

A Systems Biology Consideration of the Vasculopathy of Sickle Cell Anemia: The Need for Multi-Modality Chemo-Prophylaxis

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Abstract: Much of the morbidity and mortality of sickle cell anemia is accounted for by a chronic vasculopathy syndrome. There is currently no identified therapy, interventional or prophylactic, for this problem. For two reasons, development of an effective therapeutic approach will require a systems biology level perspective on the vascular pathobiology of sickle disease. In the first place, multiple biological processes contribute to the pathogenesis of vasculopathy: red cell sickling, inflammation and adhesion biology, coagulation activation, stasis, deficient bioavailability and excessive consumption of NO, excessive oxidation, and reperfusion injury physiology. The probable hierarchy of involvement of these disparate sub-biologies places inflammation caused by reperfusion injury physiology as the likely, proximate, linking pathophysiological factor. In the second place, most of these sub-biologies overlap with each other and, in any case, have multiple points of potential interaction and transactivation. Consequently, an approach modeled upon chemotherapy for cancer is needed. This would be a truly multi-modality approach that hopefully could be achieved *via* employment of relatively few drugs. It is proposed here that the specific combination of a statin with suberoylanilide hydroxamic acid would provide a suitable, broad, multi-modality approach to chemo-prophylaxis for sickle vasculopathy.

INTRODUCTION

The overall pathobiology of sickle cell anemia is exceedingly complicated [1]. As part of this, a chronic arterial vasculopathy causes much of the non-infectious morbidity and mortality of this disease. This communication will describe what we believe would be the most logical and effective prophylactic therapy for this vasculopathy syndrome, for which there is no currently accepted therapy. First, to elucidate the rationale for the recommendation, we discuss the relevant etiologic processes. When available, we cite previous thorough reviews.

This presentation will highlight two critical features of this pathobiological process: [1] that it is comprised of multiple participating sub-biologies, and [2] that these overlap and are highly inter-connected, with multiple positive feedback loops between sub-biologies, so that the biology of one cannot be simply viewed without perception of the others. This concept is schematically illustrated by the network diagram shown in (Fig. 1). As a consequence, our main thesis is that devising an effective chemo-prophylaxis for sickle vasculopathy will require approaches exhibiting multi-modality benefits.

SICKLE CELL ANEMIA OVERVIEW

Sickle cell anemia is a crippling disease characterized by hemolytic anemia, chaotic vasoocclusive episodes, and chronic organ damage. The condition is caused by a single-base mutation in the sixth codon for the beta globin chain,

which results in formation of the mutant sickle hemoglobin (HbS). Homozygotes for HbS have sickle cell anemia; heterozygotes have sickle trait. This mutant hemoglobin exhibits three abnormal molecular behaviors: it reversibly polymerizes upon red cell deoxygenation, thereby causing reversible sickling, a distortion of the red cell; it exhibits modest instability, leading to abnormal precipitation of cytoplasmic hemoglobin, concomitant oxidant generation, and red cell membrane defects; and it comprises a charge change that alters the assembly kinetics of the $\alpha\beta$ heterodimers required to form functional hemoglobin tetramers [1]. Around five thousand years ago, the gene mutation was preferentially selected for in regions that then had endemic malaria. It reached the United States *via* the slave trade, and it is most frequently seen in those of African ancestry.

SICKLE VASCULOPATHY

It has been recognized for decades that there are certain vascular complications of sickle disease [2]. For example, about 10% of children develop clinical ischemic stroke, at an average age of 5 years, due to thrombosis occurring over an area of vessel wall disease at the Circle of Willis, the medium-to-large arteries at the base of the brain [3]. Pulmonary arteriopathy, often with *in situ* thrombosis, occurs in up to 30% of the adults [4, 5]. Also involved with vasculopathic lesions are the kidney, the spleen, the penis, and the umbilical cord [6]. Overall, the clinical complications that result from these areas of vasculopathic involvement are ischemic stroke, pulmonary hypertension, chronic renal disease, autosplenectomy, priapism, fetal wastage and growth retardation (Table 1).

Interestingly, it only recently has been recognized that these vascular lesions are probably linked through a unifying

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Likely Hierarchy of Sub-Biologies

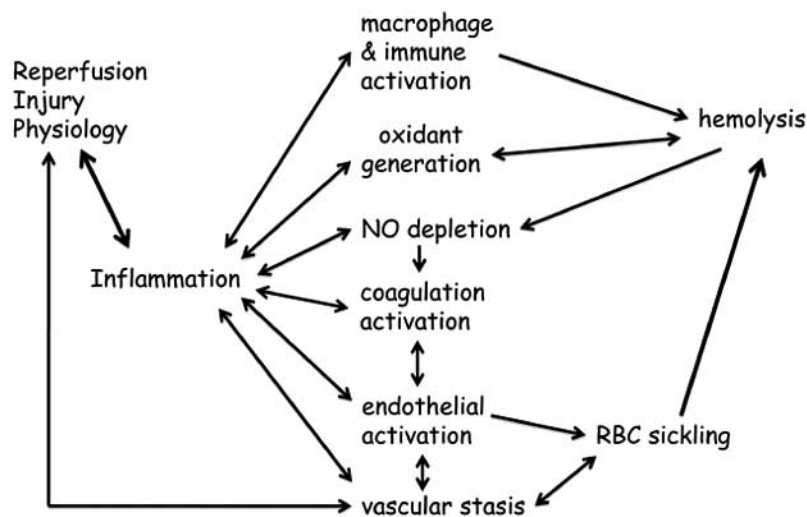


Fig. (1). Probable hierarchy of the major sub-biologies participating in development of sickle vasculopathy. Modified from Kato GJ, Hebbel RP, Steinberg MH, Gladwin MT; Vasculopathy in Sickle Cell Disease: Biology, pathophysiology, genetics, translational medicine and new research directions. [Meeting Report] *Am. J. Hematol.*, 2009.

pathogenesis with a common underlying histopathology, a true vasculopathy syndrome [6]. Such lesions tend to exhibit intimal hypertrophy with proliferative changes and a disrupted internal elastic lamina [3, 7]. Additionally included sometimes in pulmonary vasculopathy are the plexiform endothelial lesions typical of pulmonary hypertension. Lesions are non-random in occurrence, and are associated with a chronic, systemic inflammatory state. Note that this assembly of characteristics is very similar to the atherosclerotic lesion in the general population, with the exception of an absence of the fatty streak in sickle subjects (who have low plasma lipids) [6].

It is notable that the state of understanding of the sickle vasculopathic lesion is roughly comparable to that of the atherosclerotic lesion in the mid-1960s, before the importance of the fatty streak had been fully recognized and before the elegant and extensive use of animal models had identified the critical role of the blood monocyte in lesion development [8, 9]. The geographic location of the lesions is different in atherosclerosis and sickle disease. However, we can

speculate that this may be accounted for by differences in the role of lipids, participation of the additional stresses imposed by the anemia of sickle disease (hypoxia and increased wall shear stress), as well as a different proximate etiology to the accompanying inflammatory state. That is, instead of vascular wall inflammation triggered by deposition of dietary inflammatory lipids [8], sickle vasculopathy occurs in the context of a systemic inflammatory state caused by reperfusion injury physiology [6], in which some inflammatory mediators can actually trigger endothelial generation of the same inflammatory lipids associated with atherosclerosis [10]. Much more research is needed to fill out the current, nascent understanding of sickle vasculopathy.

THE ENDOTHELIAL BIOLOGY OF SICKLE CELL ANEMIA

In normal physiology, the role of the endothelium is extensive [11]. Particularly important aspects are: its provision of physical definition to the intravascular space; its regulation of blood and vessel wall pro- versus anti-coagulant balance; its central role in inflammation and adhesion molecule biology; its endocrine/autocrine/paracrine actions as a distributed signaling network; its regulation of vascular flow and pressure; its regulation of vessel wall permeability; and, fundamentally, its residence at the interface of multiple biological processes (coagulation, inflammation, stasis, vaso-regulation) such that it inevitably provides linkages between them.

In the spectrum of endothelial biology, there is a conceptual continuum from a “quiescent” endothelium under normal conditions (which actually is far from truly quiescent), to it being “activated” or even “dysfunctional”. Typical aspects of the endothelium deviated from its basal state would include: enhanced elaboration of inflammatory mediators

Table 1. Chronic Vasculopathy in Sickle Cell Disease

Geographic Region	Clinical Effect
Circle of Willis	ischemic stroke
pulmonary artery	pulmonary hypertension
renal artery	chronic renal disease
penis	priapism
umbilical cord	fetal wastage & growth retardation
spleen	splenic infarction

and cellular activators; disrupted endothelial signaling functions; increased display of adhesion molecules; and conversion to a pro-coagulant phenotype [11]. The “dysfunctional” state, as distinguished from just an “activated” state, is difficult to define, but it can be conceptualized as a shift from a state of usefulness to a state of harmful function. The latter two states seem to be the case in sickle cell anemia [6]. Indeed, in this disease, perturbed endothelial biology comprises a fundamental and fascinating aspect of its overall pathophysiology.

In this background context, the endothelium directly participates in causation of the chronic vasculopathy of sickle cell anemia, and research interest has focused upon a wide variety of pathobiologies. Potential contributory factors include: polymerization-induced red cell sickling; a systemic inflammatory state; the abnormal display of endothelial adhesion molecules for red blood cells and white blood cells; activation of the coagulation system; deficient NO bioavailability, plus NO consumption; an accompanying vascular instability with up-regulation of non-NO vasoregulators; disruption of the endothelium’s normal signaling function; an excessive oxidative stress state; vascular stasis; and a state of recurrent ischemia/reperfusion (I/R) (Table 2). Additionally, there must be innumerable genetic influences on endothelial biology, although these are only beginning to be defined. Although the justifiable reductionist tendency of most investigators leads them to be proponents of one or another of these specific sub-biologies, our view is that they are all interrelated in complex fashion, so that a systems biology perspective is required (Fig. 1).

Critically, the latter point defines the constraints upon potential therapeutic approaches to sickle vasculopathy. Hence, the following section will review both the roles that these disparate sub-biologies may individually play in sickle vasculopathy, and the manner in which they overlap and interact.

THE PARTICIPATING VASCULOPATHIC SUB-BIOLOGIES

Red Cell Sickling

The cellular biophysics of the mutant HbS has been defined in elegant detail [12]. Briefly, deoxygenation of HbS causes an explosive autocatalytic formation of polymer, re-

sulting in red cell sickling. Notably, however, there is a finite delay time between a drop in oxygen tension and the onset of actual HbS polymerization. This delay time is exquisitely sensitive to initial conditions, as it shows a ~40th power inverse dependence on mean cellular HbS concentration, MCH_SC (Fig. 2) [13]. And it varies markedly from cell to cell, due to heterogeneity in MCH_SC, so individual cells can have polymerization times varying from 1 ms to >100 seconds, and a 15% increase in MCH_SC would cause a nearly five log decrease in delay time [14].

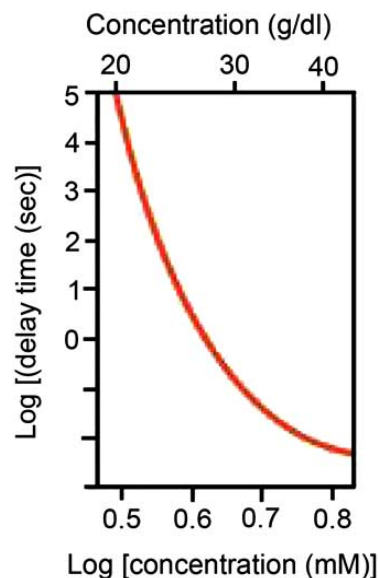


Fig. (2). Delay time versus concentration. The polymerization rate-limiting delay time (vertical axis, shown as log of the delay time) between (total) deoxygenation and onset of hemoglobin polymerization is steeply proportional to the inverse of the concentration of hemoglobin (shown in mM). Obtained with permission from Eaton WA and Hoffrichter J, Hemoglobin S gelation and sickle cell disease; *Blood* 1987, 70, 1245-1266.

Surprisingly, the actual physiological implications of polymerization kinetics are often not fully appreciated. These kinetics are studied by utilizing, for example, temperature jump (alters hemoglobin solubility) or near-instantaneous and total deoxygenation, events that do not occur in real physiology. Rather, the rate of deoxygenation under physiological conditions is actually gradual, increasing as the red cell traverses the microcirculation. This, in turn, constrains the rate of polymerization so that it approximately parallels the rate of deoxygenation [15]. Therefore, *in vivo* a cell is unlikely to exhibit significant polymer formation in less than a whole second (Fig. 3), which happens to be the normal capillary transit time for a red cell. Consequently, for most sickle red cells it takes longer for them to sickle than they actually spend traversing the vessels of critical diameter [16].

Notably, presence of any normal HbA or fetal HbF (for genetic reasons or due to pharmacologic or gene therapeutic re-engineering of the erythron’s hemoglobin synthesis program) has a powerful anti-sickling effect [1], such that in-

Table 2. Endothelial Biology in Sickle Cell Disease

Inflammatory state Increased adhesion Coagulation activation
Biodeficiency of NO & NO consumption Excessive oxidation Reperfusion injury physiology
Disturbance of long-range signaling
Endothelial activation and dysfunction Disturbed vasoregulation

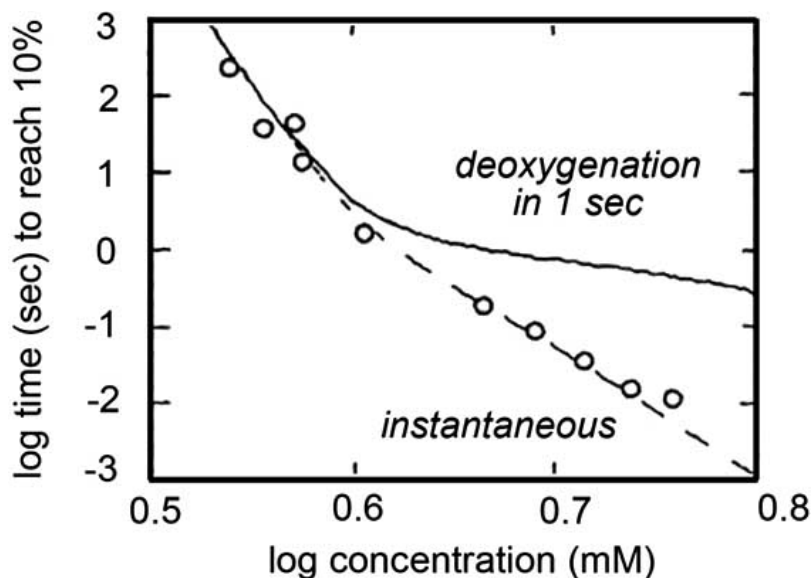


Fig. (3). Polymerization rate is limited physiologically by rate of deoxygenation. The kinetics of hemoglobin polymer formation (shown here as the log of the time it takes to reach 10% of the complete polymerization reaction) is dependent on starting hemoglobin concentration (per Fig. 2) but is severely limited by the physiologic rate of deoxygenation. The dashed line assumes near-instantaneous (<1 msec) deoxygenation; the solid line assumes that (full) deoxygenation takes about 1 second. Thus, physiologically the delay time would always be about 1 second or longer. Obtained with permission from Ferrone FA, Oxygen Transits and Transports; In Sick Cell Disease: Basic Principles and Clinical Practice; Ed: Embury SH, Hebbel RP, Narla M, Steinberg MH; Raven Press, Ltd., New York, 1994; pp 89-98.

duction of elevated HbF by medication, or introduction of HbA by gene therapy, are potential therapeutic avenues for sickle cell anemia.

Relationship to other Sub-Biologies

For most red cells to sickle, their microvascular transit time needs to be lengthened. If this constraint is met, the cells that do contain polymerized HbS become deformed and have proportionately diminished deformability [17] and could directly precipitate vasoocclusion. Such critical transit time delay is undoubtedly provided by the various factors that determine stasis, one reason why the inflammatory state and adhesion biology of sickle vascular pathobiology are of paramount importance.

Hemolytic Anemia

Greatly accelerated red cell destruction (hemolysis) is a hallmark feature of sickle cell anemia, with the half-life of an average sickle red cell being ~7 days rather than the normal 60 days.

Intra-vascular Destruction

Approximately one-third of sickle red cell destruction is accounted for by intra-vascular hemolysis, whereby cellular hemoglobin is released in substantial amounts (~34 ml of red cells per day) directly into the plasma [18]. Intravascular hemolysis is largely caused by proximate red cell sickling, although there is some contribution from red cell fragility [17]. Across the spectrum of the sickling disorders the degree of anemia (a reflection of hemolytic rate) correlates with the (calculated) tendency of red cells to contain polymerized hemoglobin [19]. Red cell sickling also causes liberation of Hb-laden microparticles into the blood [20], so that

about one-half of cell-free plasma hemoglobin is actually confined within these [21].

Extra-vascular Destruction

About two-thirds of sickle red cell destruction occurs outside of the vascular lumen, accounting for ~68 ml of red cell additional loss per day [18]. This derives from excessive erythrophagocytosis of red cells by tissue macrophages, that is triggered by aldehyde modification of the red cell membrane plus attachment of antibody [22]. There also is some contribution from ineffective erythropoiesis [23]. In both cases, imperfect engulfment of red cells can result in leak of cell-free hemoglobin into the plasma. But direct intravascular hemolysis is probably the greatest contributor to plasma hemoglobin content.

Relationship to other Sub-Biologies

Cell-free hemoglobin in the plasma can efficiently consume NO [24], so this may contribute to the NO biodeficient state of sickle patients. Further, hemolysis indirectly contributes to abnormal red cell/endothelial adhesion, by virtue of the accompanying compensatory reticulocytosis, increased production of younger red cells that exhibit increased adhesion molecule expression [6]. In addition, plasma hemoglobin could also contribute to oxidative biochemistry. Indeed soluble hemoglobin can interact with biomembranes, if they are exposing phosphatidylserine, and denature and transfer heme and free iron to the membrane environment, leading to membrane-associated Fenton chemistry [25].

Due to consumption by its binding of free hemoglobin, the haptoglobin level of sickle patients is virtually zero [26], allowing elevated levels of unscavenged free hemoglobin.

When hemoglobin is bound by haptoglobin, it is still able to redox cycle, but the haptoglobin itself absorbs the resulting oxidant [27]. There is no such buffering benefit for redox cycling of the excess plasma free hemoglobin, which will tend to exist as methemoglobin. Similarly, free methemoglobin S loses its heme very rapidly [28], so that hemopexin levels are depleted [29] and there is an excess of redox-capable free heme (which is very hydrophobic and can seek membrane environments for stability, and there reek oxidative havoc [25].

The “Hyper-Hemolytic Phenotype”

It has vigorously been promoted that the spectrum of clinical involvement in sickle cell anemia actually comprises two distinguishable phenotypes: an occlusive phenotype (with occurrences of osteonecrosis, acute chest syndrome, stroke and vasoocclusive pain crisis) and a distinct “hyper-hemolytic” phenotype (with occurrences of pulmonary hypertension, priapism, leg ulceration) [26, 30] (Fig. 4). It is then suggested that the former process is caused by microvascular occlusion, reperfusion injury, and inflammation; while the latter is caused by hemolysis and consequent NO deficiency.

However, this distinction is rather artificial because the fundamental cause of both occlusion and hemolysis is, in fact, the HbS mutation and red cell sickling. Thus, identifying a “hyper-hemolytic” state is a bit like saying that patients with more severe sickle disease are more severe. It has been believed for decades that sickle patients with lower hemoglobin levels have more severe disease. That subgroup of patients is important, not because they comprise a “new” sickle phenotype, but because their higher level of free plasma hemoglobin can provide a mechanism (but only one of seven or so) to cause NO depletion. However, it should be noted that a direct link between hemolysis and deficient NO

and a disease complication has only been demonstrated experimentally for pulmonary hypertension; the other disease events included in the syndrome only have a correlative association with degree of hemolysis.

Stroke

Note that the promoters of the distinct phenotype concept tend not to consider stroke to be part of the vasculopathy syndrome, simply because it does not correlate with hemolysis and plasma hemoglobin [26, 30]. But this circumstance by no means negates the presence of stroke in the vasculopathy syndrome. Rather it probably simply reflects the role of endothelial heterogeneity. That is, the responsiveness of cerebrovascular system, compared to other vascular beds, may be less influenced by low NO and more influenced by something else, e.g., increased thrombin. And note that there is a difference between development of Circle of Willis vasculopathy and precipitation of an actual clinical stroke, which generally requires the additional participation of coagulation and thrombosis.

Inflammatory state

Sickle cell anemia comprises a state of systemic inflammation [6]. The evidence for this is summarized in Table 3. Notably, the elevated levels of biological mediators are, in general, present not only in sickle subjects undergoing acute events (e.g., the acute painful episode due to vasoocclusion) but also in those in so-called “steady state” and between acute events. There are a number of clinical correlates of this inflammatory state in sickle cell anemia, e.g., leukocytosis as a predictor of mortality and morbidity [31] and of a worse predicted course for children [32].

Transgenic sickle mice share this inflammatory state with sickle humans in having: leukocytosis; increased peroxynitrate deposition and increased oxidant generation; abnormal

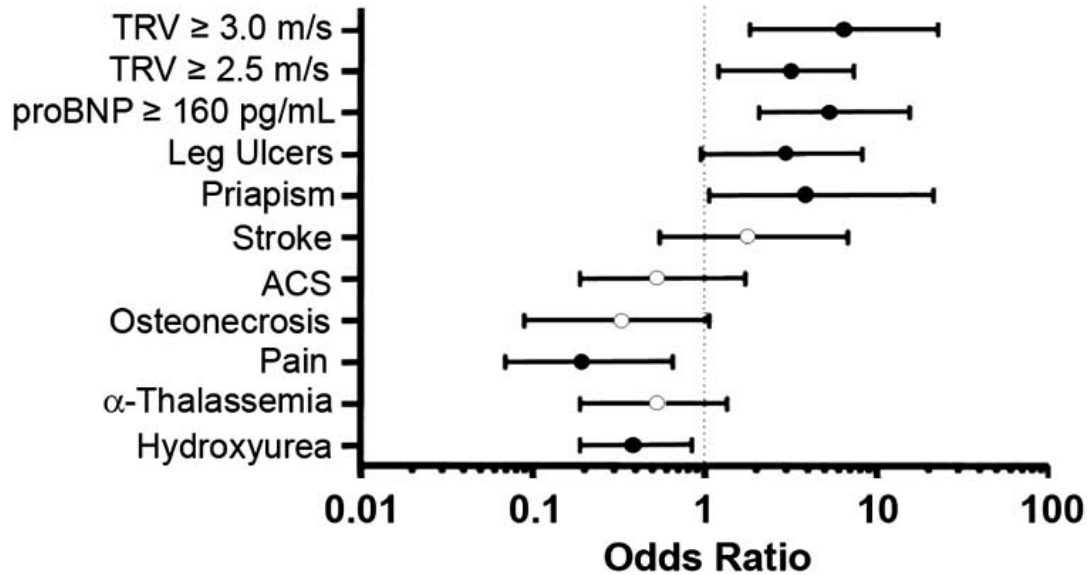


Fig. (4). Hemolytic phenotype. This displays the odds ratio that various clinical facets of sickle patients are associated with increased hemolysis. Obtained with permission from Taylor JG 6th, *et al.*, Chronic hyper-hemolysis in sickle cell anemia: association of vascular complications and mortality with less frequent vasoocclusive pain. *PLoS ONE* 2008, 3, e2095.

Table 3. Inflammatory State of Sickle Cell Anemia

↑ number of granulocytes & monocytes in blood
↑ activation of granulocytes and monocytes activated phenotype of circulating endothelial cells (pro-adhesive, pro-coagulant, pro-oxidative)
↑ soluble VCAM and P-selectin activation of the coagulation system
↑ levels of inflammatory mediators (e.g., IL6, CRP, TNF α , IL1 β)
↑ levels of acute phase reactants (e.g., CRP, phospholipase-A2, ferritin)
↑ levels of endothelial cell perturbants *
↑ microparticles from monocytes, platelets, endothelial cells, red cells sickle mice have an inflammatory state

* which include, but are not limited to: hypoxia, thrombin, IL2, IL4, IL8, endotoxin, TGF β , thrombospondin, G-CSF, GM-CSF, endothelin-1, 12-HETE, peroxynitrite, serum amyloid a, PGE2, fibrinogen, leukotriene B4, homocysteine, CD40 Ligand.

interaction of blood leukocytes with endothelium (decreased rolling velocity, increased rolling flux, increased firm adhesion); deposition of xanthine oxidase on vessel walls; activation of the pre-eminent inflammatory transcription factor, NF κ B (typically components p50 and p65), and an activated phenotype of tissue endothelial cells [6].

We will not here devote space to a discussion of the mechanisms of inflammation in general, since the nature of the inflammasome is currently understood in great detail, for example as exemplified by sepsis [33], and much is known about the endothelial biology of this [34]. We believe that activated monocytes are a promoter of endothelial inflammatory phenotype in sickle disease because of their role in endothelial TF expression [35] and because they have been shown to activate endothelial NF κ B [36]. It seems highly likely that the inflammatory milieu, particularly the activated state of blood monocytes, underlies development of vasculopathic lesions in sickle disease [6], just as it does in atherosclerosis [8, 9].

Relationship to other Sub-Biologies

A fundamental question is whether inflammation is a cause of vascular disease or a result of vascular disease. The likely answer is that it is both, with a vicious cycle occurring, with inflammation and vascular occlusion reciprocally stimulating each other (Fig. 1). Certainly, inflammation biology overlaps with many other sub-biologies. It is often associated with coagulation activation [37]. It is promotive of abnormal expression of adhesion molecules for both red cells and white cells [6, 33]. It depletes NO and alters vasoregulation [34]. By several mechanisms it would promote stasis. And it is a fundamental consequence of reperfusion injury physiology [38], the basic contextual reality of the sickle patient.

Coagulation Activation

The coagulation system shows a high degree of activation in sickle cell anemia. Both platelet and plasmatic coagu-

lation activation are observed for patients in steady-state, and are generally worsened in conjunction with an acute vasoocclusive event [39,40]. Evidence of activation is seen over the whole spectrum of the system (Table 4). In sickle mice, elevated levels of thrombin:antithrombin complexes have been noted [41], so the mouse appears to have activation as well, although more detailed aspects of coagulation have not been addressed in sickle mice.

Table 4. Coagulation Activation in Human Sickle Cell Disease*

↑ prothrombin F1.2 (reflects conversion of prothrombin to thrombin)
↑ thrombin/antithrombin complexes (reflects ↑ thrombin generation)
↑ fibrinopeptide A (reflects ↑ proteolysis of fibrinogen by thrombin)
↑ fibrin(ogen) fragment E ^a (reflects formation and degradation of fibrin)
↑ D-dimer (reflects degradation of cross-linked fibrin)
↓ contact system factors (reflects intrinsic pathway activation)
↑ factor VII turnover (reflects external pathway activation)
↓ thrombin sensitive factors (reflects ↑ thrombin action)
↓ proteins C and S (reflects ↑ consumption)
↑ platelet activation
↑ whole blood tissue factor levels (reflects trigger expression)
↑ platelet-monocyte aggregates in blood (reflects platelet activation)
↑ number of tissue-factor positive microparticles (reflect tissue TF expression)

* This table is modified from that in Francis RB and Hebbel RP. "Hemostasis" in Sickle Cell Disease: Basic Principles and Clinical Practice, Raven Press, Ltd., New York, 1944; pp 299-310.

In this sub-biology, tissue factor (TF) is the acknowledged trigger of system activation. Reflecting mostly blood monocyte status, the whole blood TF level is elevated in sickle patients [42], and their circulating endothelial cells exhibit abnormal TF expression [43], reflecting the status of the endothelium in the vessel wall. Further, sickle blood contains increased numbers of TF positive microparticles released from both activated endothelial cells and monocytes [21]. Regarding direct observation, there are virtually no direct tissue data on TF expression in sickle humans except that we did observe endothelial TF expression in the lung of a single sickle patient after she died from thromboembolism (Fig. 5). We are not aware of any direct measurement of TF on sickle monocytes by FACS.

Using sickle transgenic mouse models, we have documented abnormal expression of TF on endothelium of the pulmonary veins [44], where it is expressed in the more severe mouse models (S+SAntilles, hBERK, and BERK) even at ambient air without any imposed stress. In mild-phenotype NY1DD mice, TF expression is very low just like in normal mice, but it becomes more highly expressed on the pulmonary endothelium, a true phenotype switch, if they are exposed to transient hypoxia followed by reoxygenation (H/R

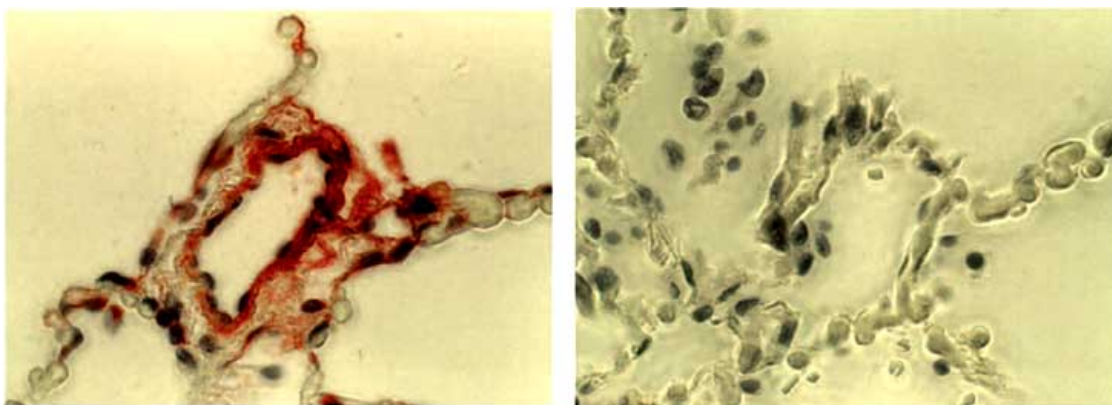


Fig. (5). Pulmonary endothelial expression of tissue factor is evident (left) in this patient who died of thromboembolism. Obtained with permission from Solovey A and Heibel RP, Tissue factor expression in sickle cell anemia. *J. Lab. Clin. Med.* **2001**, 137.

stress, which induces ischemia reperfusion in the sickle, but not the normal, mouse). Such data are shown in (Fig. 6). Whether this pattern and occurrence of endothelial TF expression occurs in humans is not known. However, it is noteworthy that the pulmonary venous endothelium resides immediately upstream of the vascular bed that most commonly endures thrombosis in sickle patients, the Circle of Willis. Also, activated coagulation presumably contributes to the thrombosis *in situ* that tends to occur in conjunction with sickle pulmonary hypertension [45].

Relationship to other Sub-Biologies

The endothelial cell comprises the biologic interface of the coagulation and inflammatory biologies, the reason why so many inflammatory disorders are associated with coagulopathy [37]. Indeed, the major activators of coagulation are

the cytokines released during inflammation. Naturally occurring anticoagulants dampen the cytokine inflammatory signaling and make the endothelial cell less responsive to inflammation. Conversely, down-regulation of anticoagulant pathways (true of sickle patients) activates both inflammatory and coagulation pathways. This complex dance is highly relevant to vicious cycles in sickle disease (Fig. 1).

Importantly, study of sickle mice has allowed us to directly demonstrate that endothelial tissue factor expression is specifically dependent upon the NFκB p50 component that resides in the peripheral blood mononuclear cells (but not the p50 which resides in the vessel wall) [35]. It is evident that the blood monocyte is a direct player in the vessel wall perturbation so characteristic of this disease. Indeed, there are increased platelet-monocyte aggregates in sickle plasma

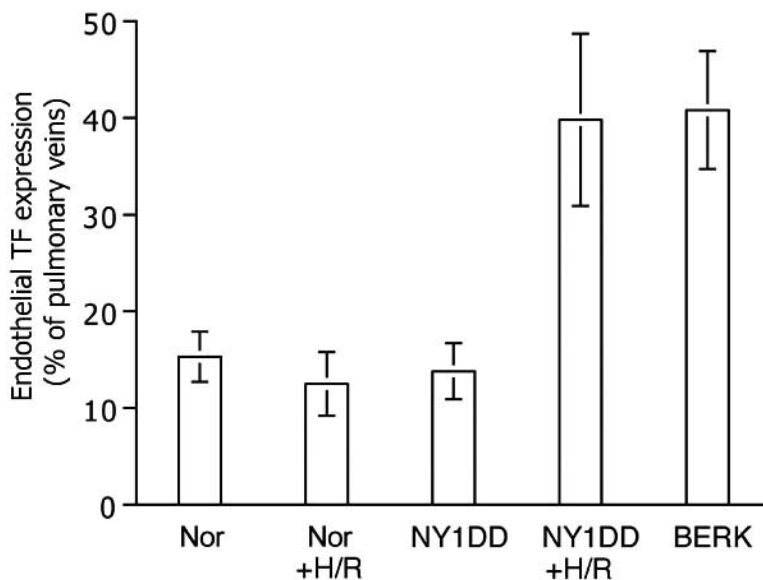


Fig. (6). Endothelial TF expression. This shows extent of tissue factor expression by pulmonary vein endothelium in sickle mice (vertical axis). TF expression is low in normal control mice (Nor), and is unchanged in the mild-phenotype sickle mouse (NY1DD). After exposure to hypoxia/reoxygenation (H/R), the normal mouse is unchanged by the NY1DD mouse converts to high-level TF expression. The severe phenotype sickle mouse (BERK) displays high TF expression at ambient air, without exposure to H/R. Obtained with permission from Solovey A, *et al.* Endothelial tissue factor expression in sickle mice is augmented by hypoxia/reoxygenation and inhibited by lovastatin. *Blood* **2004**, 104, 840-6,.

[46], which constitute evidence for platelet activation and an actual etiologic factor in vascular disease [47].

The dysfunctional endothelium of sickle disease feeds directly into the relevance of coagulation for other sub-biologies. Normally, the NO elaborated by endothelial cells would inhibit platelet activation and adhesion, down-regulate endothelial adhesion molecules, inhibit TF expression; furthermore, the functionally normal endothelium elaborates prostacyclin a vasodilator and inhibitor of platelets; it expresses an ectonucleotidase that breaks down ADP (a platelet activator); it expresses thrombomodulin, which responds to thrombin to activate coagulation inhibitor protein C and EPC-R (which promotes this reaction); and it expresses anti-thrombin III and TFPI, both inhibitors of coagulation. A NO biodeficient state promotes a dysfunctional endothelium, which in turn tends to promote a hypercoagulable state.

We have demonstrated *in vivo* using sickle mice that endothelial eNOS normally down regulates endothelial TF expression, so reduction in eNOS promotes increased TF expression [48]. Hence, we believe that the coagulopathy of sickle disease derives primarily from inflammation (the primary stimulus) and deficient availability of NO (impaired down-regulation) [35, 48].

At the distal end of the coagulation cascade, thrombin generation is critical, not only because it is the final effector in the process of blood clotting, but also because it is the most biologically relevant activator of platelet activation, and it is a major endothelial perturbant, presumably *via* its PAR1 (proteinase activated receptor) thrombin receptor. For example, thrombin is one known stimulus for further TF expression, another vicious cycle. Others include: endotoxin, IL1 β , TNF α , oxidized LDL, monocytes, some infections,

complement fixation, oxygen radicals, and others. PARs help regulate vascular tone, inducing vasorelaxation that is NO dependent in larger vessels and NO independent in smaller vessels [49]. PAR stimulation (e.g., by thrombin) worsens vascular intimal remodeling (shown in restenosis models) [49]. Thrombin generation to excess directly relates to inflammation and adhesion biology in that thrombin is a direct stimulus for rapid endothelial expression of P-selectin.

TF expression may also be related to adhesion biology, since it has preliminarily been reported that phosphatidylserine-exposing sickle red cells can induce endothelial TF *in vitro*, part of the injury response of endothelium to adherent red cells [50]. Additionally, adherent platelets are a source of activating molecules such as IL1 β and RANTES [51].

In aggregate, these observations indicate that sickle cell disease essentially fulfills all aspects of Virchow's triad: blood stasis, hypercoagulable blood, and abnormal vessel wall (Fig. 7). Of interest, it has recently been shown that even sickle trait individuals have somewhat activated coagulation [52] and have a two-fold increased propensity for thromboembolism [53].

Oxidation Biology

Sickle cell anemia involves excessive oxidative stress [6, 54]. The molecular biochemistry of oxidant generation at the red cell's cytoplasmic/membrane interface, caused by the instability of HbS, has been defined in great detail [25]. This provides an instructive model for what happens when iron gains abnormal access to a biomembrane. Whether the generation of oxidants from red cells has any direct effect on the vascular wall is unknown, but it may since these red cells are abnormally adherent to—and juxtaposed with-- endothelium,

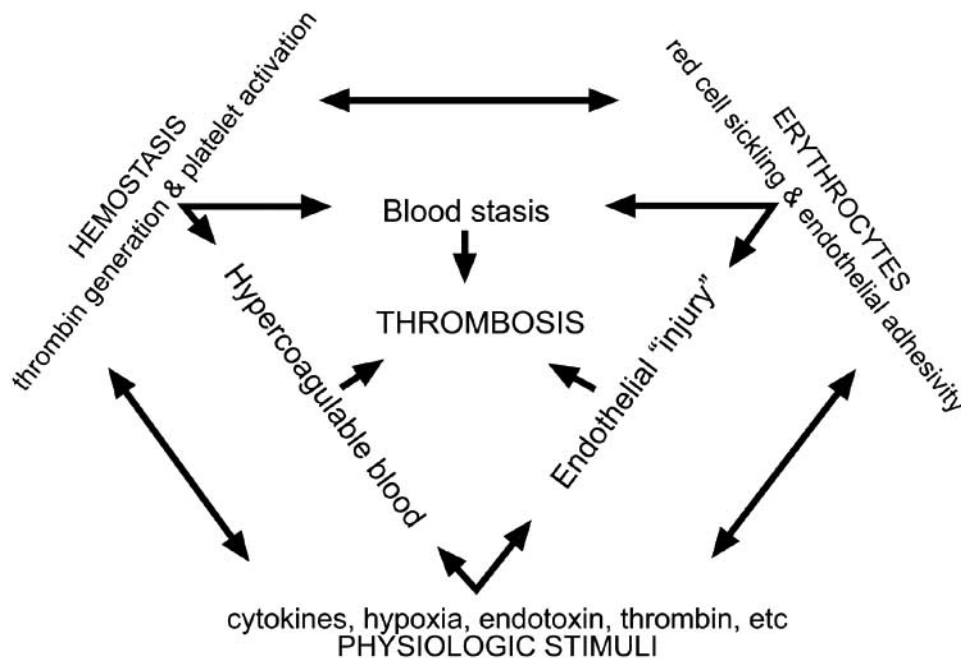


Fig. (7). Virchow's triad. This classical triad provides a conceptual context for understanding risk of thrombosis. The Fig. illustrates how its conditions are met in the sickle context. Obtained with permission from Francis RB and Hebbel RP, Hemostasis; In *Sickle Cell Disease: Basic Principles and Clinical Practice*; Embury, S.H.; Hebbel, R.P.; Narla, M.; Steinberg, M.H. Eds.; Raven Press, Ltd., New York, 1994; pp 299-310.

and such adhesion does induce an injury response on the part of the endothelium [6, 55].

However, there are many additional sources of oxidant stress that probably impact the vessel wall and its endothelial/plasma interface. These include: abnormal amounts or locations of bioactive iron that can participate in Fenton chemistry [25]; multiple enzyme systems (activity of NADPH oxidase of endothelial cells [56], exogenous xanthine oxidase abnormally deposited on endothelial surface [57,58], uncoupling of eNOS [59], abnormal myeloperoxidase deposits [60]; diminished NO availability for reaction with superoxide; and oxidant generation from endothelial-adherent or circulating leukocytes [61]. All of these sources of oxidant have been supported experimentally in the sickle context, largely *via* studies of sickle mice.

Biomarkers of Oxidation

Blood from sickle humans, even in “steady state”, contains high amounts of TBARS and other carbonyls (reflecting lipid peroxidation) [62], and lowered amounts of vitamin E (presumably reflecting consumption by oxidants) [63]. Sickle LDL are deficient in vitamin E and exhibit increased oxidizability [64]. Circulating endothelial cells from sickle patients exhibit excessive expression of HO-1 (reflecting activity of oxidative systems) [65]. Human gene expression studies of peripheral blood monocytes reveal evidence for oxidative stress [66]. As observed in sickle mice, there is an elevation of ethane expiration (reflection of whole body lipid peroxidation) and of salicylate hydroxylation (reflection of blood hydroxyl radical generation) [67] (Fig. 8); whether this is true of humans is unknown.

Role of HO-1

An important piece of the oxidant stress puzzle is how the sickle patient defends or adapts to excessive oxidative

stress. An understanding of these processes could potentially underpin therapeutic approaches. In response to an inflammatory onslaught of heme, organisms have evolved an elegant mechanism to deal with excessive heme burdens, the induction of heme oxygenase 1 (HO-1). In fact, sickle cell disease represents a prime example of HO-1 upregulation in response to hemolysis. It is hypothesized that HO-1 upregulation, may inhibit --and modulate resolution of --vasoocclusion. This effect may be through the enzyme's products, CO and biliverdin, and its linkage to ferritin [68]. In this manner, HO-1 reduces oxidant stress, inhibits NF κ B and down-regulates endothelial adhesion molecules. HO-1 additionally inhibits MCP-1 expression and inflammation [65]. The adaptive response of the sickle patient (an increase in HO-1) may be too little too late to handle the excessive heme burden in sickle disease. This may argue for a therapeutic strategy aimed at further increasing HO-1 level/activity above that normally achieved physiologically.

Relationship to other Sub-Biologies

Oxidants can impact the endothelium and its function in multiple ways. Among these would be peroxidation of membrane lipid which can alter membrane fluidity, lipid raft formation, and the function of membrane proteins. Oxidants are activators of NF κ B and AP1, the pre-eminent transcriptional regulators of the inflammatory response and of tissue factor expression. Relevant to vasculopathic lesions, the inflammatory stimulant IL1 β causes endothelial cells to liberate the same oxidized lipid species [10] that are implicated as proximate triggers of the inflammatory process in atherosclerosis [69]. Finally, oxidation biology is an activator of multiple other sub-biologies: inflammation, coagulation, and stasis. And in the sickle mouse oxidation is reported to have significant inhibiting effect on NO signaling by increasing NO consumption *via* superoxide (probably a major contribu-

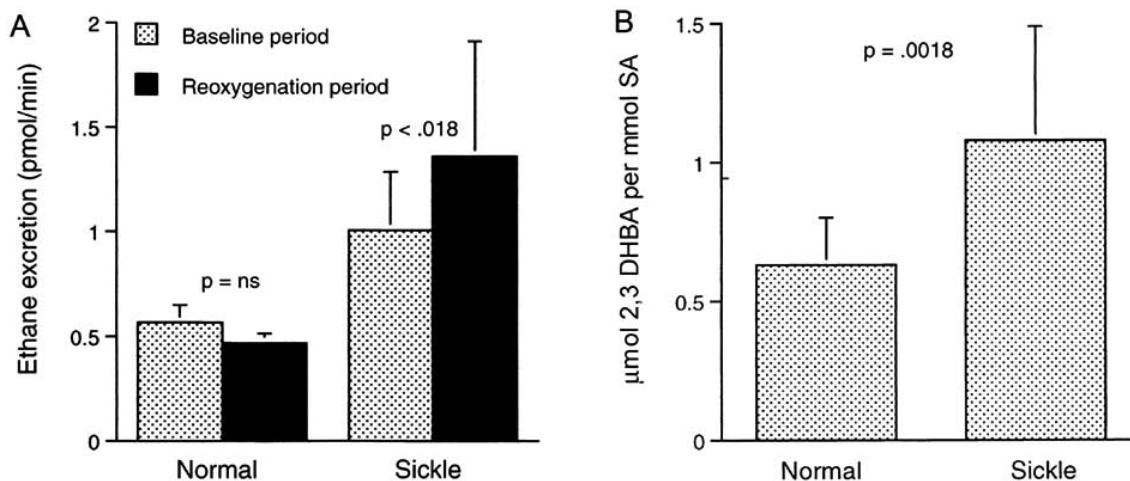


Fig. (8). Biochemical footprints of excessive oxidation. Data obtained from the NY1DD sickle mouse model. **Panel A.** Amount of ethane measured in expired gas (measure of body lipid peroxidation) is low for normal control mice and elevated for sickle mice under ambient air conditions (left). After H/R stress, amount of ethane is increased for normal mice, but increased further for sickle mice. **Panel B.** Amount of blood conversion of salicylate to 2,3DHBA (indicator of body hydroxyl radical generation) is low for normal mouse and elevated for sickle mouse, both studied under ambient air conditions. Obtained with permission from Osarogiagbon RU *et al.*, Reperfusion injury pathophysiology in sickle transgenic mice. *Blood* 2000, 96, 314-20.

ABNORMAL CELL-CELL INTERACTIONS

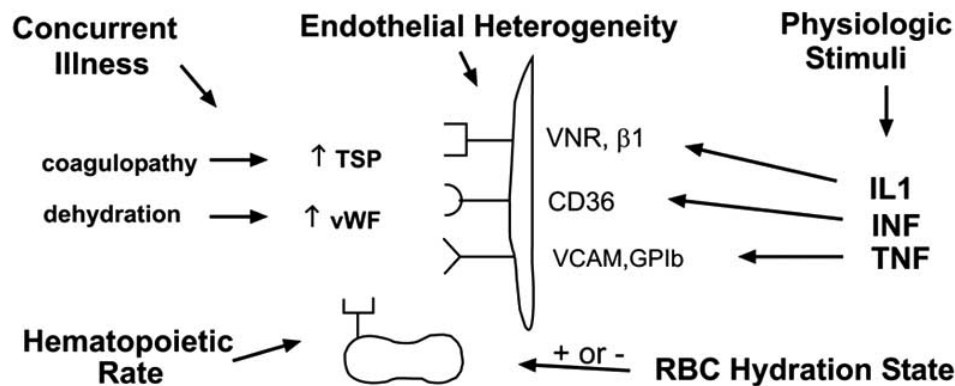


Fig. (9). Red cell adhesion to endothelium. Sickie red cells are abnormally adherent to vascular endothelium, as studied *in vitro* and *in vivo*. This scheme emphasizes that, although multiple mechanisms have been proposed and supported experimentally, most involve receptor structures on both red cell and endothelial cell, as well as a plasma bridging molecule of some kind. Obtained with permission from Hebbel RP and Mohandas N, Sickie Cell Adherence; In Sickie Cell Disease: Basic Principles and Clinical Practice; Embury, S.H.; Hebbel, R.P.; Narla, M.; Steinberg, M.H. Ed; Raven Press, Ltd., New York, 1994; pp 217-230.

tor [70] and by lowering eNOS activity [58]. Oxidative stress causes inflammatory endothelial dysfunction, adhesion molecule induction, and barrier deterioration.

Adhesion Biology

Sickie red cells are abnormally adherent to endothelium [6, 71]. Multiple mechanisms have been implicated, most involving an adhesive structure on the red cell (e.g., CD36, $\alpha 4\beta 1$) and a counter-receptor expressed by the endothelial cells (e.g., $\alpha v\beta 3$, VCAM1); some additionally involve a bridging molecule in the plasma such as thrombospondin and von Willebrand Factor (Fig. 9). The levels of these adhesion molecules, and degree of expression (and/or avidity) of the adhesion receptors on both red cell and endothelium, can fluctuate along with clinical conditions, in particular platelet activation and inflammation [6]. Also, the hemolytic rate is relevant because it leads to greater proportion of young red cells, which have increased expression of some of these adhesive structures.

Since sickie red cell adhesivity to endothelium correlates with clinical vasoocclusive severity [72], it was proposed that this adhesion might be the trigger which slows down microvascular flow to fulfill the delay time requirement so that sickling can occur [72,73]. Elegant mouse experiments support this conjecture by demonstrating that adhesion of red cells is proximate, and is followed by a propagation phase characterized by log jamming of poorly deformable cells, followed by sickling [74].

Regarding leukocytes, intravital microscopy has shown that there is increased white cell interaction with vessel wall in sickie mice [61]. Predictably, this is greatly augmented after establishment of ischemia reperfusion in these animals (Fig. 10). Sickie mice have increased expression of adhesion molecules on endothelium [75]. These adhesive structures,

of course, are well known to mediate leukocyte/endothelial interactions [76]. Others have observed endothelial adhesion of multi-cellular formations of white cell/red cell aggregates [77]. Since leukocytes are far larger, stiffer, and stickier than red cells, we have suggested that these may play a primary role in slowing microvascular flow and, ultimately, in causing vasoocclusion. Notably, antibodies to P selectin inhibit both red cell and leukocyte adhesion to endothelium in some sickie models [61, 78].

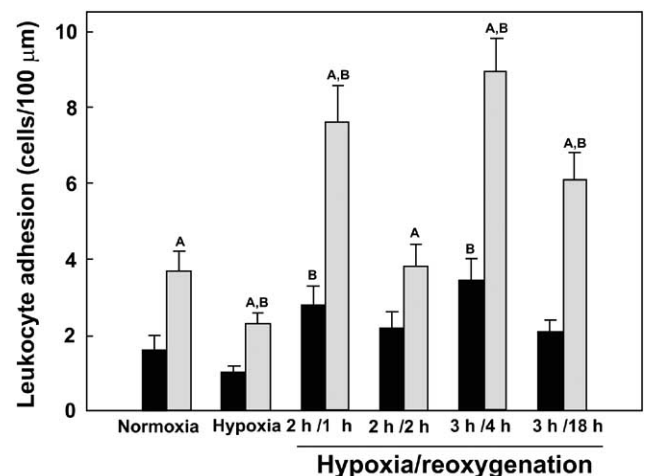


Fig. (10). Leukocyte adhesion to endothelium *in vivo*. Normal (black bars) and sickie (grey bars) mice were examined by intravital microscopy after exposure to hypoxia/reoxygenation (H/R). Times at hypoxia and reoxygenation are indicated in hours. Sickie mice have leukocytosis under all conditions and show increased response to hypoxia itself and a greatly exaggerated response to H/R. Obtained with permission from Kaul DK and Hebbel RP, Hypoxia/reoxygenation causes inflammatory response in transgenic sickie mice but not in normal mice. *J. Clin. Invest.* **2000**, *106*, 411-420.

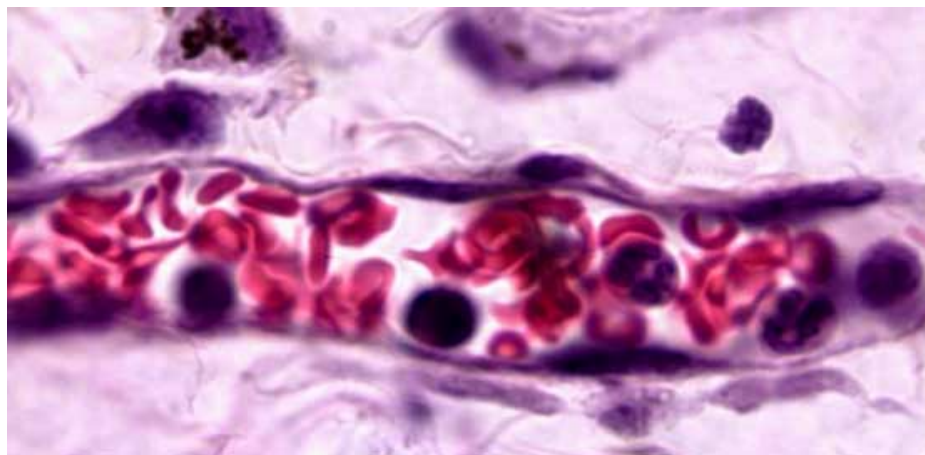


Fig. (11). Cell/cell adhesion. In sickle biology, as shown here during stasis of flow, there is abnormal adhesion of both red cells and leukocytes to endothelium. Obtained with permission from Kalambar, V.S.; *et al.*, Microvascular blood flow and stasis in transgenic sickle mice: utility of a dorsal skin fold chamber for intravital microscopy. *Am. J. Hematol.* **2004**, *77*, 117-125.

One important caveat is that endothelial adhesion molecule expression is highly heterogeneous, varying from organ-to-organ and even from large-to-small vessels in any given organ [75]. Thus, the precise mechanism(s) of adhesion involvement for both red cells and leukocytes may vary from patient-to-patient or even with geographic location or time for a given patient (Fig. 11).

Relationship to other Sub-Biologies

Interaction of red cells with endothelium can be a vicious cycle, since adherence of red cells causes an injury response to the endothelium [6, 55]. Thus adhesion participates in endothelial dysfunction. The interaction of platelets and leukocytes and multi-cellular aggregates can precipitate stasis and bidirectional activation of endothelium and blood cells. The major role of adhesion biology is discussed under Stasis below. Enhanced adhesion biology is a major aspect of an inflammatory state.

Stasis and VEGF

Since slowed microvascular flow is necessary for promotion of sickling, the various mechanisms underlying stasis undoubtedly are pre-eminent factors in sickle vascular pathobiology. Stasis could derive from red cell or white cell or multi-cellular aggregate adhesion to endothelium, possibly from coagulation activation, from dysfunctional vasoregulation, and from heightened whole blood viscosity. Stasis has been observed in skin capillaries in sickle humans (where it seems to be caused by red cell/endothelial interaction [79]), as well as in sickle mice in multiple vascular beds [74, 77, 78]. And, stasis increases greatly in sickle mice during a period of ischemia/reperfusion [80]. Different mechanisms have been supported for stasis in different models, particularly VCAM [80] and P-selectin [78]. Because stasis and inflammation are both causes and consequences of ischemia/reperfusion, this comprises another vicious cycle.

Stasis leads to local hypoxia, a cause of endothelial dysfunction and NO deficiency. Stasis would disrupt shear-dependent endothelial responses, e.g., NO, PGI₂ and tPA all

are partly shear dependent. Stasis also allows interaction of platelets and fibrin monomer with the endothelium locally. Possibly of greatest importance, hypoxia stimulates VEGF (vascular endothelial growth factor) [81]. Indeed, VEGF levels are increased in plasma of sickle patients, and there is an inverse correlation between VEGF level and percentage of circulating endothelial cells that are apoptotic (Fig. 12) [82]. The latter may indicate that the elevated VEGF level exerts a somewhat sparing effect on the vessel wall endothelium of sickle patients. It does suggest that the blood of the sickle patient has a pro-angiogenic/anti-apoptotic tone to it [82]. VEGF induces a variety of genes, including COX2 and Egr-1, the latter being an important regulator of TF expression.

Clinically, the elevated VEGF level in sickle patients may contribute to the tendency of some sickle disease patients to develop retinopathy [2] and to the development of plexiform lesions in association with the pulmonary hypertension of sickle disease [5]. In addition the permeabilizing property of VEGF may help explain the vigorous edema formation that accompanies development of the acute chest syndrome in sickle patients [1].

NO and Vasoregulation

Sickle disease is a state characterized by biodeficient NO, eNOS uncoupling, and enhanced NO consumption. Experimentally, there certainly is evidence for disruption of vasoregulation in sickle patients, first called to attention by Belhassen [83] who found impaired flow-mediated dilation (Fig. 13) and apparently normal responses of forearm blood flow to L-NAME and SNP. Essential confirmation [84-86] further suggested that the abnormality of vasoregulation was much more apparent in men than in women. [This is reminiscent of the protective role that estrogen seems to play in reperfusion injury physiology [87]. Sickle mice [88-90] exhibit dysfunctional endothelium with impaired endothelial-dependent vasodilation but intact endothelial-independent vasodilation, and deficient basal NO bioavailability.

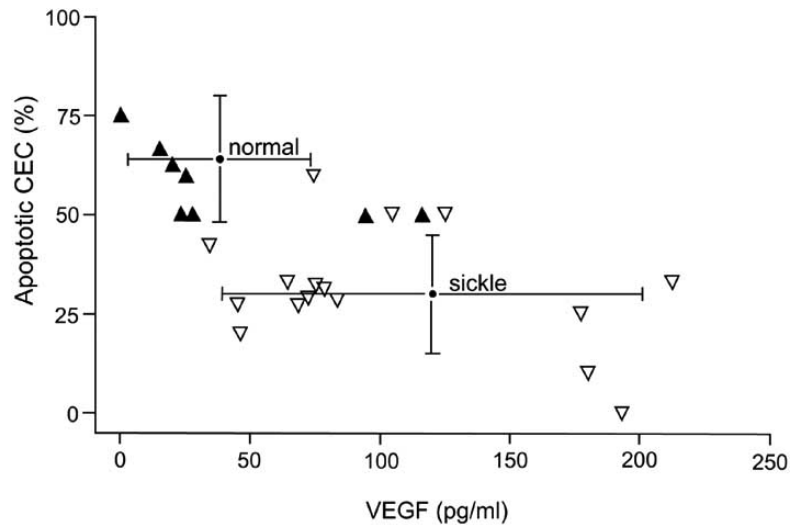


Fig. (12). VEGF in sickle disease. Plasma level of VEGF in sickle patients is shown on horizontal axis. Degree of apoptosis concurrently displayed by that patient's circulating endothelial cells (CEC) (vertical axis) is inversely proportional to VEGF level. So elevated VEGF might provide an endothelial sparing signal in sickle patients. Obtained with permission from Solovey A, *et al.*, Sickle cell anemia as a possible state of enhanced anti-apoptotic tone, survival effect of vascular endothelial growth factor on circulating and unattached endothelial cells. *Blood* 1999, 93, 3824-30.

The Perfusion Paradox of Sickle Cell Disease

Regional hypoperfusion and tissue ischemia in microcirculatory beds, arising as a consequence of vasoocclusive processes, are among the defining clinical and pathologic issues in sickle disease [91]. This represents not only the occlusive effect of sickled red cells, but also the vasoconstrictive effects of species such as endothelins, thromboxanes, and isoprostanes which are generated in increased amounts in the steady state and during acute vasoocclusive events [91].

Ironically, the systemic circulation in sickle disease, in its steady state, is characterized by hyperperfusion and vasodilation: the cardiac output in sickle disease is significantly increased and is accompanied by increased perfusion of certain organs and limbs. Such increased systemic perfusion is

attended by reduced systemic vascular resistances, and cannot be ascribed to anemia *per se*. Interestingly, during sickle vasoocclusive crisis, the increase in cardiac output and the reduction in systemic vascular resistances in sickle disease are both exaggerated.

Thus, as previously stated, "[Sickle cell disease] exhibits a curious coexistence of contrasting perfusion profiles in the circulatory system: hypoperfusion is endemic in microcirculatory beds occluded by hemoglobin S-containing erythrocytes while hyperperfusion characterizes the systemic (macro) circulation and a number of regional vascular circuits" [91]. These divergent changes in vascular perfusion are exemplified by the renal circulation: whole kidney blood flow is increased in sickle cell disease, whereas regional perfusion to the renal medulla is significantly compromised and, indeed, contributes to the recognized concentrating and other

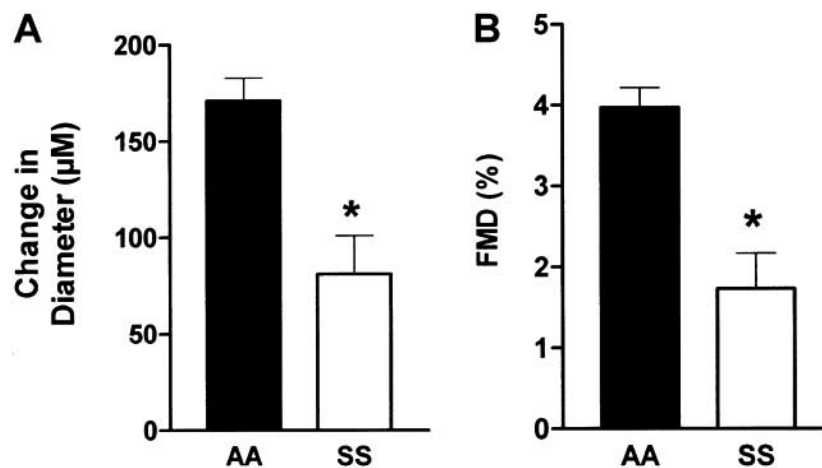


Fig. (13). Deficient vasorelaxation. Sickle patients (SS) have abnormally low arterial relaxation compared to normal controls (AA), as measured by arterial diameter (A) and flow mediated dilation (B). Obtained with permission from Belhassen L, *et al.*, Endothelial dysfunction in patients with sickle cell disease is related to selective impairment of shear stress-mediated vasodilation. *Blood* 2001, 97, 1584-1589.

tubular defects, and papillary necrosis that occur in sickle disease.

Decreased Nitric Oxide Bioavailability

The basis for reduced systemic vascular resistances in sickle disease remains unresolved, and particularly so as regards the extent to which the eNOS/NO system contributes to this hemodynamic alteration. Studies of vascular reactivity in murine sickle models demonstrate that there is an impairment in endothelium-dependent vasorelaxation and vasorelaxant responses to NO-generating agents; that eNOS expression, depending upon the vascular segment, is either induced or unaltered; and that vascular wall content of cGMP can be increased [92,93]. In studies of human sickle subjects, certain subsets of patients can exhibit an impaired blood flow response to NO donors; an attenuation in the reduction in blood flow following the administration of an inhibitor of NOS; and a reduced involvement of NO in mediating endothelium-dependent vasorelaxation [84-86].

Such findings have led to the view that the contribution of NO to vascular responses is impaired in sickle disease, and that there is reduced bioavailability of NO as well [94, 95]. The former has been described as “NO resistance”. However, this is a misnomer, since this situation presumably stems from NO consumption by the combination of cell-free (plasma) hemoglobin and the production of excessive oxidant (superoxide) (Fig. 14). Hence, rather than “NO resistance” it should be called a state of “NO consumption”.

It should be pointed out, however, that an impaired vascular reactivity to NO is characteristic of states attended by increased generation of NO. For example, eNOS over-expressing mutant mice demonstrate reduced blood pressure, increased vascular cGMP, and reduced vasorelaxation re-

sponses to acetylcholine and NO-generating agents. So the sickle picture is complex.

Mechanism(s) Underlying NO Biodeficiency

Notwithstanding the current vigorous promotion of the notion that plasma free hemoglobin is responsible for the NO biodeficient state of sickle disease [24, 95], it is notable that there actually are multiple independent ways to achieve NO biodeficiency that also have been implicated in sickle disease [94]. These relate either to increased consumption of NO, or decreased generation of NO.

Increased scavenging of NO by superoxide can occur in the sickle context, which involves an abnormal imbalance between superoxide and NO generation [59, 94]. Indeed, sickle mice display enhanced vessel wall oxidation [61, 94]. The increased superoxide generation itself can be from increased HbS autooxidation in red cells [25], activated leukocytes [6], endogenous endothelial xanthine oxidase, heightened activity of NADPH oxidase [56], abnormal deposits of plasma xanthine oxidase on the endothelial cell surface [57], and abnormal deposition of myeloperoxidase in tissue [60].

Alternatively, NO can be limited by its enhanced consumption, which occurs in sickle disease due to the activity of cell-free plasma hemoglobin; evidence is strong that the amounts of free hemoglobin in sickle plasma are, indeed, high enough to exert this effect [24]. Whether this is “the” explanation of reduced bioavailability in sickle disease is less certain, but this consuming effect could explain the observations of unexpectedly low impact of NO donating drugs.

In the sickle context, it is believed that amount of eNOS enzyme may actually be increased in the vessel wall [92]. This may vary from organ to organ [90, 92]. Yet, in parallel,

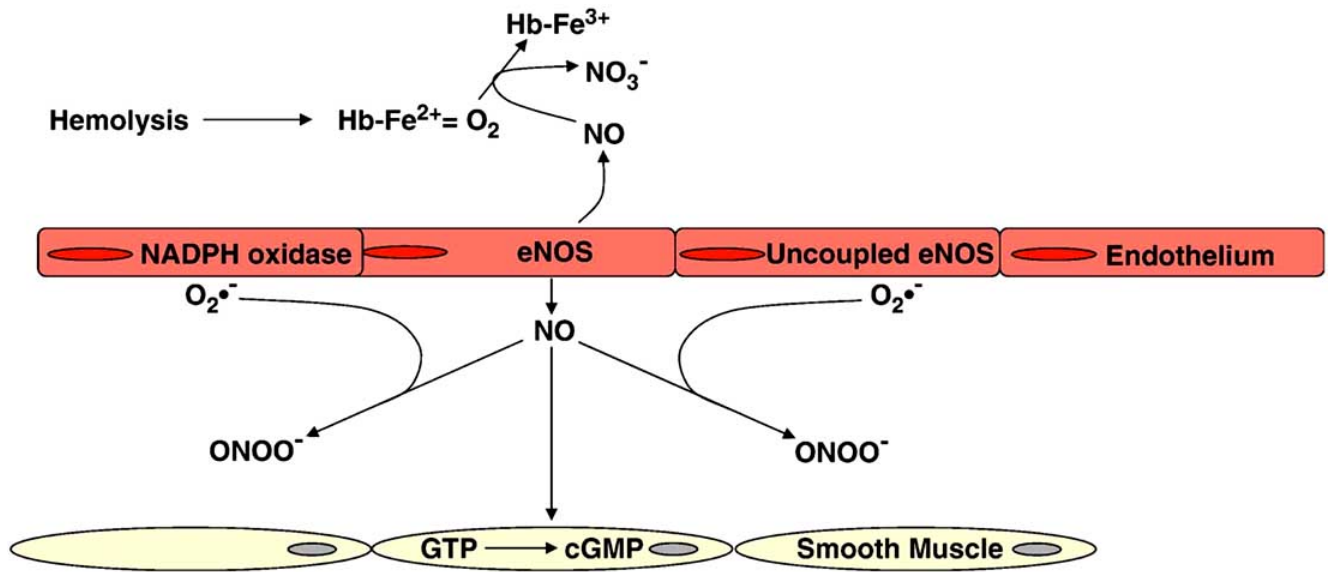


Fig. (14). The complex interrelationship between the oxidant superoxide and NO generated from endothelial eNOS. Superoxide is generated to excess in the sickle context, via multiple mechanisms, and this serves to consume NO. It is a contributor to the biodeficiency of NO in sickle disease. Obtained with permission from Aslan M and Freeman BA, Redox-dependent impairment of vascular function in sickle cell disease. *Free Radic Biol. Med.* 2007, 43, 1469-83.

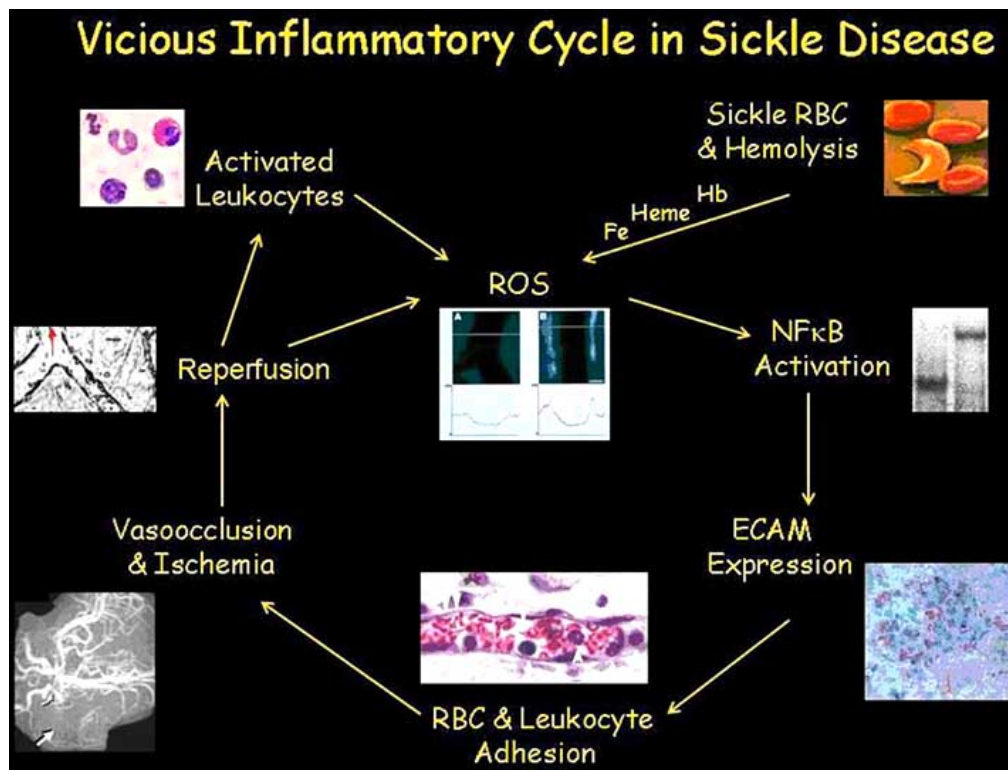


Fig. (15). The vicious cycle of reperfusion injury and inflammation. Shown at top left and top right, respectively, activated leukocytes and the presence of red cells, representing an iron-replete environment (obtained with permission from Belcher JD and Vercellotti GM. Heme oxygenase-1: A potential modulator of inflammation and vasoocclusion in sickle cell disease. In Heme Oxygenase: The Elegant Orchestration of its Products in Medicines [ed.: LE Otterbein and BS Zuckerman], Nova Science, New York, pp 97-112, 2005). Panels from upper middle and going clockwise are intended to show the following. Generation of reactive oxygen species, as evidenced by enhanced dihydrorhodamine fluorescence (obtained with permission from Kaul DK and Hebbel RP, Hypoxia/reoxygenation causes inflammatory response in transgenic sickle mice but not in normal mice. *J. Clin. Invest.* **2000**, *106*, 411-20). Enhanced CAM expression on endothelium of glomerular capillaries (previously unpublished image, A Solovey and RP Hebbel). Stasis and leukocyte and red cell adhesion to endothelium (obtained with permission from Kalambur VS, *et al.*, Microvascular blood flow and stasis in transgenic sickle mice: utility of a dorsal skin fold chamber for intravital microscopy. *Am. J. Hematol.* **2004**, *77*, 117-125). Occlusion of the middle cerebral artery (obtained with permission from Hillery DA and Panepinto JA, Pathophysiology of stroke in sickle cell disease, *Microcirculation* 11:195-208, 2004.) Restoration of flow allowing reoxygenation (obtained with permission from Kaul DK, *et al.*, *In vivo* demonstration of red cell-endothelial interaction, sickling, and altered microvascular response to oxygen in the sickle transgenic mouse. *J. Clin. Invest.* **1995**, *96*,2845-53).

decreased production of NO occurs *via* at least four mechanisms that impair eNOS activity. Amount of the endogenous metabolite ADMA (asymmetric dimethylarginine), a potent inhibitor of eNOS activity, is increased in sickle patients [96]. Similarly, lowered levels of ApoA-1 in sickle plasma can affect NOS activity, as there is evidence indicating that this molecule possibly exerts downstream influences on NOS activity.

Other situations cause an uncoupling of eNOS [90] by which it produces superoxide rather than NO [97]. This can occur because of insufficient amounts of cofactor tetrahydrobiopterin (THB4) [98]. This is critically important, because states in which eNOS protein level is actually increased, such as sickle disease, have heightened need for THB4 to support the function of increased enzyme activity. In addition, sickle patients tend to have limited arginine availability, reflected in diminished plasma arginine [99] that is possibly due to dietary reasons in part, but mostly due to their elevated plasma arginase (released from the lysed red

cells) activity which consumes arginine [100]. In the patient with renal disease, amount of arginine generation from citrulline may be diminished. Uncoupling can also occur as a consequence of eNOS oxidation and eNOS monomerization [90].

Vascular Instability

Vascular homeostasis requires a coordinated and appropriate modulation of expression and/or activity of vasorelaxants and vasoconstrictors such that adequate tissue perfusion is preserved. The reduced NO bioavailability that occurs in sickle disease thus limits the homeostatic capability of the vascular system and the extent to which it can rely on increased production of NO to offset the vascular effects of vasoconstrictors, such as endothelin, thromboxanes, and isoprostanes, the latter tonically increased in sickle disease.

It is notable that the reduced NO bioavailability is accompanied by up-regulation in other vasorelaxant systems, such as prostaglandins and the HO-1/CO system, even in the

unstressed, crisis-free state [91]. It is tempting to speculate that such augmentation in non-NO/eNOS vasorelaxant systems represents a compensatory response which attempts to redress the reduced bioavailability of NO. Indeed, the administration of arginine to sickle mice restores NO bioavailability and simultaneously reduces the up-regulation of non-NO systems [92].

This raises at least two substantial issues. First, the fact that these vasorelaxant systems are induced in the unstressed state may imply that there exists little additional capability for further induction of these systems during times of stress and crisis. Second, the HO-1/CO system is designed as an inducible system, and it is recognized that sustained up-regulation of this system may be harmful. Indeed, in certain settings inhibition of HO-1 activity actually has a protective effect on sickle mice [101].

Vascular adaptive responses are thus perturbed in sickle disease. This compromise in homeostatic mechanisms renders the vasculature in sickle patients vulnerable to stress, thereby imparting an inherent vascular instability in sickle patients. In particular, we wonder about the safety of red cell transfusion in the sickle patient, since this is accompanied by massive early red cell destruction and inevitable release of a substantial hemoglobin load which would further enhance NO consumption; thus transfusion in the sickle patient may entail risk of vascular pressure and/or flow disturbances.

Relationship to other Sub-Biologies

The reduced NO bioavailability in sickle disease not only compromises vascular responses but also sets in train a number of pathobiologic changes including a procoagulant profile; increased platelet adhesion to the endothelium; increased adherence of leukocytes to the endothelium and leukocytic recruitment to tissues; and upregulation of proinflammatory transcription factors such as NF- κ B and attendant inflammatory pathways.

Reperfusion Injury

Given that the fundamental characteristic of sickle disease is reversible red cell sickling and occlusion, one would expect sickle cell anemia to comprise the paradigmatic example of ischemia/reperfusion physiology, also referred to as "reperfusion injury". This is a well-established pathobiological paradigm [38] in which much damage to the vessel wall and tissue occurs due to the re-introduction of oxygen upon establishment of reperfusion, rather than being just due to the vascular occlusion itself. Metabolic changes accompanying ischemia allow an excessive burst of oxidative stress during the reoxygenation period. This exerts multiple effects, such as NF κ B activation, and it is characterized by an intensely inflammatory state [38].

A major aspect of reperfusion injury physiology is the characteristic development of microvascular dysfunction that is greatly exaggerated compared to effects of hypoxia alone. It exerts slightly different effects depending upon whether it involves arterioles, capillaries, or venules. But its general features are strikingly excessive oxidant production (from

within the endothelium, from adherent activated leukocytes, and from circulating xanthine oxidase bound and concentrated on endothelial surface glycosaminoglycans). This state is accompanied by production of inflammatory cytokines and mediators (e.g., MCP-1), increased activation of leukocytes, increased expression and/or avidity of endothelial and leukocyte adhesion molecules, vascular plugging by adherent leukocytes, and deficiency of NO production by endothelium resulting in abnormal endothelial-dependent vasodilation.

There is experimental evidence that reperfusion injury does occur in sickle transgenic mice. Such mice reveal biochemical footprints of excessive oxidant generation even at ambient air and absent any exogenous stress, as discussed above (Fig. 8). In parallel, intravital microscopy reveals that these mice have leukocytosis, with abnormally increased interaction with endothelium [61] (Fig. 10). This abnormal leukocyte/endothelial interaction seems to involve P-selectin dependent processes.

Notably, the transient exposure of sickle mice to hypoxia followed by reoxygenation (H/R) leads to additional oxidative stress (Fig. 8) [67], such that vascular walls exhibit increased dihydrorhodamine fluorescence, and there is even greater leukocyte adhesion and transmigration [61]. Also, sickle mice exposed to H/R demonstrate increased expression of endothelial tissue factor [44]. Significantly, this is not true of normal mice, for which the exposure to H/R only has the physiological consequences of transient hypoxia exposure. In dramatic contrast, H/R exposure of sickle mice promotes actual ischemia/reperfusion (I/R), because the percentage of sickled red cells dramatically increases during the H period [67], so that the statistical chance for vasoocclusion is greatly enhanced, thus perhaps approximating acutely occlusive clinical sickle disease.

Long-Range Signaling in the Vasculature

The vascular/tissue injury and microvascular dysfunction consequent to reperfusion injury is not confined to the local geographic location of the occlusion [38]. Indeed, sickle disease represents a striking example wherein impaired perfusion in regional vascular beds can instigate aberrant and adverse effects in distant vascular beds, as observed in sickle mice [102]. For example, ischemia induced in -and restricted to-- both kidneys instigated vascular congestion and sickling in the lungs. These long-range adverse vascular effects of localized ischemia are accompanied by a systemic inflammatory response, and studies have implicated activation of the endothelin system as a critical pathobiologic pathway underlying this phenomenon [103]. Such long-range vascular signaling in sickle disease enables localized vascular insults to provoke adverse effects in the systemic circulation and in vascular beds far removed from the site of the original and localized lesion. Long-range signaling thus constitutes a significant pathway that extends and accentuates vascular injury [38].

In the general context of reperfusion injury, the lung is by far the most sensitive and frequently involved target organ in

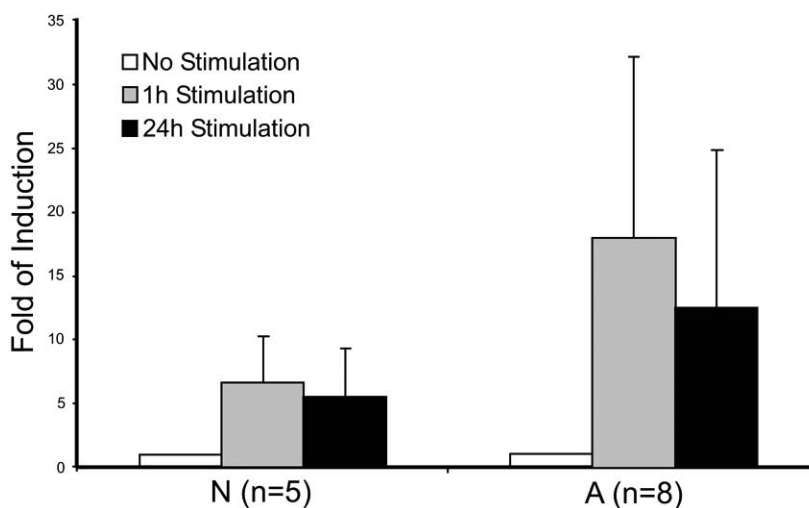


Fig. (16). Endothelial RelA response. BOEC (blood outgrowth endothelium) were stimulated *in vitro* with IL1 β and TNF α . The resulting RelA (NF κ B component p65) response of the BOEC from sickle patients that do have Circle of Willis disease by independent criteria (A, for at-risk) showed exaggerated RelA response, compared to BOEC from sickle patients without Circle of Willis disease (N, for not-at-risk). This suggests that the sickle patients who have Circle of Willis vessel disease exhibit exaggerated inflammatory responses, due to their own accumulation of genetic polymorphisms affecting gene expression. Obtained with permission from Milbauer LC, *et al.*, Genetic endothelial systems biology of sickle stroke risk. *Blood* **2008**, *111*, 3872-9.

receipt of injury from these long-range effects [38]. Indeed, we have observed endothelial TF expression to be restricted to pulmonary veins, and this pattern is reproduced by other models of systemic inflammation: LPS, TNF α , peritonitis [35].

Genetic Influences

The wealth of sub-biologies involved in the vasculopathy of sickle disease predicts that a great variety of genetic polymorphisms potentially could affect physiology and thereby impact on its vascular pathobiology.

To date, a single study has attempted to specifically assess genetically determined differences in endothelial biology as participants in regulation of clinical phenotype. To accomplish this, we developed and validated a method to obtain cultures of endothelial cells from blood outgrowth endothelial cells (BOEC) [104] and globally survey their gene expression levels by microarray [105]. We found no significant single-gene differences between sickle children with Circle of Willis disease (which comprises the strongest risk factor, and probably causal factor, of ischemic stroke in sickle children) and those without it. However, using a biological systems based analysis, we did find that the overall sub-biology of inflammation signaling is significantly activated in the with-disease subjects, with particular implication of genes proximate to NF κ B p65 [105].

This led us to hypothesize that the at-risk subjects would have exaggerated signaling responses to the inflammation that is ever-present in the sickle subject. Consistent with this, cell biologic study of the BOEC from these subjects did reveal an exaggerated RelA response to TNF/IL1 stimulation on the part of the at-risk group (Fig. 14). Follow-up analysis of other transcriptional regulators has confirmed that the

pro/anti inflammatory regulatory balance is tipped towards the pro direction in the endothelial cells in sickle cell disease (unpublished data, 2009: RP Hebbel, LC Milbauer, J Enenstein).

Genetic studies using more traditional approaches have started appearing that are relevant to the vasculopathy of sickle disease. One large SNP study of a large number of subjects (>1300) used Bayesian network analysis, and among other things identified an association of clinical stroke and SELP [106], just as it is in the general population; the importance of P selectin to the inflammatory and reperfusion injury paradigms in sickle vasculopathy was addressed above. Similarly, a small study (<100 subjects) of sickle patients with known large vessel disease in the brain and found that the TNF (-308) G/A polymorphism presents a >3- fold increased risk for large vessel disease [107]. This, of course, is consistent with the importance of inflammatory signaling. Another study observed an association between polymorphisms of KLOTHO (related to NO biology) and priapism in sickle patients [108]. And another small SNP study found evidence of association between pulmonary hypertension in sickle patients and genes in the TGF β superfamily [109]. Overall, the status of genetic association studies in sickle disease has recently been excellently summarized [110].

In our opinion, additional systems-biology informed studies will need to be done to establish the most useful picture of the role of inter-individual differences in endothelial function, which would then influence design of therapy. We believe that systems-biology based analysis will be more informative as to the genesis of vasculopathy than will identification of ever more SNP associations. However, a strong argument has been made favoring conduct of genome-wide

association studies in preference in smaller, targeted association studies that employ pre-determined gene sets [111]. We accept this argument.

PROBABLE HIERARCHY OF SUB-BIOLOGIES & THE ROLE OF A SYSTEMS BIOLOGY OUTLOOK

Given the multiple ways in which the participating sub-biologies overlap and interact, it would be exceedingly difficult, if not impossible, to actually sort out a precise hierarchy of participants in a system where they all are important and where there are multiple vicious cycles. However, logic and the principle of parsimony argue that reperfusion injury is very likely to be the proximate cause of the various participating sub-biologies leading to vasculopathy (Fig. 1). Although relatively limited data are available on these sub-biologies in the sickle literature *per se*, there is a wealth of information about each of these processes in the general biomedical literature. This can be drawn upon to assess the likely ways that the identified sub-biologies interact with one another. Considering all these data, it seems most likely that the other implicated sub-biologies develop downstream from inflammation, itself derived from reperfusion injury physiology. Thus, reperfusion injury provides a linking pathophysiological explanation for the diverse vasculopathic effects seen in sickle patients.

The schematic network diagram shown in (Fig. 1) addresses the systems biology of sickle vasculopathy at its most basic level, i.e., by recognizing participation of multiple components that are interconnected in multiple ways. From a network perspective, the components and connections would be regarded as nodes and arcs, respectively. A methodological start to addressing the complexity of such relationships has been made by Steinberg and colleagues, who used Bayesian network modeling to evaluate relationships of certain lab findings and certain clinical events to occurrence of death in sickle patients [112]. This produced promising results, although these are compromised by potential investigator bias in terms of choice of clinical events and laboratory parameters to be included in the model. Nonetheless, it may be that such approaches can be employed to assess the exceptionally complex systems biology of sickle vasculopathy, in terms of the involved biological processes themselves. Again, the present effort is merely an attempt to argue that investigators need to devise methods that recognize and approach this complexity, and to get away from the natural reductionist tendencies that most of us have, inclinations that lead to focusing on aspects of a single sub-biology rather than dealing with its complex interactions within a universe of multiple sub-biologies.

PRINCIPLES FOR CHEMO-PROPHYLAXIS

The most important principle for guiding chemoprophylaxis should be to recognize the participation of multiple sub-biologies and their highly interacting and overlapping nature (Fig. 1). Most can transactivate others, and each can be promoted by multiple factors. For this reason, a therapy designed to attack only one or two of these sub-biologies predictably will not be efficacious. Rather, multiple modalities

of action are necessary. Of course, any effective therapy must be safe, well tolerated, and have only acceptable side effects. Ideally it would be something that could be used in childhood and lifelong.

The role of endothelial heterogeneity in sickle disease is only beginning to be explored. Our studies of sickle mice have indicated that the display of adhesion molecules and of tissue factor is not the same in all vessel beds [35, 44, 75]. Hence, interpretation of experimental studies must be tempered with an understanding that results might be different if a different organ was being studied. For example, extensive studies of reperfusion injury in the general literature have revealed that the details of how this process is executed can vary somewhat from organ-to-organ [38].

Adding another layer of distressing complexity, the exact nature of ongoing pathogenesis may differ from time-to-time in given individuals, or from person-to-person. Fundamental modulators of the many participating sub-biologies can wax and wane, corresponding to the pathophysiological milieu and clinical events.

For all of these reasons, we do not believe that application of a single modality therapy (e.g., using a NO donor) makes sense. Rather, multi-modality therapy is needed. Ideally, for reasons of safety and simplicity, this should take the form of only one or two agents that display powerful multi-modality effects. The literature already identifies some candidate therapies, although in our opinion only three bear serious consideration as fulfilling the apparent need for a truly multi-modality therapy: statins, arginine, SAHA.

Hydroxyurea

Originally intended to increase HbF level and thereby lower risk for polymerization and sickling, this drug actually has additional interesting effects. It lowers leukocyte count, it reduces myeloperoxidase, it can be an NO donor [113], it diminishes the count of (highly adhesive) reticulocytes, it may lower endothelial adhesion molecule expression and red cell adhesion to endothelium, and it improves level of red cell hydration (an anti-sickling effect per Fig. 2). It already is widely used for attempted pain prophylaxis in sickle patients, both adult and children. The possibility that it affects spermatogenesis, although it is not leukemogenic has been raised [114].

NO

Nitric oxide can be inhaled, or derived from NO donor drugs [115]. It has multiple beneficial effects, including presumed repletion of NO bioavailability, and platelet and inflammatory and adhesive inhibition. However, it must be recognized that NO also can be destructive. For example, its administration could lead to formation of enhanced peroxynitrite in presence of an unimpeded inflammatory environment associated with superoxide generation. Certainly, administration of anything expected to boost eNOS activity would have to be utilized concomitantly with tetrahydrobiopterin to avoid eNOS uncoupling (probably already a problem in sickle disease).

Anti-Inflammatory Drugs

This is clearly a good idea, although by itself would presumably be suboptimal as a therapy. We previously demonstrated that sulfasalazine, a very strong NF κ B inhibitor, has endothelial-modifying effects (down-regulation of adhesion molecule expression, although not of TF expression) that argue for its use [75]. We recently identified a host of other NF κ B inhibitors that are effective in diminishing the NF κ B-dependent endothelial TF expression in the sickle mouse: lovastatin, 4H-andrographolide, isohelenin, curcumin, didox, trimidox [35].

Anti-Oxidants

An argument for this approach is strong, but is limited to only one sub-biology. We previously noted, however, that chelation of red cell membrane iron by L1 (a membrane-permeable chelator capable of scrubbing iron from membranes) improved red cell survival in a thalassemia model [116], so it likely would do so in the sickle context as well. Repletion of consumed tocopherols would make great sense, since membrane oxidative processes underlie a host of sickle red cell membrane defects [25].

Other Single-Modality Approaches

A variety of other approaches can be justified by the individual contribution of the target biology to overall pathophysiology. Examples include, anti-adhesive therapy for red cells and/or white cells, red cell hydrating strategies, increasing HbF or other anti-sickling drugs. But all such approaches have the inherent shortcoming that they predictably are too narrow in expected efficacy, in terms of the sub-biology that is targeted, to offer significant beneficial effect. The probable exception to this, however, would be a truly effective, clinically usable inhibitor of HbS polymerization (which does not exist yet).

Apo A-1 Mimetic

Drugs such as L-4F inhibit atherosclerosis in LDL-receptor null mice, and they prevent LDL-induced uncoupling of eNOS, so they may well have efficacy for sickle vasculopathy [89,117]. In fact, L-4F was observed to improve vasodilation and decrease deposits of XO on pulmonary endothelium in sickle mice. L-4F also prevents LDL from causing eNOS uncoupling. Apo A-1 levels are low in sickle patients [118], and it may comprise a biomarker for pulmonary hypertension risk in sickle patients [119].

Statins

These have already been suggested for sickle stroke prevention [44] because they diminish expression of endothelial tissue factor expression in sickle mice. Statins have a variety of salubrious vascular protective effects, wholly unrelated to lipid-lowering effects, that would be beneficial for endothelial cell function [120]. For example: they restore bioavailability of NO, derived from endothelium, *via* multiple mechanisms that include improvement of eNOS activity. They prevent the reductions in eNOS that are caused by hy-

poxia and inflammation. They increase concentration of tetrahydrobiopterin, a critical cofactor to keep NOS coupled. They diminish superoxide production, as well as production of inflammatory cytokines. That they reduce endothelin production is relevant to the participation of non-NO mechanisms of vasoregulation in sickle disease. They inhibit endothelial cell expression of selectins (E and P) and CAMs (ICAM-1, VCAM-1). On the coagulation front, they clinically reduce incidence of thromboembolism in the general population, decrease endothelial TF expression, favor fibrinolysis and increase surface thrombomodulin. Of great relevance to sickle vasculopathy chemo-prophylaxis, it was demonstrated that a stain offered significant protection against cardiovascular events (including strokes) in apparently healthy individuals (in the general population) without hyperlipidemia but with elevated levels of C-reactive protein [121] (a combination that typically is the case in human sickle patients).

Arginine

As the substrate for eNOS, administration of this has been considered for application to sickle disease [88,122]. Indeed, arginine is deficient in sickle patients, and this undoubtedly is a cause of eNOS uncoupling. Arginine supplementation of sickle mice increases blood arginine level, lowers plasma free hemoglobin (and, less convincingly, maybe diminishes hemolysis), improves NO bioavailability. As a consequence, its administration concomitantly lowers the compensatory increase of non-NO vasoregulators. Arginine also lowers lipid peroxidation, observed in the liver, and increases anti-oxidant capability *via* increasing glutathione. Most notably, it improves microvascular function. This is an attractive spectrum of likely benefits, but it seems to be a variant of NO administration, in that it largely misses the critical inflammation/reperfusion injury axis that is critical to development of sickle vasculopathy. There have not yet been human studies that employed adequate dosing.

HDAC Inhibition

Trichostatin A and suberoylanilide hydroxamic acid (SAHA) are specific inhibitors of histone deacetylases (unlike butyrate). They thus lead to histone hyperacetylation, and directly affect transcriptional regulators, thereby altering expression of multiple genes [123]. In recent studies of sickle mice [124], we observed that TSA and Zolinza® both have multiple independent effects that may be beneficial for sickle disease: inhibition of VCAM1 (anti-adhesive and anti-inflammatory), inhibition of vascular stasis; inhibition of tissue factor expression by endothelium and monocytes, iron chelation, and induction of gamma globin and HbF, an anti-sickling hemoglobin. This would seem to offer a nearly full spectrum of beneficial modalities for vasculopathy prevention. Certainly, much additional work on this agent is required.

Notably, SAHA (as Zolinza®) is already approved for human clinical use (in cutaneous T cell lymphoma) and it is actively being studied in other oncologic settings. A particu-

lar additional reason to employ Zolinza® would be that the expected reduction of red cell sickling would diminish the elevated hemolytic rate characteristic of sickle disease. This would lessen plasma free hemoglobin and diminish risk for excessive NO consumption. And it would presumably reduce the accelerated consumption of lipid by the erythron (the hypothesized mechanism for lowered lipids in the sickle patients); the latter effect would help buffer the low-lipid patient from the potential lipid lowering effect of statins. Finally, being hydroxamic acids like hydroxyurea, it is possible that TSA and SAHA are functional NO donors, *via* the same chemistry that allows derivation of NO from hydroxyurea [113]. Unfortunately, no data yet exist on the use of this drug in the sickle setting.

CONCLUSION AND THERAPEUTIC RECOMMENDATION

The pathogenesis of sickle vasculopathy is complex and certainly involves multiple sub-biologies (Fig. 1). It seems certain that inflammation triggered by reperfusion injury physiology is the proximate, ongoing and recurrent stimulus for the long-term process that leads to vessel disease in the sickle patient. For the reasons delineated above, we believe that multi-modality chemo-prophylaxis using a statin and Zolinza® might offer the best choice for this purpose in sickle patients. This, of course, needs safety, efficacy, and tolerability study, as well as pilot scale study, before it can be contemplated for clinical application. It, of course, could be that side-effects of such drugs yields an adverse risk/benefit ratio.

In terms of future studies, what are most needed are: [a] additional approaches to the systems biology of sickle pathophysiology, and [b] assembly of a data set containing repeated, longitudinal measurements of various sub-biology activation markers in single patients, so that their complex fluctuations can be properly appreciated. Most of the data reported herein were obtained as single time point measurements and are, therefore, less informative than optimal.

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