Hereditary Hemolytic Anemias

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Inherited hemolytic anemias are characterized by increased destruction of red blood cells (RBCs). They are designated spherocytic or nonspherocytic based upon the shape of the RBC when viewed under a microscope. Spherocytosis is one of the most common inherited diseases with an incidence of 1:2500 to 1:5000 and has been described in all populations worldwide[1-3]. Spherocytic (or elliptocytic or pyropoikilocytic) RBCs have increased fragility due to abnormalities of the components of the RBC membrane skeleton. Classical biochemical and electron microscopy studies have resulted in the current model of the RBC membrane skeleton which involves a network of proteins organized around a hexagonal array of spectrin tetramers cross-linked by short actin filaments [4]. Accessory proteins (ankyrin, bands 4.1, 4.2) form connections between spectrin and integral membrane proteins and other accessory proteins are involved in the spectrin-actin cross-links (adducin, dematin, tropomyosin, tropomodulin). The membrane skeleton is crucial to the red cell, providing both support and flexibility as cells move rapidly through the circulation and traverse narrow capillaries [5, 6]. Defects in membrane skeleton proteins cause mild to severe hemolytic anemia [7] and even hydrops fetalis [8].

Inherited nonspherocytic hemolytic anemia (distinguished by lack of spherocytes and normal osmotic fragility) is attributed to various RBC enzyme deficiencies. The most common enzyme abnormalities are glucose 6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK). G6PD deficiency affects over 200 million people worldwide. More than 300 genetic variants of G6PD have been described and have a wide variety of effects on enzyme activity and clinically significant hemolytic anemia [9]. G6PD A- is present in about 10-15% of African American males and causes decreased enzyme activity in RBCs. Other G6PD variants have reduced catalytic activity and marked instability or are produced at a lower level (G6PD Mediterranean). This is seen in up to 5% of persons of Mediterranean or Asian ancestry. The severity of hemolytic anemia in G6PD mutations depends upon the level of enzyme activity in the RBC and the severity of the oxidant challenge to the RBCs. Treatment with an oxidant drug is sometimes the precipitating cause of a hemolytic episode. Other clinical events that induce oxidant stress, such as infection, diabetic ketoacidosis, or severe liver injury, may precipitate a hemolytic event. Newborns with G6PD Mediterranean or other variants can exhibit jaundice. The G6PD A- variant may cause significant jaundice in premature infants.

Mutations in pyruvate kinase are the second most common cause of inherited nonspherocytic hemolytic anemia. While over 130 mutations have been described, two mutations seem to predominate in the Western hemisphere (1529A and 1456T)[10, 11]. The 1529A mutation in particular seems to predominate in the USA (41.6%) and Northern European areas (41%). Mutation 1456T is probably the most common in Southern Europe (32% in Spain, 29% in Italy and Portugal), where mutation 1529A is rare. Both mutations are missense mutations resulting in an amino acid substitution (1456T changes 486Arg to Trp and 1529A changes 510Arg to Gln).

Current diagnosis for hereditary hemolytic anemias utilizes a variety of tests that include osmotic fragility, flow cytometry, Coombs test to rule out

immunohemolytic anemia and biochemical testing for enzyme activity. For approximately 30-50% of patients, tests are performed for all known RBC defects but a specific defect is never identified, suggesting that there are many uncharacterized genetic abnormalities.

New Approaches for diagnosis

A. EMA flow cytometric assay

While osmotic fragility has long been considered the gold standard for diagnosing membrane related hemolytic anemias (spherocytosis, elliptocytosis, etc.), the test is labor intensive and has recently been replaced with a flow cytometry based assay at the University of Washington School of Medicine and other institutions [12, 13]. This assay is based upon binding of eosin-5-maleimide (EMA) to RBC surface proteins and the observation of decreased binding in patients with hereditary spherocytosis [14]. The binding of EMA has been demonstrated to occur mostly on band 3 (Lys-430 on the first extracellular loop) and Rh-related proteins [15]. While the sensitivity and specificity of this screening test are reported as 92.7% and 99.1% it is also noted that some patients with ankyrin deficiency did not show a decrease in EMA binding. Ankryin is one of the major genes that is mutated in hereditary spherocytosis [1, 16] so it would be important to know whether ankyrin deficiency is not being detected in this screening test.

B. Automated mass spectrometry

Recently developed mass spectrometry technology represents a giant leap in our ability to analyze the protein composition of a cell. This is possible due to advances in high throughput tandem mass spectrometry and automated data analysis methods[17, 18]. Briefly, a complex mixture of proteins is proteolyzed to generate peptide fragments which are separated by liquid chromatography and analyzed by a tandem mass spectrometer. Protein identifications are made using powerful software packages that match the observed peptide fragments with those predicted for proteolyzed proteins in the public sequence database. “Discovery” tandem mass spectrometers can be used to comprehensively profile a sample for its constituent proteins (hundreds to thousands). “Targeted” instruments can be used to rapidly screen a sample for a specific set of protein biomarkers (ten to twenty).

Methods for quantitative comparison of protein profiles

Several approaches have been taken to obtain quantitative comparisons between biological samples using automated mass spectrometry. One approach is metabolic labeling with a stable isotope, where all proteins of the organism or cells are labeled [19]. This approach is not a practical approach for human samples and can be costly for mouse models, requiring several generations of mouse breeding to ensure complete labeling.

The second approach is chemical labeling of proteins after isolation from cells or organisms (ICAT or iTRAQ). This approach limits the scope of proteins analyzed because of labeling only a subset of proteins with reactive groups (e.g. the sulfhydryl groups that are labeled with ICAT). It is also costly and sample to sample variation can be high.

Interest has grown in the development of label-free comparative approaches that would circumvent the need for metabolic labeling or chemical labels. Our collaborator at The University of Washington, Mike MacCoss, is a pioneer of this approach and has developed the software to perform label-free quantitative comparisons between biological samples [20]. This approach determines differences in abundance of peptides from their intensities in spectra. It is ideal for analyzing samples from humans because no labeling is involved.

Over the past three years, we have optimized methods to isolate RBCs from whole blood to 99% purity, greatly reducing contamination with proteins from platelets, white blood cells, and serum. Additionally, sample prep and run conditions have been optimized which allow for the identification of 400 to 600 proteins from sub-microgram amounts of RBC ghost protein on a LTQ linear ion trap mass spectrometer. I will describe our initial results using automated mass spectrometry for analyzing RBCs from mouse models of hemolytic anemia.

Mouse models of inherited hemolytic anemia

Mouse models have contributed significantly to our understanding of hemolytic anemia[21],[22]. Targeted disruptions or spontaneous mutations have been described in the genes for many of the well-studied components of the RBC membrane skeleton (for example, spectrin[23], ankyrin[24], band 3[25], protein 4.2[26], band 4.1, adducin [27, 28]). These models differ in disease severity and in their secondary effects and represent a valuable

resource for studying the global effects of individual gene mutations.

The mouse models have been under-utilized to date because normal versus disease protein differences have not been globally and comprehensively analyzed. Past studies of protein changes in the RBC have been hampered by the limitations of methods for studying complex systems and limitations in detecting low abundance protein components. Newly developed mass spectrometry technology is an ideal approach for analyzing changes in RBCs associated with inherited hemolytic anemias. The RBC is particularly amenable to quantitative mass spectrometry due to its relatively limited protein complexity (lack of nucleus and organelles) and its ease of purification. My lab is the first to apply label-free differential mass spectrometry to the analysis of RBCs from mice and humans with inherited hemolytic anemia.

For our first application of label free analysis to comprehensively detect protein differences between normal RBCs and RBCs from mice with inherited hemolytic anemia, we chose the mouse with targeted disruption of beta adducin, previously well studied in my lab**.** The beta-adducin knock-out (*Add2*-KO) mouse is a model of compensated hemolytic anemia. Adducin is present in human RBCs as a mixture of α/β heterodimers [29] while mouse RBCs also have the γ subunit at low levels [27, 28, 30]. Similar to patients with hereditary spherocytosis, RBCs from beta-adducin null mice are osmotically fragile, spherocytic, and dehydrated compared with wild type.

For both the wild-type and *Add2*-KO mouse, proteins were qualitatively identified using 6 reverse phase MS/MS runs. In total, 572 proteins were identified for the normal RBC using SEQUEST and postprocessing with Percolator (peptide spectrum match q-value <0.005, ≥ 1 peptide per protein). Likewise, 710 proteins were identified for the *Add2*-KO RBC. The difference in proteins identified (572 in wild-type versus 710 in *Add2*-KO) is most likely due in part to differences in circulating RBC subtypes. In mice and patients with hereditary hemolytic anemias, the body attempts to compensate by increasing RBC production. These animals and patients commonly have elevated reticulocytes (immature RBCs), the immediate progenitor of mature RBCs. Thus, a blood sample collected from an *Add2*-KO mouse will have elevated reticulocytes and is predicted to have more reticulocyte-specific proteins detected by mass spectrometry.

Protein differences detected between wild-type and *Add2*-KO RBCs

The normal and *Add2*-KO RBC μLC/MS data were analyzed by CRAWDAD [20]to identify protein differences. Data sets consisted of a total of six technical replicates per genotype (three technical replicates for each of two biological replicates). The CRAWDAD alignment of the data resulted in the identification of 298 μLC/MS regions that have a different abundance between samples. Of the detected difference regions, 44.3% were successfully mapped to MS/MS peptide identifications at a q-value of 0.01 or less. A total of 38 proteins were identified with changing levels at criteria of 1 peptide per protein. From this, 7 proteins were found to be decreased in the *Add2*-KO RBC while 31 proteins were found to be increased.

Further studies are in progress to confirm these protein differences identified by comparative mass spectrometry. Intriguing results suggest new protein interactions that are important to the normal architecture of the RBC membrane skeleton. We are now beginning to apply this technique of comparative proteomics to the study of families with inherited hemolytic anemia. We will perform direct comparisons of diagnostic testing methods including flow cytometry, enzyme activity assays, and automated mass spectrometry. We hope that our studies will lay the foundation for quantitative analysis of patient samples and the future development of a rapid, clinical diagnostic tool.

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