Minireview

Sickle cell vaso-occlusion: The dialectic between red cells and white cells

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Impact statement

Sickle cell disease (SCD) is a disease of abnormal blood flow, in which genetic modification of hemoglobin results in red blood cell alterations that are primarily responsible for the acute painful vaso-occlusive episodes and, eventually, organ damage that severely diminish the quality and expectancy of life of patients with the disease. Sickle red blood cells (SRBC) are significantly modified by, and contribute to, imbalanced redox physiology, accelerated erythropoiesis, and the rheological changes associated with SCD, thereby playing a fundamental role in vaso-occlusive processes. Clear contributions by chronic inflammation, pan-cellular adhesion, thromboinflammation, oxidative stress, and reduced nitric oxide bioavailability have all been established in SCD pathophysiology; moreover, anti-P-selectin therapy and other approaches that abrogate the interactions of the SRBC and other blood cells with the endothelium represent important current and future strategies for treating patients with this devastating disease.

Abstract

The pathophysiology of sickle cell anemia, a hereditary hemoglobinopathy, has fascinated clinicians and scientists alike since its description over 100 years ago. A single gene mutation in the *HBB* gene results in the production of abnormal hemoglobin (Hb) S, whose polymerization when deoxygenated alters the physiochemical properties of red blood cells, in turn triggering pan-cellular activation and pathological mechanisms that include hemolysis, vaso-occlusion, and ischemia-reperfusion to result in the varied and severe complications of the disease. Now widely regarded as an inflammatory disease, in recent years attention has included the role of leukocytes in vaso-occlusive processes in view of the part that these cells play in innate immune processes, their inherent ability to adhere to the endothelium when activated, and their sheer physical and potentially obstructive size. Here, we consider the role of sickle red blood cell populations in elucidating the importance of adhesion vis-a-vis polymerization in vaso-occlusion, review the direct adhesion of sickle red cells to the endothelium in vaso-occlusive processes, and discuss how red cell- and leukocyte-centered mechanisms are not mutually exclusive. Given the initial clinical success of crizanlizumab, a specific anti-P selectin therapy, we suggest that it is appropriate to take a holistic approach to understanding and exploring the complexity of vaso-occlusive mechanisms and the adhesive roles of the varied cell types, including endothelial cells, platelets, leukocytes, and red blood cells.

Keywords: Acute vaso-occlusive crisis, adhesion, blood flow, erythrocytes, sickle red blood cells, leukocytes

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Introduction

Sickle cell disease (SCD) is ultimately a disease of abnormal blood flow, in which genetic modification of the hemoglobin protein results in red blood cell (RBC) alterations that are primarily responsible for vaso-occlusive mechanisms resultant from cellular activation, cell-cell adhesive interactions, and inflammation. Caused by a single base-pair substitution in the *HBB* gene (encoding β globin), the sickle mutation produces abnormal sickle hemoglobin (HbS) and in homozygosity results in sickle cell anemia (SCA).[1] Compound variations of sickle cell inheritance, denominated as sickle cell disease (SCD), result from the co-inheritance of the beta S allele with other β-globin mutations. [2] Polymerization of HbS, under conditions of deoxygenation, leads to the formation of deoxy-HbS fibers in the RBC, altering the properties of these cells and leaving them less deformable and often sickle shaped.[3] The ensuing and complex pathophysiology of SCD vaso-occlusion involves multiple molecular mechanisms beyond polymerization and sickling, which we will cover in this review.

The clinical course of SCD is characterized predominantly by painful vaso-occlusive episodes that vary in frequency and severity, can often lead to hospitalization of the patient,[4] and are the feature of SCD that are the greatest concern of patients with the disease.[5] Other important and varied complications of the disease, including acute complications and end-organ damage are the result of abnormal blood flow leading to ischemia and consequent inflammatory processes.[6] Currently available treatments for SCD include hematopoietic stem cell therapy, which can be curative but is not available for all;[7] hydroxyurea therapy, which is a cytostatic drug with anti-polymerization and anti-inflammatory properties;[8] and drugs that have been developed specifically based on SCD pathophysiology but are relatively new additions to the therapeutic options for SCD, such as L-glutamine supplementation, crizanlizumab, and Voxelotor.[9]

Vaso-occlusion: What, where, and how?

Vaso-occlusion, central to the pathology of SCD, consists of the obstruction of a blood vessel such that the velocity of blood flow considerably slows or stops. In SCD, vaso-occlusive events arise predominantly in the microvasculature and are generally believed to occur mainly in the venules,[10,11] given the slower flow rate and lower oxygen concentrations in these post-capillary regions, but there is evidence that in some organs vaso-occlusion arises in arterioles, provoking the formation of "bottlenecks" and deregulation of blood flow.[12]

Initiation and propagation of vaso-occlusion

Molecular and physical triggers drive the initiation and propagation of cellular recruitment and aggregation in the vasculature. Known such stimuli in individuals with SCD include infections, hypoxia, dehydration, stress, acidosis, cold temperature, and even pain itself.[13–15] In mouse models of SCD, inflammatory molecules (such as tumor necrosis factor-α (TNF) cytokine, hemolysis products, lipopolysaccharide), agonists of endothelial and platelet protease active receptors such as thrombin, immunomodulatory epinephrine, and physiological stress (that induces autonomic nervous system reactivity[16]) have all been employed to induce and study vaso-occlusive mechanisms.[12,17–22]

Components of vaso-occlusion

The obstruction of the microvasculature in SCD is a direct result of cellular recruitment to the vascular wall and multicellular aggregate formation in the blood vessel lumen and vascular dysfunction, in conjunction with SRBC sickling and rheological changes.[23] The adhesion of blood cells, that is SRBC as well as leukocytes and platelets, to the vascular wall is the consequence of cell membrane molecular alterations and endothelial cell activation (see Table 1) that are caused by physical changes in HbS-containing RBC[24,25] and by molecular triggers generated during the inflammatory processes that characterize SCD pathophysiology.[26]

SRBC and the rheological component of vaso-occlusion

As a direct effect of their HbS content, SRBC suffer cellular dehydration, HbS autooxidation, and membrane alterations.[27] Dehydration of SRBC results in extreme elevations of intracellular concentrations of HbS and seismic increases in HbS polymerization and sickling.[28–30] In addition, pathological roles for adenosine signaling, erythrocyte hypoxic metabolic programming, and hypoxia-induced impairments in phospholipid metabolism may all participate in HbS deoxygenation and consequent SRBC sickling.[31] In turn, SRBC sickling reduces SRBC deformability, increases blood viscosity, and is an iconic effector of impaired blood rheology and therefore SCD vaso-occlusion.[32,33] Increases in blood viscosity under normal circumstances can elevate nitric oxide (NO) production by the endothelium through shear-stress dependent mechanisms.[34] However, as a result of the vascular dysfunction associated with SCD, increases in blood viscosity can instead trigger vaso-occlusive processes in individuals with SCD.[34,35] Additionally, the autonomic nervous system (ANS), a significant regulator of microvascular blood flow, may be dysfunctional in SCD, many patients demonstrating peripheral vasoconstriction without significant increases in heart rate.[36] Thus, modulation of the SRBC microvascular transit time in small vessels by rheological factors[11] can facilitate SRBC sickling and obstruction of blood flow in small vessels. Of importance, stoppage of microvasculature blood flow can also alter the adhesion of cells to the blood vessel wall.[14] For instance, all three selectins (P-, E-, and L-selectin) lack adhesivity in the absence of shear stress.[37,38]

Molecular mechanisms driving vaso-occlusion

Major molecular mechanisms that induce cellular changes and the initiation and propagation of vaso-occlusion include oxidative stress and inflammatory processes. Enhanced NADPH activity and HbS autooxidation in SRBC contribute to the increased production of reactive oxygen species (ROS) in RBC and the oxidation of membrane proteins.[39–43] Oxidative stress also has a role in vesiculation and membrane shedding[44] by SRBC, which produce large numbers of microparticles that carry the pro-inflammatory hemoglobin products that play a role in cellular activation and the initiation of vaso-occlusion.[45,46]

Accordingly, intravascular hemolysis is a major source and generator of inflammatory molecules in SCD. SRBC have a significantly reduced half-life compared to normal RBC,[23] leading to extravascular and intravascular hemolysis, the latter of which accounts for up to 30% of red cell lysis.[23,47,48] Besides making an important contribution to hemolytic anemia and accelerating erythropoiesis, intravascular hemolysis is a major source of the inflammatory signals that take part in the initiation and propagation of vaso-occlusion in SCD. Initially, oxidative reactions of the hemoglobin that is released from ruptured cells into the circulation inactivates vascular NO, thereby causing endothelial dysfunction and endothelial cell activation as a result of the anti-inflammatory properties of NO. Additionally, cell-free hemoglobin, and its released heme

Table 1. Involvement of cell populations in the vaso-occlusive process in sickle cell disease.

Cell population	Properties	Role in vaso-occlusion	Major molecular interactions	Refs
Lower density red blood cells	- Increased adhesive properties - Secondary recruitment to vascular wall mediated by leukocytes - Participation in heterocellular aggregates	- Adhesion to activated endothelium - Physical obstruction of microvessels - Augmentation of SRBC transit time	Major SRBC molecules involved in adhesion: CD36, $\alpha4\beta1$ integrin, ICAM-4, PS, P-selectin binding determinants, Lu/BCAM	235, 21, 236, 142
Higher density red blood cells	- Less deformable, sickling-prone cells	- Increased likelihood of sickling and trapping in cellular agglomerates - Reduction of blood flow	Fewer molecular interactions, but evidence of Lu/BCAM-dependent interactions	107, 108, 124
Leukocytes	- Increased leukocyte counts in SCD - Innate immune cells - Adhere to endothelium of the microvasculature, especially when activated - Large cells - Participation in heterocellular aggregates	- Potential trigger of physical obstruction of microvessels - Augmentation of SRBC transit time - Contribute to inflammatory mediator production in SCD	Major molecules involved in adhesion: Selectins and selectin ligands (PSGL-1, E-selectin ligand), $\beta2$ integrins, especially Mac-1 and LFA-1	10, 19, 237
Platelets	- Platelet activation is associated with SCD - Participation in leukocyte/platelet/RBC aggregates - Participation in innate immunity	- Platelets are recruited to the vascular wall when it is damaged - Contribute to vaso-occlusive mechanisms in microvessels by activating endothelium and leukocytes, and participating in aggregate formation - Major producer of inflammatory mediators in SCD	Major platelet molecules involved in aggregates and adhesion: P-selectin, $\alpha_{iib}\beta_3$, GPIbα, $\alpha_5\beta_1$, $\alpha_2\beta_1$, ICAM-2	85, 171, 62, 175
Endothelial cells	- Present surface adhesion molecules when activated - Participation in immune responses	- Tether and capture SRBC, leukocytes, and platelets to the microvascular wall - Contribute to inflammatory mediator production and thromboinflammation in SCD	Major endothelial surface molecules involved in cell capture: P-selectin, E-selectin, ICAM-1, VCAM-1, vWF, CD36, $\alpha_v\beta_3$, GIb-IX-V Subendothelial matrix proteins: Laminin, fibronectin, fibrinogen	235, 148, 62, 196, 238,239

ICAM: intercellular adhesion molecule; Lu/BCAM: Lutheran/Basal cell adhesion molecule; PS: phosphatidylserine; PSGL-1: P-selectin glycoprotein ligand-1; SCD: sickle cell disease; SRBC: sickle red blood cell; VCAM: vascular cell adhesion molecule; vWF: von Willebrand factor.

group are recognized as erythrocytic damage-associated molecular patterns (DAMPs) that activate innate immune responses with consequent generation of pro-inflammatory cytokine processing and oxidant reactions.[49] Heme release also plays a role in direct activation of the endothelium, mobilizing von Willebrand factor and translocating P-selectin from storage in Weibel Palade bodies to the endothelial surface, and in the expression of other adhesion molecules that promote cellular recruitment and consequent vaso-occlusion.[50,51]

SCD also generates a prothrombotic state;[52–55] platelet activation is an important consequence of hemolysis, due to the reduction in vascular NO (an inhibitor of platelet aggregation), erythrocyte-derived ADP and heme release,[56–58] and dense SRBC phosphatidylserine exposure,[59] which enhances thrombin generation.[60,61] Activated platelets readily adhere to the vascular wall, participate in heterocellular aggregate formation and contribute to the release of the pro-inflammatory milieu that drives the vaso-occlusive process,[12,53,62,63] although the overall pathophysiological relevance of platelet activation may be called to question by the consistent failure of platelet-inhibiting compounds to ameliorate pain crises.[64–70]

Finally, a major inducer of vaso-occlusive processes is vaso-occlusion itself. In a vicious cycle, constant vaso-occlusive processes in the microvasculature can lead to oxidative damage, ischemia-reperfusion injury, generation of inflammatory responses, and activation of innate immune pathways. Vaso-occlusion disrupts vascular flow, promoting tissue ischemia and, thereby, hypoxia with consequent RBC sickling, DAMP and other inflammatory molecule generation, and further endothelial activation with P-

selectin expression.[71–75] In turn, the reoxygenation of the vasculature, upon resumption of blood flow,[76] generates more damaging ROS,[76,77] which together with enhanced inflammatory pathway activation[26,78,79] generates further vaso-oclusive processes. Hypoxia itself is used as a trigger for vaso-occlusion in murine models of SCD.[75,80]

Are vaso-occlusive mechanisms the same in different tissues and organs?

It is probable that the profile of triggering and propagating mechanisms and also cellular involvement may differ depending on the organ or tissue. In the kidney, for example, vaso-occlusion may be more frequent in the renal medulla, which is more hypoxic, hyperosmotic, and acidic[81] than in the hyperperfused renal cortex,[82] although these sickling dynamics may be changed by acute kidney injury.[82] The bone marrow and the liver both have hypoxic and sinusoidal environments that promote SRBC sickling,[83,84] and cellular obstructions comprised of erythroid and myeloid cells and nucleated erythroid precursor cells have been reported in the bone marrow of SCD mice.[83] In contrast to other organs, the lung is highly oxygenated and, at least in mice with SCD, a defining feature of pulmonary vaso-occlusion may be the formation of occlusive neutrophil-platelet aggregates in the arterioles rather than in the venules.[12] The expression of the P-selectin adhesion molecule on the surface of endothelial cells and platelets appears to play a significant role in lung vaso-occlusion,[85] and may contribute to acute chest syndrome,[12,86] one of the most common causes of death in patients with SCD.

Insights from differential roles of SRBC populations in vaso-occlusion

In addition to the paradigmatic HbS polymerization and SRBC sickling considered above, other SRBC changes are important to SCD,[4,87] including abnormalities of redox, hemoglobin stability, oxygen affinity, metabolism, membrane integrity, adhesivity, and cation homeostasis. A focus on SRBC dehydration and its effects on HbS polymerization and SRBC sickling provides insights into other vaso-occlusive processes resultant from abnormal cation homeostasis.

The broad clinical heterogeneity among patients with SCD is commonly attributed to variability in the amounts of fetal hemoglobin (HbF) within SRBC,[88,89] A separate powerful determinant of SRBC pathobiology is abnormal SRBC dehydration,[30] which increases cell density, intracellular concentrations of HbS (mean cellular Hb concentration; MCHC),[28] HbS polymerization, and cell sickling.[29] Density of RBC, as measured by density gradient separation,[90] is much greater and more heterogeneous in SRBC than in normal RBC.[91,92] Those SRBC that are most dehydrated are the most dense and have the highest MCHC,[28] which decreases the deformability of even oxygenated SRBC as measured by laser scattering viscometry,[93] and increases immensely the rate of HbS polymerization in deoxygenated SRBC.[94] These relationships are essential tenets of the polymerization paradigm that presumes to account for the totality of SCD clinical features. Leading authorities have attributed all the clinical heterogeneity of SCD to differences in SRBC density because of the powerful effect that MCHC has on HbS polymerization and SRBC sickling;[95] indeed the densest SRBC are the most sickling-prone. This paradigm predicts a direct association between the number of dense SRBC and the clinical severity of SCD.[96,97]

However, attribution of the severity of all facets of disease to HbS polymerization is challenged by the indirect relationship of the severity of hemolytic anemia and vaso-occlusive crises (VOC). In a large study of the natural history of SCD, milder anemia was associated with more frequent VOC.[98] Similar associations of milder anemia and more frequent VOC were observed with coexistent α thalassemia and SCD[99,100] and in therapeutic trials of the cell-hydrating, polymerization-inhibiting drug Senicapoc,[101] which together indicated opposite effects of polymerization on the degree of hemolytic anemia and the frequency of VOC.

One astute analysis concluded that the initiation of VOC is not directly related to the major determinants of HbS polymerization, SRBC density or HbF levels.[102] A seminal report had found no correlation of the number of dense SRBC with the frequency of VOC,[99] which subsequently was supported by the finding of a paradoxical association of α thalassemia, a coinherited condition associated with fewer dense SRBC,[92] with increased VOC.[100] These observations demonstrated that SCD could not be understood by simple extrapolations from polymerization paradigms alone. A crucial discovery critical to elucidating the relevance of disparate sickle cell pathophysiologies was that low-density SRBC populations had greater adhesivity in micropipet and flow-adhesion assays compared to high-density SRBC.[103–105]

Designation of the most-dense SRBC as the most sickling-prone, least adhesive subpopulation and of the least dense SRBC as the most adhesive, least sickling-prone subpopulation provided the foundation for reinterpreting the polymerization paradigm. For example, a study in children with SCD found a positive correlation between the fraction of better hydrated, more deformable SRBC and the incidence of VOC,[27] and a study in adults with SCD found a positive correlation between incidence of VOC and the fraction of better hydrated, more deformable SRBC and a negative correlation with the percentage of more dense SRBC.[106] Additional evidence that polymerization alone did not explain VOC was provided by the discovery that patients with greater numbers of more deformable, less dense SRBC had more frequent VOC compared to those with greater numbers of less deformable, more dense SRBC.[107]

A touchstone study of different sickle cell pathophysiologies and their effects on the flow of human SRBC populations in a rat vascular flow system discovered that the most dense, sickling-prone SRBC fraction neither stuck within the vasculature nor caused stoppage of flow, that the least dense, least sickling-prone SRBC fraction stuck within vessels but did not cause stoppage of flow, and that serial infusion of the two fractions with the least dense followed by

the most dense resulted in initial adhesion of the former followed by complete stoppage of flow by the latter.[108] These findings indicated that adhesion of stickier SRBC is the initiatory step in vaso-occlusion and that physical trapping of more sickling-prone SRBC is the second step.

Crucial to these considerations are the delays that occur in SRBC deoxygenation and HbS polymerization during transit through the low oxygen environment of small blood vessels, which usually delay sickling until SRBC have entered large blood vessels where they do no harm, thereby mitigating the detrimental effects of occluded blood flow in small blood vessels.[94,109] Adhesion of the less dense, stickier SRBC prolongs their transit time as well as the transit time of nonadherant sickling-prone SRBC, resulting in polymerization and sickling in small vessels where occlusion of blood flow elicits tissue damage.

Correlations of VOC severity with adhesive properties of SRBC have established the relative importance of SRBC adhesion compared to HbS polymerization on SCD severity.

Adhesive interactions of SRBC with the endothelium

Abnormal blood flow is the cause of most of the major clinical consequences of SCD.[94,110–113] Even asymptomatic patients have impaired blood flow, and exacerbations thereof result in acute VOC.[114–116] The frequency of VOC correlates with mortality in adult patients,[98] is a major determinant of quality of life of patients with SCD,[117] and is the feature of disease about which patients have the greatest concern.[5]

Abnormal sickle cell blood flow is fundamentally related to adhesion of SRBC to the vascular endothelium. The first studies of SRBC adhesion revealed that SRBC have greater adhesion to cultured endothelial cells compared to normal RBC,[118] and subsequent studies disclosed that the adhesivity of SRBC correlated with the vaso-occlusive severity of patients with SCD.[119] Further perspective for this observation was derived from the appreciation of vaso-occlusion as a two-step process initiated by SRBC adhesion to the vascular endothelium.[108]

SRBC adhesion mediates chronic impairment of sickle cell blood flow in unperturbed experimental animals and in asymptomatic patients with SCD[21,120] and initiates acute vascular occlusion in experimental animals[21,75] and in patients with SCD.[27,99,106,107]

Numerous adhesion molecules on SRBC and endothelial cells and in the plasma, including integrins, members of the immunoglobulin superfamily, and others, participate in SRBC adhesion (Table 1).[121–123] Recently, oxidation of RBCs was found to induce post-translational modification of the Lu/BCAM adhesion molecule on the surface of dense SRBCs, signifying that this molecule could mediate adhesion of this specific RBC population to laminin on blood vessel walls, even in the absence of sickling.[124] Many of the molecules involved in the tethering of SRBC to the endothelium also mediate the molecular cascade of leukocyte-endothelial adhesion during inflammation,[125,126] which is an apt model for SRBC adhesion. That cascade is initiated by and dependent upon the endothelial cell adhesion molecule, P-selectin.[127,128] Of particular importance to the inflammatory processes is the P-selectin dependence of slow rolling adhesion of leukocytes, which is a necessary antecedent for their firm adhesion and extravasation.[129–131]

Endothelial P-selectin is critical to the adhesion of SRBC to vascular endothelium

The expression of P-selectin on platelets and endothelial cells is generally regarded as acute and transient following exposure to agonists.[131] However, in certain systems,[132] including sickle cell mouse models [133] and asymptomatic patients with SCD,[120] P-selectin is expressed chronically on the vascular endothelium. Mechanisms of chronic P-selectin expression include increased transcription[134,135] and reutilization involving invagination and recycling of the molecule onto the cell surface.[136] Chronically elevated P-selectin expression on the surface of the vascular endothelium in sickle cell mouse models has been shown to be inducible to higher levels by use of an endothelial cell agonist.[21]

P-selectin is the only selectin that has been shown to support the direct adhesion of SRBC to endothelial cells,[137] and unpublished results from the Vanguard Therapeutics, Inc. laboratory have shown that SRBC adhere *in vitro* to P-selectin but not E- or L-selectin. Under both static and flow conditions SRBC adhere abnormally to recombinant P-selectin and to cultured endothelial cells that have been activated to express P-selectin.[137,138] Most importantly, P-selectin has been demonstrated to support the initial contact and slow rolling adhesion of SRBC.[138]

The adhesion of SRBC to endothelial cells creates a positive feedback mechanism by increasing intraendothelial ROS levels,[139] which in turn induces P-selectin transcription[140] and expression of P-selectin on the endothelial cell surface.[141] P-selectin binding determinants are found in greater amounts on SRBC membranes than on normal RBC membranes, contain sialyl LewisX, exist on glycoproteins and glycolipids, and exist on both sickle-reticulocytes and older SRBC.[142] SRBC adhesion and increased intraendothelial ROS also induce transcription and expression of VCAM-1,[143,144] which may account for the elevated plasma levels in patients with SCD of soluble VCAM-1 (sVCAM-1), a marker of endothelial injury, activation, or stimulation.[145–147]

Leukocytes as participants in the vaso-occlusive process

The discovery of leukocyte adhesion in a mouse model of SCD[148] has led to an avalanche of research into this phenomenon despite unmistakable digressions from physiologic and pathophysiologic norms in this original report. These include a 1:4 capture/minute ratio of SRBC vs. WBC, failure to detect vaso-occlusion, and an elevated baseline serum TNF concentration of 15 pg/mL for this mouse model[149] even before treatment with an additional 500,000 pg. of TNF. While fascinating, this observation does

not nullify established evidence that SRBC adhere directly to the vascular endothelium in vaso-occlusion *in vivo* independently of leukocytes.[21,150–153]

While leukocytosis had long been known to be a common feature of SCD,[154] a role for leukocytes in the pathophysiology of SCD and vaso-occlusive processes was not appreciated until observations of significant associations between white cell counts with disease severity and early SCD-related death.[155–157] In addition to their role in inflammatory mechanisms, leukocytes play a substantial mechanical and obstructive role in vaso-occlusive process. *Ex vivo* assays first demonstrated the increased adhesive properties of neutrophils, which adhere abnormally to extracellular matrix components and endothelial cell layers.[158–160] Leukocytes are large (human neutrophils are about 12–15 μm in diameter) rigid cells, and stimuli that elicit their interactions with the walls of microvessels can slow blood flow, thereby increasing the RBC transit time in the vessel and promoting RBC sickling; *in vivo* studies show that the percentage of temporarily static leukocytes and the time of their stasis in a vessel correlates inversely to RBC velocity.[161] Microvascular imaging of sickle cell mice[75] detected that adherent leukocytes can capture circulating SRBC, further exacerbating vessel obstruction,[148,162] and an *in vitro* flow model of vaso-occlusion confirmed the capacity of human SCD leukocytes adherent on endothelial cell layers to capture SRBC.[163] A positive feedback is suggested by proximity of the vessel wall and activated leukocytes augmenting SRBC adhesion to the endothelium[164] and stimulated human SRBC promoting leukocyte recruitment to the vessel wall.[165,166] The molecular interactions that mediate the recruitment and adhesion of leukocytes to the vessel wall largely involve endothelial surface selectins (P-selectin and E-selectin) and ICAM-1, which bind with selectin ligands and β_2 integrins (Mac-1 and LFA-1) on the leukocyte surface (see Table 1).[19,74,167,168]

Neutrophils are the most abundant leukocytes and circulate in an activated state in patients with SCD, displaying an increased expression and functionality of the β2 integrins on their surface, due in part to endothelin B receptor mediated signaling and TNF stimulation.[169,170] In addition to their increased propensity for adhesion, neutrophils form heterocellular aggregates with platelets via interactions involving the neutrophil surface;[10,171–173] a subset of effector neutrophils that express high levels of surface CXCR4[174] and have enhanced Mac-1 integrin activity play a major role in the formation of these aggregates in SCD.[175] Neutrophils also form aggregates with SRBC, sometimes involving platelet "bridges",[171] and SCD mice that are deficient in the Mac-1 integrin have a depletion of neutrophil-RBC aggregates and reduced vaso-occlusive processes in response to trauma and cytokine stimulation.[176] The importance of heterocellular aggregate formation in SCD pathophysiology is illustrated by the aforementioned role that neutrophil-platelet aggregates appear to play in vaso-occlusive mechanisms in the pulmonary circulation of SCD mice.[12,177] A role for activated monocytes also is implicated in SCD pathophysiology,[149] as supported by reports of endothelial cell activation by SCD monocytes and the formation of aggregates between this leukocyte type and both platelets[178] and RBC.[179,180] In contrast, patrolling monocytes have actually been shown to protect against vaso-occlusive processes by scavenging endothelial-adherent SRBC.[181]

Finally, leukocytes are key to mounting innate immune responses and the generation of many of the molecules, including cytokines, chemokines, and growth factors, that participate in the inflammatory mechanisms that drive vaso-occlusive processes in sickle cell disease.[26,149] Neutrophils in particular are critical innate immunity effector cells, and the release of neutrophil extracellular traps in response to heme molecules and other inflammatory stimuli[182] may be a factor in painful vaso-occlusive episodes and acute chest syndrome.[183] Individuals with SCD also present intestinal injury and increased gut permeability that may augment intestinal barrier translocation of lipopolysaccharide,[184] a bacterial membrane component and major activator of innate immune signaling. It may be relevant that the microbiota appears to regulate neutrophil heterogeneity and aging in SCD, while the depletion of the microbiota in mice with SCD decreases vaso-occlusive events.[174]

As such, the inflammatory nature of leukocytes, their adhesion to the endothelium of the microvasculature, and their participation in SRBC adhesion and heterocellular aggregate formation appear to contribute to increasing the SRBC transit time in small vessels, thereby triggering the sickling of dense SRBC to promote vaso-occlusion.

Therapeutic approaches for reducing both red and white cell adhesive mechanisms in SCD

As mentioned, current therapeutic options for SCD are limited. Approaches for reducing/abolishing HbS production and polymerization include HSCT, gene therapies and the recently—USA Food and Drug Administration (FDA) approved Hb-affinity modulator, Voxelotor.[185–187] Herein, we discuss some of the drugs and biological agents currently in development or use for SCD in the context of the pathophysiological mechanisms described herein.

Hydroxyurea therapy

Hydroxyurea is frequently used as therapy for SCD as it reduces the incidence of hospitalization, acute pain, acute chest syndrome, and transfusion frequency, and prolongs life among other effects in patients with SCD.[188–192] This cytostatic compound acts principally by inducing the production of HbF, thereby reducing HbS polymerization.[193] However, hydroxyurea also has extensive effects on both cellular adhesivity and inflammatory pathways, due both to the downstream effects of HbS polymerization inhibition and due to its direct anti-inflammatory, myelosuppressive, and NO-dependent signaling effects.[189,194,195] Of note, hydroxyurea therapy is associated with significant reductions in the expression and activity of adhesion molecules on the surface of reticulocytes, SRBCs, leukocytes, and the endothelium in SCD.[196–201] However, hydroxyurea therapy inexplicably has been associated with an extraordinary

degree of patient noncompliance, with 85.9% of initial prescriptions not being filled in one study.[202]

Anti-adhesion therapies

We have mentioned that P-selectin expression on activated endothelial cells plays a major role in tethering rolling SRBC and leukocytes in SCD, and it also contributes to the formation of heterocellular aggregates involving platelets.[12,74,137] Crizanlizumab is a monoclonal antibody that neutralizes the activity of P-selectin. In a phase 2 trial, higher doses of crizanlizumab reduced the incidence of SCD VOC,[203] and this biological agent is now FDA-approved as Adakveo for use in patients aged 16 years or older with SCD but with the requirement of extensive post-marketing clinical testing for the presence and immunogenic effect of anti-drug antibodies (ADAb). Informal high-level comparison of median VOC frequency, time to first VOC, and time to second VOC has determined that Adakveo is at least as effective at reducing the frequency of VOC as was hydroxyurea in its original study.[203,204] The use of Adakveo requires administration by monthly intravenous infusion which is a challenge to patient compliance, is susceptible to inducing ADAb that have the potential to neutralize drug activity or cause serious reactions such as anaphylaxis, and requires ready venous access that is often unavailable in patients with SCD.

The pan-selectin antagonist, Rivipansel (GMI-1070), was developed with the aim of treating, not preventing, VOC in SCD by decreasing leukocyte interactions with the endothelium and the formation of heterocellular leukocyte aggregates. Its blocking activity is 100-fold less for P- and L-selectin than for E-selectin. The aim of treating existent VOC by blocking the initiatory vaso-occlusive molecules, selectins, is challenged by the action of numerous vaso-occlusive mechanisms by the time symptoms of VOC are detected. Phase 2 studies demonstrated that GMI-1070 improved clinical outcomes including time to resolution of pain crisis, opioid use and length of hospital stay.[205–207] However, a phase 3 clinical trial (NCT02187003) that further assessed the effectiveness and safety of Rivipansel for the treatment of pain crisis in hospitalized SCD patients failed to achieve proposed study outcomes, namely reductions in the time patients spent in hospital or opioid use for pain management. A subsequent retrospective analysis revealed that patients who received the agent earliest in their VOC may have had effective reductions in duration of therapy,[208] which has rekindled interest in its use for treating sickle cell VOC.

Modified heparins have anti-adhesive properties that show potential for use in SCD therapy. Low molecular weight heparins (LMWHs) can inhibit collagen-induced platelet activation[209] and the adhesion of SRBC to endothelial VCAM-1 by blocking erythrocyte VLA-4 integrin function and by blocking P-selectin mediated adhesion of SRBC.[120,210,211] Similarly, sevuparin a modified heparin, which retains heparin's anti-adhesive properties while decreasing its anticoagulant activity, also inhibits the adhesion of human SRBC to endothelial cell layers and prevents vaso-occlusion in a mouse model of SCD.[212] The efficacy and safety of this molecule was recently evaluated in a phase 2 study for the treatment of acute VOC in subjects with SCD (NCT02515838), although to our knowledge results have not yet been published. Other anti-cell adhesion approaches that have been evaluated in SCD include single dose intravenous immunoglobulin, and poloxamer 188.[205,213–215]

Anti-inflammatory therapies

By reducing the inflammatory milieu capable of cellular activation, anti-inflammatory drugs may be important for abrogating the adhesive interactions of SRBC, leukocytes, and platelets with each other and the endothelium. A vast number of anti-inflammatory approaches are currently in pre-clinical and clinical development for SCD. Blocking the action of the sentinel cytokine, TNF, could be particularly useful for reducing endothelial activation and therefore the initiation and propagation of vaso-occlusion in SCD. In SCD mice, etanercept, a fusion protein that binds to and inhibits the action of TNF, ameliorated endothelial activation, blood biomarkers of inflammation, and hypoxia/reoxygenation-triggered vaso-occlusive processes, among other beneficial effects.[149]

Neutralization of cell-free hemoglobin and heme may counteract the effects of hemolysis; infusions of haptoglobin or hemopexin have successfully prevented inflammatory mechanisms, vaso-occlusion, and acute chest syndrome onset in SCD mouse models.[18,216] CSL889, a plasma-derived hemopexin therapy, has recently received FDA and European Commission designation as an orphan drug and a phase 1 clinical trial of CSL889 hemopexin infusion therapy is currently underway (NCT04285827).

Preclinical studies have shown that the amplification of NO-cyclic guanosine monophosphate (cGMP)-dependent signaling using cGMP-modulating drugs, in combination with hydroxyurea, or not, can abrogate endothelial activation, leukocyte recruitment and leukocyte-RBC interactions in mice with SCD.[195,217,218] However, a recent phase 2 clinical trial in sickle cell anemia for olinciguat, a stimulator of soluble guanylate cyclase (sGC; the enzyme that catalyzes cGMP production), was discontinued for failure to achieve study endpoints (NCT03285178).[219] Inhibitors of phosphodiesterase 9 (PDE9; an enzyme that specifically degrades intracellular cGMP and is highly expressed in hematopoietic cells)[220] have completed or are in phase 1/2 clinical trials for use in SCD individuals (NCT02114203; NCT04474314); IMR-687 is said to lack the potential side effects of other PDE9 inhibitors because it does not cross the blood brain barrier and it also increases HbF production in erythroid cells.[221,222] Other anti-inflammatory drugs have been recently reviewed.[26]

Antioxidants

Control of the intracellular oxidative stress generated in SCD may diminish membrane instability[223] and cellular activation[49] and consequently vaso-occlusive processes. L-glutamine supplementation was found to ameliorate the redox potential of SRBC[224,225] and was approved by the FDA in 2017 for use in treating SCD in the form of

Endari L-glutamine oral powder following findings of a randomized, double-blind, placebo-controlled, multicenter clinical trial that showed that twice-daily administration of this amino acid reduced the frequency of sickle cell crises and hospitalization;[226] however, hurdles in the initiation and adherence to L-glutamine have been reported.[227]

NADPH oxidase repression may also represent potential for the reduction of SRBC oxidative stress. Manganese porphyrins are low-molecular-weight synthetic nonpeptides that are commonly known as superoxide dismutase mimics; the administration of a single dose of these redox-active manganese porphyrins to humanized SCD mice, following the establishment of vaso-occlusive processes, was found to reverse and reduce the adhesion of SRBCs and leukocytes to venules, thus restoring blood flow and increasing the survival rate of mice.[228]

Finally, small molecule activators of pyruvate kinase-R in SRBC have been found to increase ATP levels and decrease 2,3-DPG levels in RBC,[229] which is predicted to retard SRBC sickling and to mitigate levels of anemia in patients with SCD. In early clinical studies, FT-4202 reduced anemia in 86% of patients and Mitapivat improved anemia in 55%.[230,231]

Final considerations

Recent decades have witnessed an explosion of evidence of the pathophysiologies of vaso-occlusive mechanisms in SCD, with the recognition of the fundamental roles for chronic inflammation, endothelial and leukocyte stimulation, activation of platelets and coagulation, and alterations in NO bioavailabilty. The critical role of SRBC, which are more affected by imbalanced redox physiology, accelerated erythropoiesis, and rheological modifications than by inflammatory pathways is essential to vaso-occlusive processes. At the end of the day, similar inflammatory processes and cellular activation are observed in other pathologies that display vascular inflammation,[232] but the overwhelming pathological microvascular obstruction seen in SCD also requires participation of the altered SRBC and sickling. We recognize that, in what is commonly referred to as the vicious cycle of SCD pathophysiology, inflammatory triggers generated by SRBC destruction and ischemia-reperfusion injury appear to be largely responsible for fueling the cellular alterations that result in the adhesion of both less dense SRBC and activated leukocytes to the adhesion molecule-presenting endothelium and heterocellular aggregate formation in microvessels. The ensuing mechanical obstruction of the vessel alters blood rheology, increases the SRBC transit time, and incurs trapping of the dense, less deformable SRBC population that are prone to sickling and, vaso-occlusion (Figure 1). We suggest that future studies could focus on further understanding how this mechanism adapts in the microvasculature of different organs with different characteristics and in response to different molecular and mechanical stimuli.

Drugs aiming to prevent vaso-occlusive processes in SCD, other than by inhibiting the primary HbS polymerization event, should aim to abrogate leukocyte and SRBC adhesion to the activated endothelial surface with a view to preventing the slowing of blood flow in small vessels and dense SRBC trapping. Indeed, the biological agent crizanlizumab inhibits the activity of P-selectin on activated endothelium and platelets, thereby potentially reducing the adhesion of SRBC and leukocytes to the vessel walls and the formation of heterocellular aggregates,[21,85,233] and has displayed translational success to the clinic. However, it may be that future approaches should ensure not just the prevention of cellular recruitment to the endothelium and cell–cell interactions to prevent VOC, but also the reversal of these interactions to provide badly needed approaches for the treatment of acute VOC. Additionally,

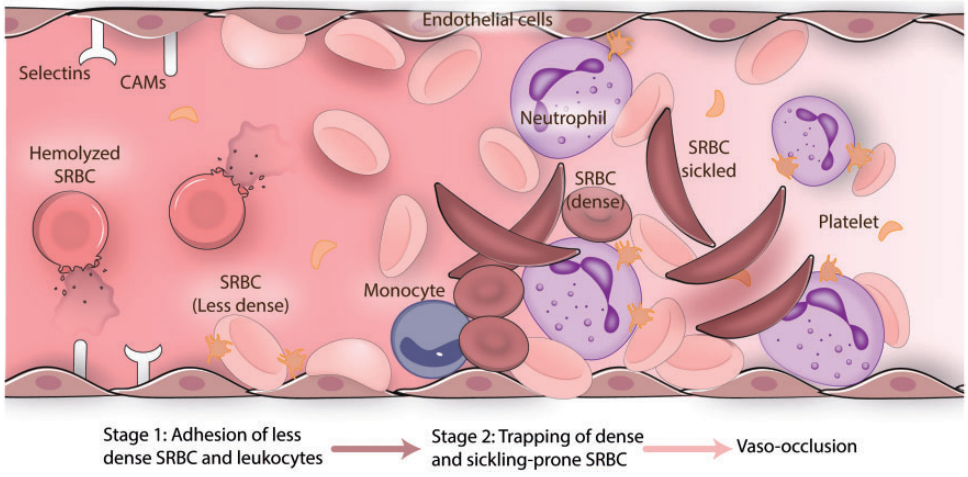

Figure 1. Proposed two-step mechanism for sickle red blood cell involvement in vaso-occlusive processes. Inflammatory mechanisms, caused by intravascular hemolysis and processes of ischemia-reperfusion, among other factors, lead to endothelial cell, leukocyte and platelet activation. Activated endothelium presents multiple adhesion molecules on its surface, including P-selectin, E-selectin and ICAM-1, which mediate cellular tethering to the vascular wall. Less-dense, deformable, less sickling-prone and more adhesive sickle red blood cells (SRBCs) are recruited to activated endothelium, as are activated leukocytes, especially neutrophils. In the microvasculature, especially venules, the mechanical obstruction of the vessel by the adhered SRBCs, and adhered leukocytes, increases the transit time of other SRBCs in the vessel, leading to the trapping of the denser sickling-prone SRBC population, as well as heterocellular aggregates, in these cellular agglomerates. Extensive cellular trapping associated with rheological alterations may result in local SRBC sickling, blood flow arrest and, therefore, vaso-occlusion. (A color version of this figure is available in the online journal.)

the chronic organ damage that is observed in individuals with SCD[234] as they age, is an increasing concern and reducing both vaso-occlusive mechanisms and associated damaging inflammatory processes may be necessary for complete therapy of this population. Such approaches may potentially combine anti-inflammatory approaches with anti-adhesive approaches, or even approaches that target other cellular and adhesive interactions.

AUTHORS' CONTRIBUTIONS

NC and SE wrote the article and have read and approved the final article.

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