Published in final edited form as:

Expert Rev Proteomics. 2010 December ; 7(6): 833–848. doi:10.1586/epr.10.88.

The proteome of sickle cell disease: insights from exploratory proteomic profiling

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Abstract

The expanding realm of exploratory proteomics has added a unique dimension to the study of the complex pathophysiology involved in sickle cell disease. A review of proteomic studies published on sickle cell erythrocytes and plasma shows trends of upregulation of antioxidant proteins, an increase in cytoskeletal defects, an increase in protein repair and turnover components, a decrease in lipid raft proteins and apolipoprotein dysregulation. Many of these findings are consistent with the pathophysiology of sickle cell disease, including high oxidant burden, resulting in damage to cytoskeletal and other proteins, and erythrocyte rigidity. More unexpected findings, such as a decrease in lipid raft components and apolipoprotein dysregulation, offer previously unexplored targets for future investigation and potential therapeutic intervention. Exploratory proteomic profiling is a valuable source of hypothesis generation for the cellular and molecular pathophysiology of sickle cell disease.

Keywords

2D-DIGE; apolipoprotein; cytoskeleton; exploratory proteomics; lipid rafts; MALDI-TOF; oxidative stress; pulmonary hypertension; SELDI-TOF; sickle cell disease

Sickle cell disease (SCD) is an autosomal recessive genetic disorder that involves a single adenine-to-thymine point mutation in the gene encoding the β -subunit of hemoglobin. This translates to a single amino acid substitution of valine to glutamic acid. Patients with SCD demonstrate marked phenotypic heterogeneity in disease expression, particularly in the prevalence and severity of its complications and its mortality rate. The fundamental pathophysiological basis of SCD is the polymerization of sickle hemoglobin in the cytoplasm of the red blood cell (RBC) [1–4]. The degree of sickle hemoglobin polymerization varies with microenvironmental changes, including oxygen saturation and anatomic location in the body, and varies over time. Most prominent of its consequences is diminished RBC flexibility, which impairs blood flow through the microvasculature. Unique to SCD are episodes of acute vascular occlusion that promote tissue ischemia and pain,

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

sometimes progressing to organ infarction. Vascular occlusion may also cause a pneumonia-like condition, known as ‘acute chest syndrome’, and bone necrosis, especially in the femoral head. Vascular occlusion due to polymerization of sickle hemoglobin remains the hallmark and virtually unique feature of SCD.

Sickle hemoglobin polymerization leads to a remarkable spectrum of biochemical, cellular and physiological pathology, which is associated with a wide array of clinical complications. Sickling appears to promote adhesion of various blood cells to the activated, adhesive vascular endothelium, which may contribute to vascular occlusion [5,6]. Sickle hemoglobin polymerization robustly induces RBC oxidant stress, with linked damage to RBC metabolism, the cytoskeleton and membrane, leading to loss of membrane integrity and hemolysis. Recently, proposed models of SCD pathophysiology have focused on understanding the variable expression of pain, infarction and a host of additional complications. Strong evidence has accumulated from one recent model, suggesting that the hemolysis induced by sickle hemoglobin polymerization promotes deficiency of nitric oxide (NO), increasing the risk of pulmonary hypertension, cutaneous leg ulceration and priapism, a painful, persistent erection of the penis [7]. These complications are reported in other hemolytic diseases as well [8]. Cerebrovascular disease and stroke are also common in SCD, with some suggestion that hemolysis may play a role in this risk [9]. However, the contribution of hemolysis to vascular dysfunction has also been disputed [10].

The field of proteomics has grown exponentially in the last decade with significant technological advances that allow high-throughput proteomic profiling of multiple patient samples, database-driven identification of proteins on a molecular level with highly sensitive and precise mass spectrometry (MS), and improved resolution of a wider range of molecular mass:charge ratios, as well as techniques that are able to complement and validate each other for improved accuracy of findings. The intent of this article is to examine what has been discovered to date using these proteomic tools, to study the RBC and plasma proteome involved in SCD and to suggest future directions of study.

Normal erythrocyte proteome

To summarize a recent comprehensive review of the RBC membrane proteome [11], the major categories of known proteins are those involved in membrane repair, maintaining RBC shape and deformability, regulation of cell volume, the transport of nutrients and intercell signaling and interaction.

The RBC membrane consists of the phospholipid bilayer and supporting cytoskeletal network, which are attached to each other by protein 4.1, protein 3 and ankyrin [12–14]. The RBC is unable to synthesize phospholipids itself, so any loss of integrity of the phospholipid membrane depends on exchange with the environment for repair – the outer layer, by incorporating free cholesterol obtained from plasma lipoproteins, and the inner layer by ATP-mediated acylation of membrane lysophospholipids [15–17].

Red blood cell shape and deformability is largely determined by the cytoskeletal network, which consists of highly coiled helical rods of spectrin tetramers, conferring flexibility and spring-like properties [18,19], and actin, which polymerizes to increase cell rigidity and *vice versa*. Dematin (protein 4.9) and α - and β -adducin heterodimers bundle the actin filaments. Adducin also acts as an actin polymer cap and, together with tropomyosin and tropomodulin, tightly controls the ratio of polymerized:depolymerized actin to affect cell malleability [20–23]. ATP-driven phosphorylation of the cytoskeletal network [24,25], calcium extrusion and various membrane-associated enzymes [26] have also been noted to mediate RBC plasticity.

Deformability of the RBC is a crucial property to facilitate its passage through capillaries, and for successful oxygen delivery to the target tissues. Two factors that have been identified as major contributors to the deformability of the cell are the reduced surface area:volume ratio [27,28] and cytoplasmic viscosity, which depends on the concentration of hemoglobin [29]. Viscoelastic properties of the membrane itself have actually only been a minor factor [30]. Owing to cell volume being an important determinant, the red cell has a number of transporters believed to have a role in volume regulation, including several different cation ATPase transporters [31–33], passive K:Cl co-transporters and calcium-dependent potassium transporters [34], aquaporins that respond to changes in osmolarity [35–37] and amino acid transporters that are also suspected to be involved in osmoregulation [38,39].

Other transporters found in the RBC membrane are responsible for RBC function (the band 3 anion exchanger facilitating proton-mediated oxygen release from hemoglobin to tissues [40]) and nutrient influx (facilitated glucose transporters providing RBCs with their primary energy source [41] and nucleoside transporters [42]).

RBC membrane proteomics in SCD

In 2005, Kakhniashvili *et al.* evaluated the sickle cell erythrocyte membrane proteome using 2D fluorescence difference gel electrophoresis (2D-DIGE; Figure 1), trypsin digest, HPLC and tandem MS (LC-MS/MS). A total of 22 proteins were identified (44 protein forms, including post-translational modifications) from sickle cell RBC membranes that had a 2.5-fold or greater difference from control RBC membranes, according to the threshold deemed statistically significant, based on normal variation of protein levels in normal healthy controls [43]. Proteins with the most pronounced increases in sickle cell RBCs included the 70-kDa heat-shock protein 8 isoform 1 and chaperonin-containing T-complex protein 1 (TCP1) – both involved in protein repair; protein 4.1; and ankyrin – both involved in cytoskeleton attachment to the phospholipid bilayer, and the α -subunit of ATP-synthase. Interestingly protein 4.1 was both increased and decreased in differing spots on the 2D gel. The two isoforms diverging in quantity had a notable difference in isoelectric point (pI) and molecular weight (MW). The isoform with a pI of 5.98 and MW of 89.2 kDa was decreased 3.2-fold in sickle cell RBCs, in contrast to the isoform with a pI that increased from 5.6 to 5.78 and a MW of 82.7 kDa, which was increased 3.3– to 6.6-fold. Ankyrin's MW was increased 3.9– to 4.9-fold in sickle erythrocytes (Table 1).

Decreases in sickle cell membranes tended to be among components of the lipid raft (flotillin-1 and stomatin) and actin accessory proteins (dematin and tropomyosin 3). The functions of flotillin-1 and stomatin have not been completely elucidated, but they are transmembrane moieties thought to be involved in signal transduction. Stomatin is known to regulate transmembrane monovalent cation flux [44].

More recently, the sickle cell membrane proteome was studied in RBCs exposed to hydroxyurea, the only drug approved by the US FDA expressly for SCD [45]. This agent is known to promote elevation of fetal hemoglobin, increasing the mean corpuscular volume suggestive of improved RBC hydration and decreasing endothelial adhesion. In this exploratory proteomic approach, the investigators sought to identify changes to the sickle cell erythrocyte membrane induced by exposure of the RBCs to hydroxyurea *in vitro*. To assess quantitative changes in protein forms and post-translational modifications, 2D-DIGE and MS/MS were applied to erythrocyte membranes from samples of sickle cell whole blood, incubated at physiologic temperatures for 15 h with and without a physiologic concentration of hydroxyurea (50 μ M). This approach yielded ten proteins of interest: a trend involving antioxidants (catalase, thioredoxin peroxidase, flavin reductase and

peroxiredoxin-2 isoform a), an oxidoreductase (aldehyde dehydrogenase), proteins involved in protein repair (chaperonin-containing TCP1 subunits δ and ϵ) and protein degradation (proteasomal α_2 subunit). In addition, key players included a structural membrane component (palmitoylated membrane protein p55), carbonic anhydrase and β -globin.

The rise in antioxidants was attributed to a compensatory response to the oxidative stress effect of hydroxyurea from its mechanism for induction of altered erythroid differentiation. The increase in protein repair and turnover constituents was suggested as a response to oxidative protein damage. Further investigation to assess the post-translational modification of catalase using 2D quantitative western blotting showed a twofold increase in tyrosine phosphorylation with hydroxyurea exposure. This observation is consistent with prior studies that showed phosphorylation of catalase by tyrosine kinases in response to oxidative stress, increasing catalase activity at a post-translational level [46–48]. It is remarkable that this set of *in vitro* hydroxyurea experiments yielded alterations in protein abundance, since the principal effects of hydroxyurea have previously been attributed to alterations in transcriptional regulation that are not possible in enucleated mature RBCs. In patients with SCD, there are large numbers of circulating reticulocytes – enucleated immature RBCs that contain abundant RNA and are translationally active – so it is possible that these *ex vivo* hydroxyurea experiments might have reflected both its translational and post-translational effects.

A follow-up study with a similar design examined the RBC membrane proteome following *in vivo* administration of therapeutic doses of hydroxyurea (400 μ m) in five patients with excellent clinical responses to treatment, compared with five untreated SCD patients [49]. The most significant increases were observed in cytoskeletal components (anion exchanger band 3, ankyrin, protein 4.1, p55, actin, tropomodulin and stomatin) and glycolytic enzymes (glyceraldehydes-3-phosphate dehydrogenase and fructose biphosphate aldolase). The increase in glycolytic enzymes was attributed to an increased demand for ATP synthesis as a result of hydroxyurea-induced oxidative stress; however, an alternate explanation might involve activation of the hypoxia-inducible factor (HIF)-1, which is known to regulate anaerobic glycolysis [50].

Decreases were observed in chaperonin-containing TCP1 subunit 2 (2.45-fold) and proteasome subunit- α type 4 (4.13-fold). Although the authors again attributed these decreases to increased oxidative stress in response to hydroxyurea, these changes may actually reflect a clinical improvement in response to treatment, considering that both of these proteins are elevated in SCD at baseline [43]. The only hydroxyurea-induced change common to both the *in vivo* and *in vitro* studies was the increase in p55. Identity was confirmed by immunoblot, which demonstrated a five–tenfold increase of p55 in two representative hydroxyurea-treated patients, as compared with untreated patients. Conclusions are limited by the lack of paired control specimens from each subject prior to hydroxyurea, providing potential confounding by variability of inherent characteristics of each individual sickle cell patient. The authors also acknowledged the limitation of the small sample size of this study. A larger confirmatory study, although possible with 2D-DIGE, would likely require incorporation of more high-throughput proteomic techniques.

As a precursor to formal sickle cell proteomic studies, Chou *et al.* published a pilot study with a primary intent to establish the utility of cleavable isotope affinity tag (cICAT) methodology as a proteomic technique [51]. Its secondary aim was to preliminarily identify structural proteins with statistically significant differences in sickle cell RBC membranes. Core RBC membrane skeletons of patients with SCD and normal healthy African–American controls were analyzed by ICAT and nano-LC-MS/MS. Samples were analyzed in three combinations: a control sample to itself (AA1/AA1), a control sample to a different control

sample (AA1/AA2) and a control sample to a sickle cell sample (SS/AA), looking at mean ratios of each of the four cytoskeletal core proteins (α -spectrin, β -spectrin, protein 4.1 and actin). Mean ratios were not significantly different from 1.0, with similar variation among ratios for each protein. Variation contributed by the cICAT method in quantifying protein amounts was calculated to be 14.1%. The authors concluded that cICAT has improved precision in the quantification of proteins over 2D-DIGE, but is limited by missing many post-translational modifications, and, thus, recommended it as a complementary technique to 2D-DIGE in proteomic analysis. The secondary analysis showed no statistically significant differences between sickle cell and normal healthy subject RBC membranes, but with only nine subjects, the experiment probably lacked sufficient statistical power to definitively rule out a difference.

Monocyte proteomics in sickle cell disease

Elevated PIGF levels correlate with the severity of vaso-occlusive manifestations in SCD [52]. PIGF also activates endothelial cell adhesion molecule expression by increasing monocyte production of IL-1 β and TNF- α [53]. This premise prompted examination of the proteome of monocytes from ten well-phenotyped adolescents with SCD by 2D-DIGE, followed by trypsin digest and LC-MS/MS [54]. Quantities of identified proteins were correlated with the vaso-occlusive crisis rate, a surrogate measure of clinical severity.

Monocyte membrane proteins most strongly correlated with disease severity were glycolytic enzyme transketolase, coronin and moesin – actin binding proteins involved in motility – and cell signaling mediator guanine nucleotide-binding protein. These proteins were all negatively correlated with vaso-occlusive manifestations.

Cytosolic monocyte proteins with the greatest positive correlation with disease severity were the far upstream element-binding protein, α -actinin, the myristic acid/tri-iodobenzoic acid/albumin complex, filamin A, integrin, the apo form of human mitochondrial aldehyde dehydrogenase chain A and leukotriene A-4 hydrolase. Negatively correlated cytosolic monocyte proteins largely consisted of those involved in protein folding, repair and turnover – heat-shock proteins (70 kDa and mitochondrial 60 kDa), TCP and chaperonin-containing TCP1 (Table 2). A mitochondrial apoptotic mediator, adenylate kinase 2 (variant AK2D), was also negatively correlated with recurrent vaso-occlusive events, as was phosphoglycerate kinase 1, an enzyme involved in glycolysis and angiogenesis. These findings are potentially useful as hypothesis-generating information, but are limited by unclear statistical significance. Validation of each candidate marker by independent assay is also necessary for definitive identification.

Sickle cell plasma proteomics

Pulmonary hypertension (PH) is an independent risk factor for mortality in SCD [55]. Our group carried out a high-throughput exploratory proteomic study searching for markers of pulmonary arterial hypertension in the plasma of sickle cell patients utilizing a variety of proteomic techniques [56]. Proteomic profiling by SELDI-TOF MS was performed on plasma samples from a cohort of 56 age- and gender-matched patients with homozygous SCD, with and without PH as determined by high versus normal tricuspid regurgitant jet velocity on a Doppler echocardiogram. The most significant mass to charge (m/z) species (Figure 2) were identified through statistical analysis using the random forest computational algorithm and stepwise logistical regression. Species of interest were then identified using protein electrophoresis, in-gel trypsin digest and LC-MS/MS with or without confirmation by MALDI-TOF MS. These results were validated by correlation with available standard clinical immunoassays. This approach suggested decreased plasma levels of apolipoprotein A-I (apoA-I) as a potential marker of PH risk in the setting of SCD. Other preliminary

markers found were increased apolipoprotein A-II (apoA-II) and serum amyloid A (SAA)-4 levels, and decreased levels of plasminogen dimer and haptoglobin dimer (Table 3). ApoA-I levels were validated by correlation with clinical assays for both apoA-I and high-density lipoprotein cholesterol (HDL-C; $p = 0.0001$; Spearman's rank correlation: 0.59). Apolipoprotein B (apoB) levels, though not detected through SELDI-TOF MS, were also measured by a clinical assay, with high levels correlating with PH ($p = 0.05$). This led to calculation of the apoB:apoA-I ratio, which was significantly elevated in PH ($p = 0.006$). In a supporting blood flow physiology study of vasomotor reactivity in SCD subjects, lower levels of apoA-I were associated with an impaired vasodilatory response to the endothelial-dependent agonist acetylcholine ($p = 0.001$), indicating association of endothelial dysfunction with lower apoA-I levels in SCD. These results taken together suggest that apolipoprotein dysregulation, manifested by low apoA-I levels, contributed to endothelial dysfunction and PH associated with SCD.

Protein function and structure alterations due to oxidative stress are thought to potentially be a contributor to the pathogenesis of PH. This premise generated a hypothesis of whether oxidative post-translational modifications of abundant plasma proteins could potentially serve as markers of disease severity, which was proteomically explored [57]. Albumin isolated from the plasma of sickle cell patients, with and without PH, revealed an increased presence of a malondialdehyde (MDA) adduct in PH, by MALDI-TOF MS and immunoblot. LC-MS/MS and x-ray crystallography further localized the adduct to the K159 residue on albumin. A similar study of albumin from patients with non-SCD-related idiopathic pulmonary arterial hypertension also showed increased post-translational modification with MDA at the same site. It was concluded that this may represent an oxidative lesion specific to the pathogenesis of pulmonary hypertension, and it could be a candidate to serve as a biomarker. This study also suggested that such post-translational modifications may be present on other abundant, as well as potentially nonabundant, plasma proteins.

A second study from our group also used SELDI-TOF MS and clinical immunoassays to profile plasma from 26 adult sickle cell patients at steady state versus during acute pain episodes (APEs) [58]. Significant differences in intensity during APEs included an elevated m/z species of 11.7 kDa and a decreased m/z species of 28.1 kDa, identified as SAA and apoA-I, respectively. The relative intensities of these m/z species correlated strongly with their respective immunoassays. Four patients with clinically severe extraosseous complications during APE were distinguished by extreme elevations of SAA (median: 413.5 vs 6.5 mg/ml). Further analysis of immunoassay data also demonstrated an inverse correlation between the two markers during APE, such that an increased SAA:apoA-I ratio ($p < 0.004$) may be an even more robust marker of APE (Table 3).

The two SELDI-TOF studies confirmed and extended previous observations regarding apoA-I and SAA in SCD [59–62]. Validation of results generated by this technique with clinical assays, high-resolution MS and immunoassays supports the validity of SELDI-TOF MS when used as a screening tool for further confirmatory immunoassays or high-resolution MS. SELDI-TOF alone lacks the resolution for independent identification of protein markers.

Sickle cell transcriptomics versus proteomics

A few studies have examined reticulocyte, erythrocyte, platelet and monocyte gene-expression profiles in the setting of SCD, as well as hydroxyurea treatment, and offer an additional perspective. Several findings from a high-throughput microarray mRNA genomic-profiling study of sickle cell whole blood coincide with recurring proteins in exploratory proteomic studies, in particular, significant upregulation of ankyrin, erythrocyte

membrane protein bands 3 and 4.1, peroxiredoxin and stomatin. Increased levels of enzyme 2,3-bisphosphoglycerate kinase mRNA supports the theory of stimulation of glycolytic pathways in SCD, suggested by proteomic data. Other differentially expressed transcripts included selenium-binding protein, exportin, spermine oxidase, chemokines, interleukin receptor and peptidyl arginine deaminase [63].

A similar study design assessing platelet mRNA expression identified 100 differentially expressed genes in SCD, major categories being arginine and nitrogen metabolism, redox homeostasis, cell growth, adhesion and signaling pathways. Most notably, there was a significant increase in mRNA of arginase II and ornithine decarboxylase, both enzymes being involved in converting arginine to polyamines. The absence of SCD platelet proteomic studies limits direct comparisons between this study and proteomic data, but the finding of increased redox proteins glutathione peroxidase 4, thioredoxin reductase and superoxide dismutase continues to support the recurrent SCD theme of a response to oxidative stress [64].

Mononuclear gene-expression profiles were investigated in 27 patients with steady state SCD (ten hydroxyurea-treated, 17 untreated) and 13 healthy controls, identifying a highly specific leukocyte transcriptional response in SCD. A total of 112 genes were identified as being differentially expressed in SCD by global transcriptional microarray analysis, major categories involving heme metabolism, cell cycle regulation, antioxidants, inflammatory mediators and angiogenesis. Most notable were the upregulations of hemeoxygenase 1, biliverdin reductase, cyclin-dependent kinase p21, IL-15, ECGF-1 and antioxidants glutathione peroxidase, thioredoxin and thioredoxin peroxidase. Of note, hydroxyurea did not appear to have a direct effect on leukocyte gene expression, but the study appears underpowered to definitively comment on this effect [65].

A reverse transcriptase PCR-based transcriptomic study of hydroxyurea-induced gene expression in reticulocytes of eight SCD patients on hydroxyurea for at least 3 months, compared with eight SCD patients unexposed to hydroxyurea, found upregulation of a host of metal-ion-binding proteins and transporters, RNA binding proteins, ubiquitin-protein ligases and glycolytic enzymes, the latter of which also appeared in the *in vivo* hydroxyurea proteomic study by Ghatpande *et al.* [49,66].

A second similar study of patients with homozygous SCD at steady state, 14 of whom were on hydroxyurea therapy for at least 3 months, and 28 of whom were not treated with hydroxyurea, focused specifically on the hydroxyurea-induced expression of several low-affinity reticulocyte adhesion molecules, and suggested that hydroxyurea reduces the adhesive properties of sickle cell erythrocytes and does so at the transcriptional level [67].

As demonstrated by these gene-expression studies, SCD transcriptome results overlap with exploratory proteomic results. However, each sphere additionally provides a distinct set of nonoverlapping information. This is probably due to differences in disease-associated alterations occurring at the transcriptional versus post-translational levels, as well as biases inherent to specific methodologies. Global mRNA expression studies will not detect post-translational alterations of protein levels or products, which a proteomic approach is more likely to detect. Conversely, unstable proteins with short half-lives might not yield proteomics signals as strong as the corresponding mRNA abundance.

Another important distinction between proteomic and transcriptomic analysis, as it concerns SCD, is the tissue of interest to which either is applied. It is difficult to study platelets by exploratory proteomics due to the difficulty in isolating them in abundance. Gene-expression profiling can employ PCR amplification to permit the study of minute quantities of cells.

Thus, these two methodologies are complementary and both useful in examining a global expression profile of a disease of interest. By utilizing both, the pool of candidate biomarkers to be further studied can be maximized and functionally compartmentalized to an extent, allowing for more comprehensive hypothesis generation. The study of SCD in particular, stemming from a simple point mutation at the nucleic acid level but manifesting with an enormous range of phenotypic variation, conceptually emphasizes the importance of involving broad-based approaches.

Multiple globin gene-expression studies have identified polymorphic alterations within the SCD population, which is an important direction in understanding the phenotypic variation among sickle cell patients. However, since our focus is on proteins identified by the exploratory proteomic approach, we refer readers to a recent extensive review of this subject [68].

SCD proteomic interactome

In a step beyond exploratory proteomic profiling, interactome mapping attempts to define disease-specific interactions among proteomically identified candidate proteins. This additional analytic method was performed on the sickle cell erythrocyte membrane proteome [43] in the context of a preliminary erythrocyte interactome network constructed from a compilation of 751 erythrocyte membrane proteins identified by proteomic methods [69]. An extension of this analysis also attempted to quantify the impact of the SCD proteome on the erythrocyte protein interactome using a small number of different statistical clustering algorithms [70].

In these analyses, the relationships between the proteins were derived mathematically from Spearman correlations and database information, not from experimentally demonstrated interactions between the proteins of interest. Several of the SCD-altered proteins coincided with the repair or destroy box, a categorization of closely correlated proteins that share functions responsible for protein folding, repair and degradation. Several others (ankyrin 1, dematin, protein 4.9, heat-shock protein 8, proteasome subunit- α type 1 and - β type 1, chaperonin-containing TCP1, proteasome subunit- α and 26S subunit, and ATPase 6) appeared as key articulation points on the interactome map, with unclear significance.

This approach was admittedly incomplete and carried a certain degree of false discovery risk, although an attempt was made to minimize this with the use of Spearman correlation coefficients of at least 0.3 [69]. Interactome analysis may prove useful as a tool for the functional categorization of proteomically identified proteins. It is unclear at this point whether one of the interactome mapping methods is better than another, and this certainly deserves further study.

Discussion

Only a limited number of studies exist that characterize the proteome of SCD. Erythrocyte membranes have been studied most comprehensively. In these studies, some cytoplasmic proteins have surfaced as potentially significant players in SCD, although the cytoplasmic proteome of RBCs or other tissues has not yet been specifically evaluated. Proteomic profiling of sickle cell plasma has thus far been directed at finding biomarkers of known complications of SCD, namely pulmonary hypertension and APEs. A variety of methods have been used in these exploratory proteomic studies, including 2D-DIGE and ICAT in the erythrocyte proteomic studies, SELDI-TOF and MALDI-TOF MS in the plasma proteomic studies, and trypsin digest and MS/MS in all studies, for identification of m/z species of interest. Differentially expressed proteins have been quantitatively validated using immunoassays. Their complementary nature is advantageous as this allows one to examine

results trends across methods, as well as promoting nonredundant discoveries. These studies have allowed conclusions to be drawn, largely of quantitative protein alterations in SCD. Some hypotheses can be generated regarding suspected post-translational modification related to SCD, based on quantitative changes of the same identified protein bearing different pI and MW characteristics, but as yet, these putative modifications have not been rigorously validated. In examining the trends seen among the exploratory sickle cell proteomic studies to date, in the context of sickle erythrocyte proteins being described through protein biochemistry, several general pathophysiological themes are found to recur (Figures 3 & 4).

Antioxidants in SCD

Upregulation of antioxidant proteins seems to be common to most of the studies discussed in this article, most notably, increases in catalase and peroxiredoxins. A compensatory increase in antioxidants is expected in the setting of the high oxidative burden of SCD. Oxidative stress is believed to be a consequence of several mechanisms, including: hemolysis, causing NO depletion [71] and generation of oxidant species [72–78]; generation of reactive oxidative species by upregulation of NADPH oxidase and xanthine oxidase [72]; chronic ischemic reperfusion in the setting of intermittent microvascular vasoocclusion [79]; and loss of catalytic antioxidants [80–86]. Oxidative stress is thought to promote the formation of irreversibly sickled cells, which, in a vicious cycle, fuels further oxidative stress. Oxidative damage is manifested on RBC membranes via the lower presence of sulphhydryl groups, increased lipid peroxidation and deformation of the cytoskeleton [87].

Cytoskeletal components in SCD RBCs

Cytoskeletal proteins are consistently found to be altered in proteomic profiling of sickle cell erythrocytes. Cytoskeletal attachment proteins 4.1 and ankyrin were both increased significantly in SCD. In addition, levels of an isoform of protein 4.1 with an altered pI and MW were decreased, suggestive of an SCD-related alteration in a post-translational modification. Such protein alterations may be consistent with previously reported oxidative forms of protein 4.1 [88,89], β -actin and spectrin in sickle erythrocytes. No significant quantitative changes have been found in β -actin or spectrin in the exploratory sickle cell proteomic studies, but other studies have reported disulfide bridge formation by β -actin when oxidized [90], as well as glutathiolation of the cysteine residues of α -spectrin, which impedes dissociation from a ternary complex with protein 4.1 and β -actin [90–93]. These changes reduce cytoskeletal flexibility, which may contribute to sickled RBC rigidity [93]. The increase of the cytoskeletal attachment proteins ankyrin and protein 4.1 may reflect a compensatory cellular response to impaired anchoring of the phospholipid bilayer to the damaged cytoskeleton. Potentiation of this response may be reflected in the relative increase in these proteins seen in patients undergoing hydroxyurea treatment versus untreated SCD patients [49]. The function of the p55 protein is to link the plasma membrane with the cytoskeleton in a ternary complex with protein 4.1, thereby maintaining the stability of the erythrocyte membrane. Its increased expression, induced by both *in vivo* and *in vitro* hydroxyurea treatment, may hypothetically contribute to decreased RBC damage and hemolysis.

Besides protein 4.1, other actin accessory proteins show quantitative changes in the setting of SCD. Dematin and tropomyosin were both decreased by proteomic profiling, and both play a central role in RBC malleability by controlling actin bundling and the actin polymerization:depolymerization ratio. A decrease in these proteins may thus also reflect contribution to increased RBC rigidity. Proteomic analysis of RBC membranes, obtained from blood stored in blood banks by 2D-DIGE [94], revealed shifts in isoelectric points in cytoskeletal proteins after 7 days, which is thought to represent progressive degradation of

actin, dematin, and ankyrin, due to oxidation by reactive oxygen species. Why no compensatory increase in dematin expression, similar to that of ankyrin, has been observed is not clear, but may be related to the nature of dematin's dynamic function, as opposed to the more static one of ankyrin. Tropomyosin, also with a dynamic function, has a similar response to dematin.

Protein repair, protein turnover & protein folding components in SCD

A major consequence of oxidative stress is protein damage. It is therefore not surprising that several proteins involved in protein repair, folding and turnover emerged as being increased in the sickle cell erythrocyte and monocyte proteomic studies; namely components of proteasomes, chaperonin-containing TCP1 and several heat-shock proteins. Decrease of the former two proteins with *in vivo* hydroxyurea treatment could hypothetically reflect a lower net rate of protein damage, and thus a reduced demand for them. Proteasomes, heat-shock protein and the TCP1 ring complex are ATP dependent in their functions, which coincides with an increase in ATP synthase detected in sickle cell exploratory proteomic profiling [95]. Alterations in enzymes involved in glycolysis may be speculatively related to such an increase in demand for ATP synthesis, or alternatively, to a shift from oxidative phosphorylation to anaerobic glycolysis as an adaptation to hypoxia (see later). The ATP dependence of these protein-folding, turnover and repair processes may explain the upregulation of ATP synthase and, possibly, the increased metabolic rate seen in sickle cell patients. However, as stated previously, until many of these findings are validated by independent clinical assays, firmer mechanistic conclusions cannot be drawn.

Alterations in glycolytic enzymes in SCD

A recurrent pattern in the studies reviewed here is the association of upregulation of glycolytic enzymes with a favorable clinical course in SCD. In the monocyte, higher levels of phosphoglycerate kinase-1 and transketolase correlated with a lower vaso-occlusive crisis rate [54]. *In vivo* treatment with hydroxyurea, a medication known to promote clinical improvement in SCD patients, revealed upregulation of GADPH and fructose biphosphate aldolase, compared with untreated patients.

Glycolytic enzyme expression is now known to be under the control of HIF-1, the master switch of the gene-expression response to hypoxia [50,96]. HIF-1 coordinates adaption to hypoxia, shifting ATP production away from oxygen-dependent mitochondrial oxidative phosphorylation, and shunting pyruvate away from the mitochondria via activation of pyruvate dehydrogenase kinase and toward anaerobic ATP production via HIF-1-induced upregulation of glucose uptake and glycolytic enzyme genes [50]. Proteomics results indicate a three- to five-fold increase in the level of an ATP synthase subunit in SCD patients at baseline that may reflect a compensation for reduced oxidative phosphorylation synthesis of ATP. HIF-1 target genes within these pathways with proteomically identified alterations include phosphoglycerate kinase-1 and GADPH [96–98]. Fructose biphosphate aldolase, which favors gluconeogenesis, was identified as being possibly induced by hydroxyurea, which potentially benefits meeting the increased requirement for glucose under less-efficient anaerobic pathways.

Lipid raft proteins in SCD

A less obvious category of proteins altered in SCD by proteomic profiling are lipid raft components, namely flotillin-1 and stomatin. Both are known to be part of the stomatin prohibin flotillin Hflk/c (SPFH) domain-containing protein family, which is found in lipid raft microdomains in various cellular membranes and is, as yet, incompletely characterized. Lipid rafts are cholesterol-rich regions in the phospholipid bilayer. In a proposed 'lipid shell' hypothesis, SPFH domain-containing proteins form large multimeric complexes,

which bind cholesterol and form cholesterol-rich microdomains. They are then thought to actively recruit other types of proteins to these domains and mediate various protein–protein interactions. Functions of SPFH proteins have not been completely elucidated, but processes attributed to them include ion channel regulation, membrane protein chaperoning, vesicle and protein trafficking, membrane–cytoskeletal communication and mechanosensation. Flotillin-1 has specifically been cited in roles handling neuronal regeneration, adipocyte GLUT4 trafficking, cell proliferation, endocytosis, phagocytosis and signaling from the cell surface to the cytoskeleton. Stomatin has been putatively implicated in ion channel regulation [99]. Of note, proteasome activation and inactivation has been characterized as a lipid raft-compartmentalized event in macrophages [100], which suggests a possible relationship between the decrease in lipid raft components and the increase in proteasome proteins observed by exploratory proteomics in SCD.

Furthermore, in the cell membrane, the actin cortex plays a central role in organizing lipid raft sphingolipid–cholesterol assemblage potential [101], so the sickle cell-associated decrease in flotillin-1 and stomatin may be a consequence of impaired actin functioning. Impaired mechanosensation, along with poor communication between the cytoskeleton and plasma membrane, could cause inappropriate responses to environments calling for increased cell malleability, and thus contribute to RBC microvascular clumping and increased cell fragility. Decreases in lipid raft components could also be related to the hypocholesterolemia generally associated with SCD [102–104]. Low serum cholesterol levels may be a consequence of increased cholesterol consumption by membrane synthesis during increased erythrocyte production [103], but are more likely to be due to equilibration with the reservoir of RBC membrane cholesterol, which is diminished owing to anemia [105]. It is possible that the depleted cholesterol levels lead to a lower presence of lipid rafts in the RBC membrane, reflected by the proteomically identified decrease in stomatin and flotillin-1. Interestingly, stomatin levels increased following *in vivo* hydroxyurea treatment, which hypothetically suggests reversal of one or more of these potential mechanisms. It would be interesting to investigate, especially in the context of the link we established between apolipoprotein dysregulation and endothelial dysfunction, the impact of a lipid raft deficiency on erythrocyte and endothelial functioning. Signaling and interactions between erythrocytes and their environment generally remains a poorly understood subject that, with further elucidation may shed more light on the contribution of lipid raft defects to SCD pathophysiology. This presents a limitation in fully appreciating the impact and involvement of lipid rafts in SCD, but is an interesting direction for hypothesis generation and further research.

Oxidative post-translational modifications

The presence of a MDA adduct at the K157 residue of albumin in both SCD-associated PH and idiopathic pulmonary hypertension is interesting, in that MDA adducts represent an oxidative lesion and that they are a candidate biomarker for PH that warrants further clinical validation. This discovery also beckons further exploration of the existence of post-translational modifications on other abundant, as well as nonabundant, plasma proteins that may lend clues to disease pathogenesis and/or serve as biomarkers for sequelae of SCD. These approaches are technically challenging.

Apolipoprotein dysregulation associated with complications of SCD

The study of complications of SCD through proteomic profiling of plasma brought to the foreground an unexpected involvement of apolipoproteins in the pathogenesis of sickle cell-associated pulmonary hypertension and APEs. Low apolipoprotein levels were associated with each, on a chronic basis in pulmonary hypertension and more acutely in APEs. ApoA-I is known to have both antioxidant and anti-inflammatory properties. The increase in acute-

phase SAA along with the apoA-I depletion observed in both studies (by Yuditskaya *et al.* and Tumblin *et al.*) suggests involvement of a possible mechanism of apoA-I replacement by acute-phase SAA in HDL, either by direct displacement or, more likely, by competition during HDL synthesis [56,58]. This replacement would render HDL a proinflammatory particle, and inhibit its ability to prevent oxidation of apoB-containing particles, such as low-density lipoprotein, particularly in the subendothelial space. In other systemic inflammatory disorders, HDL has been observed to be less effective at preventing low-density lipoprotein oxidation as well, which is suggestive of a similar incapacitation of the normal functioning of HDL [106,107].

Apolipoprotein A-I is a key participant in the activation of endothelial NO synthase by HDL-mediated phosphorylation and thus plays a central role in increasing NO production in the presence of reactive oxygen species [108]. As such, beyond reverse cholesterol transport and atherogenesis inhibition, apoA-I protects against direct endothelial injury. HDL, in its anti-inflammatory state, also inhibits adhesion molecule expression on endothelial cells. These phenomena are of particular interest in the setting of SCD, especially with our evidence that a deficiency in apoA-I predisposes to the development of PH, and a precipitous drop in apoA-I level from steady state levels occurs during a sickle cell APE. The model of altered apoA-I levels in sickle cell patients opens a translational target for therapeutically increasing apoA-I levels in sickle cell patients to prevent the long-term complication of PH, which might increase life expectancy, as well as diminish the severity of APEs, thereby improving quality of life.

Apolipoprotein A-II was identified as being elevated in the setting of PH by proteomic profiling of sickle cell plasma. ApoA-II, along with acute-phase SAA, impairs the antioxidant properties of HDL by displacing paraoxonase-1, a HDL-associated enzyme [109,110]. Paraoxonase-1 enables HDL to accept and inactivate oxidized phospholipids from cell membranes [111]. Impairment of this function might be especially problematic in a setting of severe oxidative stress such as SCD. As evidenced by the elevation of apoA-II, this mechanism might contribute to vasculopathy and the development of PH in SCD.

The significance of the elevated constitutive SAA-4 in the setting of sickle cell-associated pulmonary hypertension is less clear as its function has not been as well characterized, but it does suggest a disorder of lipoprotein metabolism.

Expert commentary & five-year view

Exploratory proteomic methodologies have, in recent years, revealed some proteins that were previously detected via standard protein biochemistry methods to be altered in SCD, essentially validating the exploratory proteomics scientific approach. Comparisons of the RBC and plasma proteome between healthy controls and subjects with SCD, as well as among SCD patients with different complications, using these exploratory proteomic approaches have presented some fresh mechanistic insights, including alterations of apolipoprotein function in vascular dysfunction, PH and vaso-occlusive pain in SCD. Such discoveries promote new directions of thought and research regarding the pathophysiology of SCD, pathogenesis of complications involved in SCD, potential treatments targeting these proteomically identified aberrations, and earlier, and more specific, screening and diagnostic modalities.

Future investigation in this area over the next 5 years holds promise for devising new biomarkers to predict prognosis of SCD, stratify risk and provide surrogate outcome markers for future clinical trials in SCD. Such biomarkers could help to guide risky curative therapies such as hematopoietic stem cell transplantation and gene therapy, and to accelerate drug development to reduce the burden of human suffering in this insidious disease.

Acknowledgments

The authors are supported by funds from the Division of Intramural Research of the National Heart, Lung and Blood Institute (MD, USA) and the Clinical Center of the NIH (MD, USA). Gregory J Kato has received funding from the NIH (grant number 1ZIAHL006014-01).

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Figure 1. Representative 2D fluorescence difference gel electrophoresis image from sickle cell membrane proteomics study

Two different fluorescent dyes were used – Cy3 for normal healthy controls and Cy5 for sickle cell patients. The above image is the Cy3 image of the gel, showing spots from normal healthy control samples. Spots outlined in red represent spots that were decreased in sickle cell disease; those that were increased in sickle cell disease are outlined in blue. Reproduced with permission from [43].

Figure 2. Representative SELDI-TOF MS spectra from plasma from patients with sickle cell disease with and without pulmonary hypertension

The vertical axes represent intensity of peaks in arbitrary units, and the horizontal axes represent mass:charge ratio (m/z). These four spectra were obtained from plasma eluted from anion exchange resin, bound to IMAC30- Cu^{2+} matrix, and ionized by SELDI-TOF MS.

Two specimens are from sickle cell disease (SCD) patients without pulmonary arterial hypertension (A & B) and two are from SCD patients with PAH (C & D). A peak at m/z 28.1 kDa was observed at lower average intensity in SCD patients with PAH (arrows).

IMAC: Immobilized metal-affinity capture; PAH: Pulmonary arterial hypertension.

Reproduced with permission from [56].

Figure 3. Summary of major categories of erythrocyte and plasma proteins altered in sickle cell disease as identified by exploratory proteomic profiling

ApoA-I: Apolipoprotein A-I; MDA: Malondialdehyde; NO: Nitric oxide; RBC: Red blood cell; ROS: Reactive oxygen species; SAA: Serum amyloid A.

Figure 4. Physiologic and biochemical consequences of sickle hemoglobin polymerization-associated oxidant stress and hemolysis as a common pathway leading to alterations within the major protein biomarker categories
TCP: T-complex protein.

Table 1

Most notable erythrocyte proteins quantitatively or qualitatively altered in sickle cell disease as compiled from exploratory proteomic studies

Category	Function	Protein (MW)	GI no.	Change in SCD	Ref.
Cytoskeletal	Actin accessory	Ankyrin 1 (92 kDa)	105337	Increased 3.9–4.9-fold; increased threefold with HU	[43,49]
		Protein 4.1 (82 kDa); pI: 5.72	14916944	Increased 2.7–6.6-fold; increased two–threefold without HU [†]	[43,49]
		Protein 4.1 (89.2 kDa); pI: 5.98, 6-kDa larger	14916944	Decreased 3.2-fold; increased two–threefold without HU [†]	[43]
		Dematin protein 4.9 (44.1 kDa)	22654240	Decreased 2.6-fold	[43]
		Tropomyosin 3 (30 kDa)	24119203	Decreased 2.6-fold	[43]
		Anion exchanger band 3 (52 kDa)	4507021	Increased 2.5-fold with HU	[49]
		Tropomodulin (40 kDa)	135922	Increased two–threefold with HU	[49]
		Actin, β -actin (41 kDa)	1703156, 1419444	Increased 2- to 3.5-fold with HU	[49]
		Palmitoylated membrane protein p55 (55 kDa)	62898353	Increased two–threefold with HU	[49]
Membrane	Active transport	ATP synthase α -subunit (48.9 kDa)	4757810	Increased 2.6–5.1-fold	[43]
	Lipid raft	Flotillin-1 (49 kDa)	5031699	Decreased 2.8–3.3-fold	[43]
		Stomatin isoform a (27.4 kDa); pI: 6.5	38016911	Decreased 3.8-fold	[43]
		Stomatin isoform a (27.4 kDa); pI: 5.56	38016911	Increased 2.7-fold	[43]
	Vesicle transport	RAB-8b GTPase (22 kDa)	7706563	Increased 2.8-fold	[43]
Cytoplasmic	Protein turnover	Proteasome- β 1 subunit (22 kDa)	4506193	Increased 2.8-fold	[43]
		Proteasome 26S ATPase subunit 6 (39 kDa)	24430160	Increased 2.6-fold	[43]
		Proteasome- α 1 subunit, isoform 1 (34 kDa)	23110935	Increased 2.5-fold	[43]
	Protein folding	Chaperonin containing TCP1 subunit 7 (54 kDa)	5453607 [‡] , 1729870 [‡]	Increased 2.9–4.2-fold	[43]
	Protein repair	Heat-shock protein 8 (68 kDa)	5729877	Increased 7.3-fold	[43]
	Antioxidant	Peroxisredoxin 3 isoform b (22.2 kDa)	32483377	Increased 2.9-fold	[43]
		Peroxisredoxin 1 (22 kDa)	4505591	Increased 2.8-fold	[43]
	Antioxidant	Catalase (56 kDa)	4557014	Increased 3.1-fold	[43]
	Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase (36 kDa)	7669492	Increased two–fourfold with HU	[49]
		Fructose bisphosphate aldolase (39 kDa)	113606	Increased twofold with HU	[49]

Experimental MW, corresponding GI number (National Center for Biotechnology Information [NCBI] database accession number), relevant alterations in pI or MW and quantitative change in SCD or SCD-related complication are noted.

[†]Unclear which isoform represents the increase.

[‡]Representative NCBI database accession number.

GI: GenInfo identifier; HU: Hydroxyurea; MW: Molecular weight; pI: Isoelectric point; SCD: Sickle cell disease; TCP: T-complex protein.

Table 2

Most significant monocyte proteins altered in sickle cell disease, as compiled from exploratory proteomic studies.

Category	Function	Protein (MW)	GI no.	Change in SCD	Ref.
Membrane	Glycolysis	Transketolase (75 kDa)	4507521	Negatively correlated with VOC rate	[54]
	Actin-binding; cell motility	Coronin (62 kDa)	5902134	Negatively correlated with VOC rate	[54]
	Actin-binding; cell motility	Moesin (81 kDa)	4505257	Negatively correlated with VOC rate	[54]
	Cell signaling	Guanine nucleotide-binding protein (32 kDa)	5174447	Negatively correlated with VOC rate	[54]
Cytosolic	ATP-dependent DNA helicase	Far upstream element-binding protein (96 kDa)	37078468	Positively correlated with VOC rate	[54]
	Actin accessory	α -actinin (103 kDa)	4501891, 2804273	Positively correlated with VOC rate	[54]
	Unclear	Myristic acid/tri-iodobenzoic acid/albumin complex	4389275	Positively correlated with VOC rate	[54]
	Actin accessory	Filamin A (272 kDa)	116241365	Positively correlated with VOC rate	[54]
	Intra-/extra-cellular signal transduction	Integrin (168 kDa)	64654539	Positively correlated with VOC rate	[54]
	Aldehyde oxidation	Mitochondrial aldehyde dehydrogenase (63 kDa)	28949044	Positively correlated with VOC rate	[54]
	Arachidonic acid metabolism	Leukotriene A-4 hydrolase (82 kDa)	4505029	Positively correlated with VOC rate	[54]
	Protein repair	Heat-shock protein (70 kDa)	12585261	Negatively correlated with VOC rate	[54]
	Protein repair	Mitochondrial heat-shock protein (60 kDa)	129379	Negatively correlated with VOC rate	[54]
	Protein folding	TCP1, subunit- α (73 kDa)	57863257	Negatively correlated with VOC rate	[54]
	Protein folding	Chaperonin-containing TCP1, subunit 8 (69 kDa)	48762932	Negatively correlated with VOC rate	[54]
	Mitochondrial apoptotic mediator	Adenylate kinase 2 (64 kDa)	5453595	Negatively correlated with VOC rate	[54]
	Glycolysis/angiogenesis	Phosphoglycerate kinase 1 (46 kDa)	48145549	Negatively correlated with VOC rate	[54]

Experimental MW, corresponding GI number (National Center for Biotechnology Information [NCBI] database accession number) and alteration in SCD is noted. VOC rate was used as a surrogate marker of SCD severity.

GI: GenInfo identifier; MW: Molecular weight; SCD: Sickle cell disease; VOC: Vaso-occlusive crisis.

Table 3

Most significant plasma proteins altered in sickle cell disease, as compiled from exploratory proteomic studies.

Function	Protein (MW)	GI no.	Change in SCD	Ref.
Antioxidant/anti-inflammatory, HDL component	ApoA-I (28.1 kDa)	4960066 [†]	Decrease in SCD vs control	[56]
			Decrease in SCD + PH vs SCD - PH	[58]
			Decreased during acute pain episodes	
LDL/VLDL component	ApoB	178790 [†]	Decrease in SCD vs control	[56]
Ratio	ApoB:ApoA-I ratio	–	Decrease in SCD vs control	[56]
HDL component	ApoA-II (8.9 kDa)	296633 [†] , 178424 [†]	Increase in SCD + PH vs SCD - PH	[56]
HDL component, constitutive SAA	SAA-4 (13.4 kDa)	13937846 [†] , 119588821 [†]	Increase in SCD + PH vs SCD - PH	[56]
Glycoprotein precursor of plasmin	89.4 kDa [‡]	387031 [†]	Decrease in SCD + PH vs SCD - PH	[56]
Scavenges cell-free hemoglobin	Haptoglobin dimer (75.2 kDa)	386783 [†]	Decrease in SCD + PH vs SCD - PH	[56]
Uncertain identity	18.4	–	Decrease in SCD + PH vs SCD - PH	[56]
Acute phase reactant, antagonizes apoA-I	SAA	40316910 [†]	Increase in acute pain episodes	[58]
Ratio	SAA:apoA-I ratio	–	Decrease in acute pain episodes	[58]
Oxidative post-translational modification	Malondialdehyde–albumin adduct	–	Qualitative increase in SCD + PH vs SCD - PH	[57]

All proteins mentioned belong to the 'plasma' category. Experimental MW, corresponding GI number (National Center for Biotechnology Information [NCBI] database accession number) and change in SCD or SCD-related complication are noted.

[†]Representative NCBI database accession number.

[‡]Protein of uncertain identity.

Apo: Apolipoprotein; GI: GenInfo identifier; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MW: Molecular weight; PH: Pulmonary hypertension; SAA: Serum amyloid A; SCD: Sickle cell disease; VLDL: Very low-density lipoprotein.