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Vasculopathy in Sickle Cell Disease: Biology, Pathophysiology, Genetics, Translational Medicine and New Research Directions

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Abstract

Sickle cell disease has been very well characterized as a single amino acid molecular disorder of hemoglobin leading to its pathological polymerization, with resulting red cell rigidity that causes poor microvascular blood flow, with consequent tissue ischemia and infarction. More recently, an independent spectrum of pathophysiology of blood vessel function has been demonstrated, involving abnormal vascular tone and activated, adhesive endothelium. These vasculopathic abnormalities are attributable to pathways involving hemolysis-associated defects in nitric oxide bioavailability, oxidative stress, ischemia-reperfusion injury, hemostatic activation, leukocytes and platelets. Vasculopathy of sickle cell disease has been implicated in the development of pulmonary hypertension, stroke, leg ulceration and priapism, particularly associated with hemolytic severity, and reported also in other severe hemolytic disorders. This vasculopathy might also play a role in other chronic organ dysfunction in patients with sickle cell disease. These pathways present novel targets for pharmacologic intervention, and several clinical trials are already under way. The authors present their perspectives of a workshop held at the National Institutes of Health in August 2008 on vasculopathy in sickle cell disease, along with meritorious future scientific questions on the topic of vascular complications of sickle cell disease.

Introduction

Nearly 100 years ago, James Herrick published his observation of abnormally shaped red cells on a peripheral blood smear from a dental student from Grenada [1]. Although hemolytic anemia was the first complication linked in the English language medical literature to the condition called sickle cell disease (SCD), the occurrence of the vaso-occlusive pain crisis was recognized in Africa centuries earlier [2]. Pulmonary vasculopathy with cor pulmonale was recognized at autopsy in patients with SCD in 1936 [3]. For many decades, it was widely assumed that vaso-occlusion by rigid red cells leading to tissue infarction was the sole cause of organ dysfunction in patients with SCD. This concept was supplemented in the late 20th century by evidence of cellular interactions between

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circulating blood cells and endothelial cells, promoted by Mohandas, Hebbel, and Kaul, suggesting dysfunction of the blood vessel wall in SCD [4, 5].

Distinct subphenotypes of clinical complications of SCD were first suggested by Ballas in 1991, who noted that patients with frequent painful crises were distinct from those with recurrent leg ulceration [6]. Additional work by Duits and Schnog expanded concepts of vascular dysfunction, emphasizing that chronic organ dysfunction in SCD does not correlate with the frequency of acute vaso-occlusive crisis [7]. Further epidemiologic evidence from Serjeant and colleagues in 2004 helped to refine Ballas' concept of the leg ulcer versus painful crisis subphenotypes [8]. In a breakthrough, Gladwin and his colleagues demonstrated that intravascular hemolysis decompartmentalizes hemoglobin and arginase from the red cells into plasma, where they scavenge nitric oxide (NO) and its precursor L-arginine, reducing NO bioavailability, particularly in those patients with the highest hemolytic rate, and correlating with pulmonary hypertension and early mortality [9-11]. Kato and Gladwin showed that serum lactate dehydrogenase level is a useful surrogate marker of intravascular hemolysis in SCD, marking a subgroup of SCD patients with decreased nitric oxide bioavailability, pulmonary hypertension, priapism and leg ulceration [12]. Combining their own NIH cohort data with Steinberg and publications by the Cooperative Study of Sickle Cell Disease and others, they formulated a vasculopathy subphenotype comprising pulmonary hypertension, priapism, leg ulceration and preliminarily stroke, versus a viscosity-vaso-occlusive subphenotype involving vaso-occlusive pain crisis, the acute chest syndrome and osteonecrosis [13].

In order to summarize these recent scientific developments, a two day Workshop on Vasculopathy in Sickle Cell Disease was held at the National Institutes of Health in Bethesda, Maryland on August 27-28, 2008, funded by the Office of Rare Diseases, the National Heart, Lung and Blood Institute, and the Clinical Center Critical Care Medicine Department. The meeting brought together 350 participants, including 14 invited speakers, 10 session co-moderators and 36 poster presenters. The discussion focused on clinical, biological, physiological, biochemical and genetic factors involved in sickle cell vascular dysfunction, with discussion of translational therapies targeting these pathways. Finally, a subgroup of attendees participated in wide ranging discussions of priorities for future research in this area. The intent of the following report is to provide highlights of the presentations and the perspective of its authors and those attendees, with proposed future research questions of the greatest importance. Many of the questions are posed with respect to pathophysiology, but in most cases imply linked clinical research questions to translate positive findings to therapeutic advantage.

The Endothelial Biology of Chronic Vasculopathy

Vascular and endothelial biology comprise a fundamental, fascinating, and exceedingly complex aspect of the overall pathophysiology of sickle cell disease. The role of the endothelium in normal physiology is extensive [14], including: its glandular role with autocrine, paracrine, and even endocrine functions as a distributed signaling network; its task in regulating the pro- *versus* anti-coagulant balance of the blood and vessel wall; its critical function as a regulator of vascular pressure and flow; its participation in actualizing inflammation signaling through adhesion molecule biology; its control of vessel wall permeability, as well as its provision of the physical definition of the blood space. Remarkably, each of these vital physiological roles is disrupted in the context of sickle cell anemia [15].

We here focus the discussion of sickle disease vascular biology on the specific role of the endothelium in the chronic vasculopathy of this disease. In the whole spectrum of

endothelial biology, there is a continuum from the endothelium being in a “quiescent” state (which, in fact, is remarkably active in physiology), to it being “activated,” to it even being “dysfunctional”. The latter is essentially an operational concept, whereby the endothelial physiology has shifted from a state of physiologic usefulness to a state of some degree of harmful function [14]. The latter two states seem to be the case in sickle disease.

Within this background context, it seems logical that the diffuse loci of chronic vasculopathic disease are linked, in terms of pathophysiology. Candidate contributory factors include: polymerization-induced red cell sickling; a systemic inflammatory state [15] with abnormal expression of adhesion molecules for white blood cells and red blood cells [16-18]; activation of the coagulation system in terms of both platelets [19] and plasmatic coagulation, from its proximate end [20] through its amplification and effector aspects [21]; a biodeficiency of NO [11] plus NO resistance [22], a sign of vessel wall disease; and accompanying vascular instability with up-regulation of non-NO vasoregulatory systems [23, 24]; stasis (due to sickling and/or abnormal blood cell adhesion to endothelium and/or thrombotic activity) causing reperfusion injury physiology [25, 26]; disruption of the signaling function of endothelium [15]; and, of course, largely undefined -- but indisputably likely -- genetic influences on endothelial biology [27-30]. To all this, one must add excessive oxidative stress, which could result from multiple sources: abnormal amounts or locations of bioactive iron, heme, and hemoglobin; several endothelial- or vascular wall-based enzyme systems (xanthine oxidase, NADPH oxidase, myeloperoxidase, and electron leakage from mitochondria); and oxidant generation from circulating, or endothelial-bound, white cells or red cells. A role for each of these sources of oxidant stress has been supported experimentally in the sickle context, and they are discussed below.

The understandable tendency for investigators each to focus on, and study, a single one of these implicated factors has arguably impeded development of an adequate understanding of the true pathophysiology of chronic vasculopathy. Nonetheless, there is considerable knowledge about the specific nature of each of these causally-implicated sub-biologies available in the general biomedicine literature. So the more limited level of information about these biologies in the specific sickle context can and must be integrated with a large amount of well-defined understanding.

This allows a logical formulation of what is (likely to be) proximate *versus* what is (likely to be) distal. By far the most parsimonious, and simply logical, construct is that reperfusion injury is the proximate event (see **Figure 1**). In this physiology, some vascular wall perturbation and damage may result from vascular occlusion (regardless of mechanism), but a much greater amount results from the re-oxygenation that occurs upon resolution of the occlusion. Even the classical formulation that occlusion results from reversible red cell sickling, argues strongly that sickle cell disease ought to be the paradigmatic example of reperfusion injury. In addition, there actually is a bit of experimental evidence to support this [25, 26]. And, of course, blood cell adhesion to endothelium would promote sickling, by fulfilling the requirement for slowed flow to accommodate the polymerization delay time [31].

Since reperfusion injury is well-known to be intensely inflammatory [32], virtually all of the implicated and potential causal factors are known to be able to be derived from such a state. This includes adhesion molecule expression, vascular stasis, enhanced red cell lysis (due to oxidant effects, red cell sickling, plus immune/macrophage activation), signaling disruption, oxidant stress, NO consumption, coagulation activation, and abnormal vascular permeability. Moreover, each of these sub-biologies interacts and, to a significant extent, overlaps with the others. Most of these sub-biologies could potentially have numerous proximate routes of activation (for example, there are at least 7 independent ways that NO

deficiency can develop in sickle disease). Yet the principles of parsimony and logic argue that it is likely that the systems biology of chronic sickle vasculopathy is derived from the multiple, interacting contributions of these several powerful factors, all activated by reperfusion injury physiology (**Figure 1**). As is illustrated in this Figure, in greatly simplified form, there are multiple positive feedback loops, comprising "vicious cycles", within and between these biologies.

Research questions. The two most important questions are:

- To what extent each of these implicated sub-biologies is causal individually, relative to each other?
- How, and to what extent, do these disparate processes interact to create the final, overall systems biology of sickle vasculopathy?

In parallel, two things will have enormous impact on such studies. First, the well-known existence of endothelial heterogeneity [14], which is reported to extend to sickle disease [33], dictates that the answers to the above questions may be different in different organs, or even from vessel type to vessel type within a single organ, or from time-to-time in a given subject, or amongst different subjects. That needs evaluation. Second, each of these biological systems will undoubtedly have multiple genetically-based variations amongst individuals, and the roles of these should be defined.

Nonetheless, some of us believe that there is currently sufficient pre-clinical data available to justify proceeding with a carefully controlled, multi-modality therapy designed to prevent chronic vasculopathy in sickle disease.

Vascular Pathophysiology of SCD

Hemolysis associated endothelial dysfunction, vasculopathy and hypercoagulability

Nitric oxide (NO) is produced by the endothelium and is a critical regulator of normal vascular function. Nitric oxide regulates basal vasodilator tone, inhibits platelet and hemostatic activation and inhibits transcriptional expression of adhesion molecules, such as VCAM-1 [34-38]. The half-life of NO in the vasculature is very short because of rapid reactions with red cell hemoglobin to form methemoglobin and nitrate [39]. In fact, the vasodilator activity of NO is only possible because all of the hemoglobin is compartmentalize within erythrocytes, which creates diffusional barriers for NO entry into the red blood cells and reduces the scavenging of NO with intracellular hemoglobin [40]. Intravascular hemolysis releases hemoglobin into plasma which will then potently scavenges nitric oxide (NO) via a dioxygenation reaction with oxyhemoglobin that converts NO to nitrate [11, 39]. Hemolysis disrupts the red cell NO diffusion barriers and results in a potent inhibition of all NO bioactivity, leading to a clinical state of endothelial dysfunction and NO resistance [41-43]. Hemolysis also releases red blood cell arginase-1 into plasma. Arginase metabolizes plasma arginine into ornithine, reducing the required substrate for NO synthesis and compounding the reduction in NO bioavailability in sickle cell disease [10]. Hemolytic rate is also linked to plasma levels of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide synthase found at three-fold higher levels in SCD than healthy controls, and even high in those with PH [44-46]. Endothelial function is also compromised in SCD patients that have lower levels of apolipoprotein A-I [47].

Chronic NO depletion may contribute to vasoconstriction, proliferative vasculopathy, pulmonary hypertension, activation of endothelial adhesion molecules such as VCAM-1, activation of platelets and the production of the potent vasoconstrictor and mitogen, endothelin [48]. Because NO is a potent inhibitor of platelet activation, this pathway is activated patients with sickle cell disease secondary to direct inhibition of NO by plasma

hemoglobin and increased intracellular platelet expression of arginase [49, 50]. Clinical studies of patients with sickle cell disease reveal correlations between the intrinsic rate of intravascular hemolysis and the levels of procoagulant factors in blood [51, 52]. A state of resistance to NO mediated by cell-free plasma hemoglobin and the development of pulmonary hypertension has also been shown in transgenic mouse models of sickle cell disease and spherocytosis and in mouse models of alloimmune hemolysis and malaria [53, 54]. A constellation of complications are more common in patients with sickle cell disease who suffer from higher rates of intravascular hemolysis. These complications are also observed to a similar or lesser extent in other hemolytic diseases and include pulmonary hypertension, priapism and cutaneous leg ulceration, suggesting that intravascular hemolysis contributes to a subphenotype of vasculopathic complications [6, 8, 13].

A central risk factor for the development of pulmonary hypertension in patients with sickle cell disease is the rate of chronic intravascular hemolysis, characterized by low steady state hemoglobin levels, high lactate dehydrogenase levels, high bilirubin levels and high reticulocyte counts [9, 12, 43]. The association between high plasma hemoglobin and arginase-1 levels has now been reported in other hemolytic diseases, such as thalassemia, paroxysmal hemoglobinuria and malaria, suggesting that hemolysis may represent a common mechanism of disease for the chronic hereditary and acquired hemolytic anemia's [48, 55, 56].

Hemolytic anemia associated pulmonary hypertension

Pulmonary hypertension is an increasingly recognized complication of sickle cell disease. Three prospective adult screening studies have similarly reported that 20% of the population has mild pulmonary hypertension, defined by a pulmonary artery systolic pressure greater than 35 mm Hg and that 10% have moderate to severe pulmonary hypertension, defined by a pulmonary artery systolic pressure greater than 45 mm Hg [9, 57, 58]. Despite pulmonary pressure increases that are much lower than those observed in patients with idiopathic or hereditable pulmonary hypertension, the prospective risk of death associated with even mild pulmonary hypertension is extremely high [9, 57-61].

It is recommended that adult patients with sickle cell disease be screened for pulmonary hypertension using transthoracic Doppler-echocardiography [62, 63]. The tricuspid regurgitant jet velocity (TRV) is used to calculate the right ventricular and pulmonary artery systolic pressures ($PASP \approx 4*TRV^2$) after adding an estimate of the central venous pressure. In patients with sickle cell disease this estimate correlates well with measured pulmonary systolic pressure by right heart catheterization [9]. A value greater for the TRV than or equal to 2.5 m/sec is approximately two standard deviations above normal [43, 64]. In SCD patients, the risk of death associated with high pulmonary artery systolic pressures rises linearly and even values between 2.5-2.9 m/sec are associated with a high risk of death with an odds ratio for death of 4.4 (95% CI, 1.6-12.2; $p<0.001$); a TRV greater than or equal to 3 m/sec is associated with an odds ratio for death of 10.6 (95% CI, 3.3-33.6; $p<0.001$) [9, 57, 58].

A mixture of retrospective and prospective pediatric SCD echocardiographic screening studies suggest that elevated TRV can be seen early in life, usually without evidence of functionally significant PH [65-73]. In general, the clinical correlates of high TRV in childhood closely mirror those of adulthood. This suggests that the high TRV cohort among children with SCD is essentially the same as the high TRV group among adults, although longitudinal studies to confirm this are not yet mature. However, it is attractive to hypothesize that high TRV in childhood may be a marker of future risk of early mortality during adulthood, and that early modification of risk factors may attenuate that risk. This would follow the successful model in SCD of using transcranial Doppler screening to

identify children at high risk for stroke, and intervening with chronic transfusion to reduce the incidence of ischemic stroke [74]. The appropriate prevention strategy for PH is not known, but treatments known to reduce both hemolysis and sickling such as hydroxyurea [69, 75], chronic transfusion, or even stem cell transplant , would be candidates that should be tested. Other drugs under investigation that target the biology of vasculopathy might also be candidates [76, 77].

Left-sided heart disease in SCD is primarily due to diastolic dysfunction (present in approximately 13% of patients), although cases of systolic dysfunction and mitral or aortic valvular disease can occur as well (the latter present in approximately 2% of patients) [9, 78-81]. The presence of diastolic dysfunction alone in SCD patients is an independent risk factor for mortality [82]. In patients with both pulmonary vascular disease and echocardiographic evidence of diastolic dysfunction are at a particularly high risk of death (odds ratio for death of 12.0; 95% CI, 3.8 to 38.1; $p < 0.001$) [82].

Right heart catheterization studies of patients with sickle cell disease and pulmonary hypertension reveal a hyperdynamic state similar to the hemodynamics characteristic of porto-pulmonary hypertension [83]. The mean pulmonary artery pressure in patients with sickle cell and pulmonary arterial hypertension is approximately 40 mm Hg and pulmonary vascular resistance approximately 250 dynes per second per cm^{-5}. The relatively low pulmonary vascular resistance is caused by the high cardiac output of anemia. Approximately 60% of catheterized patients with a TRV greater than 3.0 m/sec meet the definition of pulmonary arterial hypertension, indicating that vasculopathy primarily involves the pulmonary arterial system. In the other 40% of subjects the left ventricular end diastolic pressures are greater than 15 mm Hg, indicating a component of left ventricular diastolic dysfunction [83].

It is increasingly clear that pulmonary pressures rise acutely during vaso-occlusive crisis and even more during acute chest syndrome [84]. In fact, 13% of patients with the acute chest syndrome develop acute right heart failure, and these patients exhibit the highest risk for mechanical ventilation and death [85]. These data suggest that acute pulmonary hypertension and right heart dysfunction represents a major co-morbidity during the acute chest syndrome, and that right heart failure should be considered in patients presenting with the acute chest syndrome.

Hemolysis and cerebrovascular disease

Ischemic stroke is one of the most serious complications of SCD, with an early peak incidence at age 7 years, and another peak late in adulthood [86]. The histopathology involves a cerebral vasculopathy, characterized by intimal and medial hyperplasia, irregular and activated endothelium and *in situ* thrombosis [87], features that are remarkably similar to the lung pathology in pulmonary hypertension [88]. Identified risk factors for ischemic stroke have included elevation of systolic blood pressure and low hemoglobin level [86], and these factors have also been associated independently with pulmonary hypertension, although there are conflicting data [9, 60]. There have been other indirect and direct correlations between cerebrovascular disease and pulmonary hypertension in SCD [58, 89]. This leads to a question whether the same hemolysis-associated mechanisms linked to pulmonary hypertension also play a role in cerebrovascular disease and stroke. This has been addressed in a recent study by Bernaudin and colleagues, who have correlated markers of hemolytic severity, such as low hemoglobin or high serum LDH, to risk of stroke as indicated by high velocity of cerebral blood flow by transcranial Doppler (TCD) ultrasound examination in children with SCD [90]. Co-inheritance of α-thalassemia trait, which known to attenuate hemolysis in SCD, is protective against high TCD velocity [90, 91]. These data

suggest an overlap between the pathophysiologic mechanisms of cerebral and pulmonary vasculopathy in patients with SCD that has only begun to be investigated.

Linkage of vasculopathy to other sickle cell complications

An increasing body of data suggest that the hemolysis-associated vasculopathy may be linked to priapism and leg ulceration. Pulmonary hypertension is associated with a history of cutaneous leg ulceration [92], and with priapism in males with SCD [9]. Markers of hemolysis such as low hemoglobin, high serum LDH and bilirubin, are linked to priapism and leg ulcers [12, 93, 94]. In priapism, there is some evidence that the NO-cyclic GMP-phosphodiesterase-5 signal transduction pathway is disordered in a way that paradoxically may respond to sildenafil [95-98]. There is also evidence of association of nephropathy in SCD with hemolysis and with pulmonary hypertension. Markers of renal dysfunction such as proteinuria, elevated serum urea, creatinine and uric acid have been associated with pulmonary hypertension [9, 58, 99]. It is not clear if renal dysfunction promotes the development of vasculopathy in other organs, or whether renovascular pathology is a parallel and simultaneous target of multiorgan vasculopathy.

Possible contribution of cellular adhesion to vasculopathy

A significant body of literature has developed in the last decade suggesting that circulating cells may adhere to endothelium in sickle cell disease. These data suggest that circulating blood cells are intrinsically adhesive or activated to an adhesive state in SCD, including reticulocytes [100], neutrophils [101-106], monocytes [4, 107, 108], and platelets[50, 106, 109, 110]. Interacting factors include endothelial adhesion proteins such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin, and a long list of plasma proteins that serve as intermediaries in adhesion, such as fibrinogen, von Willebrand factor, thrombospondin, and many others. Highly elegant experiments have been performed in animal models, especially the sickle cell mouse, but virtually no validation experiments have been performed in patients with SCD, a very prominent omission. For this field to move ahead, a crucial goal would be to establish human vascular physiology experiments that can determine the validity and significance of these pathways in human SCD.

Research Questions

- What is the role of leukocytes and platelets in the pathophysiology of vasculopathy and PH?
- What are the relative contributions to PH pathophysiology of right ventricular dysfunction; left ventricular disease, such as diastolic dysfunction; ventricular interdependence?
- Are there better noninvasive (or invasive) outcome measures of cardiovascular dysfunction?
- At what level of TRV elevation is clinical intervention required? Can early intervention prevent the development of PH, and what are the best biomarkers to guide intervention?
- Are there state-of-the-art imaging techniques that can provide better measures of PH vascular physiology, possibly imaging calcium flux or iron overload features?
- What is the contribution of vasculopathy to renal disease in SCD, and what is the mechanistic overlap with PH?
- What are the relationships between splenic dysfunction and vasculopathy in SCD?

- What is the role of immunological pathways in vasculopathy in SCD?
- Is there a contribution of asthma or environmental insults, including smoking or second hand smoke exposure?

Genomics of Vasculopathy in SCD

The clinical course of patients with sickle cell anemia, a Mendelian trait, is characteristically highly variable. To find new genetic modulators of disease, genotype-phenotype association studies, where single nucleotide polymorphisms (SNPs) in candidate genes are linked with a particular phenotype, have been informative. In these studies, SNPs in several genes of the TGF-β/BMP superfamily and other genes linked to endothelial function and NO biology were associated with some subphenotypes of disease that are closely associated with the disordered NO metabolism that accompanies hemolytic anemia.

Priapism occurs in at least a third of men with sickle cell anemia and is one prominent example of NO-related sickle vasculopathy. SNPs in 44 candidate genes were examined for their association with priapism in 148 patients with sickle cell anemia with priapism and 529 patient controls that had not developed this complication [111]. Polymorphisms in Klotho (KL) showed an association with priapism by genotypic and haplotype analyses. KL directly or indirectly promotes endothelial NO production. In another study of about 200 men aged more than 18 years, 83 had a history of priapism. A candidate gene study in this smaller population failed to show an association of priapism with SNPs in with KL; nevertheless, there were associations with SNPs in TGFBR3, AQP1 and the adhesion molecule, ITGAV [112]. Further study of this population found that a single coding SNP in F13A1, the Factor XIII gene was associated with priapism with an odds ratio of 2.43 for individuals with C/C compared with the C/G genotype [112].

Pulmonary hypertension or pulmonary vascular disease has emerged as an important risk factor for premature death in patients with sickle cell anemia and is another complication thought to be associated with NO scavenging [9, 57, 58]. Its genetic basis has only recently started to be studied. Pulmonary hypertension is likely to be modulated by the effects of genes that control NO and oxidant radical metabolism, cell-cell interaction, vasculogenesis and vasoreactivity. For example, mutations in bone morphogenetic protein receptor 2 (BMPR2) and other genes have been associated with both familial and sporadic pulmonary hypertension. In 111 symptomatic patients screened for pulmonary hypertension, an association of this subphenotype was identified for genes in the TGF-β/BMP superfamily, including ACVRL1, BMPR2, and BMP6 [30]. Remarkably, this study of a patient group totally independent of patients previously reported found that BMP6 SNP rs449853, previously associated with sickle cell stroke and bacteremia [113], was associated with pulmonary hypertension, as estimated by the TRV [30]. Independent validations of this type lend additional credibility to SNP association studies.

Genetic association studies based on analysis of candidate genes have suggested genes that might be the focus of deep resequencing efforts and functional analysis in model systems to discover the mechanisms of genetic modulation. GWAS hold the promise of providing a more thorough appreciation of the genetic diversity that underlies phenotypic heterogeneity. Nevertheless, positive findings from this approach will need to be confirmed and mechanisms studied. The near-term results of genotype-phenotype studies will likely be the ability to provide better prognostication to reduce the uncertainty associated with therapeutic decisions, like the use of long-term transfusion to prevent stroke or the employment of multiple agents to lessen the adverse outcome associated with pulmonary hypertension. Ideally, and currently not refined sufficiently to be feasible, this information could be had antenatally and a personalized lifelong care plan formulated. The later-term goal of genetic

association studies is to identify genes and pathways that might be therapeutically manipulated in novel treatment approaches.

Several issues must be considered when evaluating genotype-phenotype association studies in sickle cell anemia. First, most genotype-phenotype association studies have not integrated the numerous clinical and laboratory dimensions of the disease into a single measure of disease severity. Secondly, one-SNP-at-a-time, and one-phenotype-at-a-time approaches to association studies are not likely to capture the complexity of genetic modulation. Thirdly, an impediment in genotype-phenotype association studies resides in the candidate gene-focused approach. An unbiased assessment of genotype-phenotype relationships requires GWAS where hundreds of thousands to millions of SNPs are examined, freeing the investigator of the need to identify candidate modulators, and establishing any associations a posteriori. GWAS are just beginning in sickle cell anemia where patient numbers are limited compared with cardiovascular disease or diabetes, for example. This limitation of available sample size suggests that multiple patient groups should be studied and the results pooled or subjected to meta-analysis. Small sample sizes also do not lend themselves to ‘frequentist’ analytical methods that risk excluding important associations by strict reliance on draconian p-values.

Presently, most reported studies have examined only candidate genes, and more comprehensive GWAS are just beginning. The results of these studies need to be interpreted with several caveats. Many reported SNP-association studies using various disease subphenotypes have examined relatively small numbers of patients, few studies have included independent patient groups for validation purposes, and with some exceptions, interaction among SNPs and the risk of a phenotype was not examined. Importantly, all these studies are “discovery” science; they reveal genetic associations but do not define causality. Other than speculation, these reports say nothing about the mechanism by which an associated polymorphic gene influences the disease. This will be the next important step in defining genetic modifiers and turning their discovery into novel therapeutics.

Research Questions

- What are roles of modifier genes on the vasculopathy subphenotype of SCD?
- How can SCD cohorts be assembled to best perform validation studies of genes identified from initial candidate SNP and GWAS studies?

Red Cell Biology

Factors affecting rate of hemolysis in SCD

The preceding model implicating the adverse effect of intravascular hemolysis on vascular function in patients with sickle cell disease begs the question: Why do some patients with sickle cell disease have more or less severe hemolysis than other patients with sickle cell disease? In the case of patients with hemoglobin SC disease, the presence of hemoglobin C is well documented to permit hemoglobin S polymerization, but to a reduced degree compared to the patients with homozygous S genotypes, resulting in less severe hemolytic anemia [114]. Likewise, variable expression of hemoglobin A in hemoglobin S-β+ thalassemia or hemoglobin F with hereditary persistence of fetal hemoglobin results in both less sickling and reduced hemolysis [115, 116].

Interestingly, coinheritance of α-thalassemia trait with any form of sickle cell disease also is well known to modify the phenotype of SCD, in a fascinating manner that unlinks the effects of sickling and hemolysis. α-thalassemia induces a lower mean corpuscular hemoglobin concentration, also is known to reduce hemoglobin S polymerization and consequent hemolysis [117, 118]. Paradoxically, the resulting rise in hemoglobin and

hematocrit appears to induce an increased frequency of vaso-occlusive pain crisis (VOC), osteonecrosis and in some studies, the acute chest syndrome (ACS)(reviewed in [13]). This has been rationalized by the increased viscosity of high hematocrit sickle cell blood, resulting in worsened microvascular blood flow and tissue infarction, supported by the epidemiological finding in SCD patients that higher hemoglobin level is a risk factor for increased frequency of these specific complications. This formulation suggests that simply decreasing hemolysis in SCD will not itself result reduce VOC, ACS and osteonecrosis, and may even worsen them. Speculatively, this may be why further clinical development has not been reported for a Gardos channel inhibitor that is proven to reduce hemolysis in SCD [119].

Reduced hemolysis with α-thalassemia trait in SCD has been linked to decreased frequency of most of the hemolysis-associated clinical complications: leg ulceration, priapism, and cerebrovascular disease (reviewed in [13]). There are not yet substantive data regarding the effect of α-thalassemia trait on the prevalence of pulmonary hypertension. These findings help to support the model that these complications are indeed induced by chronic severe hemolysis.

Hemolytic rate varies widely among patients with SCD, and it is likely that there are additional genetic determinants of this variation besides hemoglobin C, hemoglobin F, α and β+-thalassemia. Genomics research into this area would be very likely to provide instructive lessons regarding natural modifiers of the hyperhemolysis phenotype. It is possible that subtle versions of allelic variants in genes known to cause independent forms of hemolytic anemia such as the cytoskeletal mutations in hereditary spherocytosis, or the enzymopathies of the erythrocyte catalytic antioxidant pathways, may accelerate hemolysis in SCD. Pharmacologic interventions that regulate these modifier effects might decrease hemolysis in SCD, potentially reducing the severity of the hemolysis-associated clinical complications. However, the correlations of naturally occurring decreased hemolysis to worsening of VOC, ACS and osteonecrosis is sobering, and teaches us that simply reducing hemolysis might well trade one set of SCD complications for another. An important area for future research lies in learning more about the biology and genetics of hemolysis.

Adaptive responses to hemolysis

The adaptive response to hemolysis likely also affects the magnitude of resulting pathology. These crucial pathways include haptoglobin, the plasma protein that sequesters and clears hemoglobin via the CD163 scavenger receptor on reticulo-endothelial system macrophages [120, 121]; hemopexin, which performs a similar role for free heme; heme oxygenase-1, which performs the first reaction in heme breakdown, cleavage of the heme porphyrin ring. Heme oxygenase-1, induced in SCD [17, 122, 123], is known to provide critical vasculoprotective antioxidant function in other contexts, including the production of low levels of carbon monoxide, which signals its own vasculoprotective pathway suggested to play a role in SCD [123, 124]. Finally, the appropriate intracellular and extracellular trafficking of elemental iron is critical to preventing iron-induced oxidant stress and toxicity [125]. This point is emphasized by the epidemiological association with pulmonary hypertension in SCD with markers of iron overload, such as high serum ferritin or low serum transferrin [9]. Although these may be an epiphenomenon simply indicating sicker patients with SCD that require more frequent transfusion, the known organ toxicity of iron overload in hereditary hemochromatosis and the suspected role in endothelial dysfunction and atherosclerosis suggest a role for oxidant stress of iron overload in sickle cell vasculopathy. These are important areas for future research.

Research Questions

- What is the mechanism of hemolysis in SCD?
- How is the heme load modified?
- What is the role of iron overload on the development of pulmonary hypertension in SCD?
- How much lowering of hemolysis is needed to ameliorate linked complications?

Oxidative Stress

There are many causes of oxidative stress in SCD, all of which may contribute to vasculopathy. Hemolysis contributes directly to oxidative stress in ways that have been previously discussed: 1) cell-free plasma hemoglobin and arginase contribute to deficiency of nitric oxide, an important endogenous antioxidant; 2) free heme released from hemoglobin turnover is oxidative; 3) elemental iron released from heme turnover can generate hydrogen peroxide via Fenton chemistry [126, 127]. In addition, two enzymes known to be excessively activated in SCD both generate oxygen radicals as a byproduct of their enzymatic activities, xanthine oxidoreductase (XOR) and NADPH oxidase. In addition, under certain circumstances that may occur in SCD, nitric oxide synthase (NOS) can produce reactive oxygen species. Administration of superoxide dismutase can enhance vascular function in the SCD mouse, supporting a significant role for reactive oxygen species in endothelial function [128].

XOR activity is found at three-fold higher levels in the plasma of patients with SCD compared to healthy control subjects [129]. XOR appears to be induced to high levels by hypoxia-reoxygenation [25]. XOR impairs acetylcholine-induced vascular relaxation in the SCD mouse [129]. Interestingly, the enzyme appears to be localized primarily at the endothelial cell surface [129], and is resistant to inhibition by allopurinol or oxypurinol [130]. However, a new class of drugs, vinyl-nitro fatty acid derivatives, are potent and irreversible inhibitors of XOR [131]. These promising agents have important anti-inflammatory actions.

A significant role for NADPH oxidase has been suggested by experiments in the cerebral vasculature of the SCD mouse. Hypoxia-reoxygenation induces robust adhesion of platelets and leukocytes to the endothelium of cerebral venules [132]. This adhesion is reduced by transgenic expression of superoxide dismutase, and it is dependent upon the major subunit of NADPH oxidase. Adhesion is also reduced by deferoxamine, an iron chelator, suggesting that free iron also contributes to this phenomenon [132].

NOS can produce reactive oxygen species under conditions that may be favored in SCD. This can occur with low concentrations of the substrate L-arginine [133, 134], which have been reported with depletion of plasma L-arginine associated with increased plasma cell-free arginase levels due to intravascular hemolysis [10, 135-140]. The enzymatic activities of NOS become uncoupled when its obligate cofactor tetrahydrobiopterin is deficient or oxidized [141, 142]. These pathways are beginning to be investigated in sickle cell disease and other vasculopathies [143].

Depletion of erythrocytic glutamine and glutathione, the crucial intracellular antioxidant has been reported in SCD. Significantly, the ratio of erythrocytic glutamine to glutamate is correlated to the pulmonary arterial systolic pressure as estimated by TRV [144]. These data link the redox state of the red cell with the development of increased pulmonary pressures in SCD.

Research questions

- What are the clinically relevant mechanisms of oxidative stress in vasculopathy in SCD?
- What are the most relevant measures of oxidant stress in SCD?
- What is the effect of oxidant stress on lipid biology, coagulation, NO biology, inflammation, and the pathobiology of vascular lesions?
- What is the contribution of NOS uncoupling to vasculopathy and PH in SCD?

Conclusions

The description of the preceding pathways in vasculopathy in SCD has suggested many new potential therapeutic targets for intervention. The current status of these clinical investigations has been recently reviewed [76, 77], and will not be discussed further here. This report covers the highlights of the rapidly expanding field of vasculopathy research in SCD. Each of the areas discussed here are reviewed in greater detail in the cited reviews. This increasing body of data clearly demonstrates that the pathology of SCD is not limited to the abnormal rheology of rigid red cells produced by polymerization of sickle hemoglobin. This red cell defect interacts with a complex pathobiology of the blood vessel wall, which reflects many of the vascular dysfunction which is being characterized in atherosclerosis, diabetes, and dyslipidemia [15, 76]. This reflects interaction of SCD with overlapping pathological mechanisms of vasculopathy, indicating that SCD complications may be modified by genetic and acquired risk factors for vasculopathy. It also suggests that SCD appears to amplify the otherwise small effects of these risk factors, and accelerating the development of clinical vasculopathy, promoting cerebrovascular disease and stroke in early childhood, or pulmonary vascular disease and pulmonary hypertension in adult middle age. SCD appears to cause this accelerated vasculopathy primarily through mechanisms that are linked to hemolytic rate, manifested prominently as decreased nitric oxide bioavailability. The pathways discussed in this review are ripe for further clinical investigation, with results that will likely generalize to several other severe hemolytic anemias, such as thalassemia intermedia and major [145], with lessons even for more general vasculopathies such as atherosclerosis.

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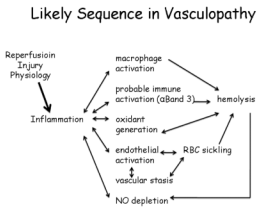

Figure 1.

This attempts to illustrate the probable hierarchy of implicated sub-biologies in sickle cell vasculopathy. There are multiple causal sub-biologies, but they are highly interconnected. This figure greatly simplifies a very complex interplay of relevant factors.