBC. The proteins involved have not been identified to date. We report that we have been able to identify the two family members

that re-acylate LPC and LPA in RBC (3,4), results that have been submitted for publication (6). We have also identified a novel form of the acyl-CoA binding protein essential in the modulation of the acyl-CoA dependent phospholipid turnover (4). Our data indicate that we will be able to define the proteins that re-acylate LPS and LPE in the coming year. Together we feel that the identification of these keymembers of lipid turnover will put us in an excellent position to define the molecular mechanisms that maintain phospholipid composition in the sickle red cell membrane.

Oxidant stress plays an important role in inactivating flippase activity and modifying scramblase activity, prerequisites for the exposure of PS, and we have reported that thiol modification will alter phospholipid transbilayer movement (7). The fact that increased cytosolic calcium plays a major role in the dysfunction of SCD RBC has prompted us to develop a method that allows us to measure calcium homeostasis in individual cells using flowcytometry (8)As alterations of cellular function are found in subpopulations of sickle RBC this approach will allow us to correlate dysfunction calcium regulation with lipid organization and activity of flippase (9).

### **C. Significance**

In the previous year we reported the development of a novel molecule; annexin V homodimer (DAV) as a superior probe to identify PS-exposing membranes. In addition, DAV protects PS RBC from sPLA2 induced hemolysis, and interferes with cellular hemostasis. The size of the novel protein exceeds the renal filtration threshold and in the current year we were able to show that DAV has a 6.5-hour half-life (beta phase) in the rat circulation as compared to 20 minutes for annexin V. DAV was shown to exert dose-dependent antithrombotic activity in rat veins and attenuates ischemia-reperfusion injury, which often complicates thrombosis. We have extended our data on this novel protein and the results are in press in the journal for Thombosis and Heamostasis (10). Our reported data suggest that Diannexin therapy may be considered in a wide range of clinical situations, in particular where PS exposure is documented such as sickle cell disease. Several avenues are currently explored towards the clinical use of this compound in collaboration with the company with whom we developed this protein, Alevita. We feel that ultimately we will be able to bring this back to the sickle cell community, as this protein interferes with the pathologic consequences of PS exposing cells. As part of the study headed by Dr. Styles in our center to define the role of sPLA2 in SCD, we have performed studies as indicated above and developed a novel sPLA2 activity assay that can be used in the clinical laboratory on a Chem. Analyzer. In addition, we are modifying an ELISA test to measure sPLA2 protein levels in the clinical laboratory. To be able to measure sPLA2, either as protein level or activity, in a clinical lab is essential to be able to use it as a prediction of ACS and to apply intervention to avoid (11). Our studies form the basis for a planned trial in the SCD clinical network (Proactive), and a recently started clinical trial to test a potent sPLA2 inhibitor with a pharmaceutical company (Anthera).