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Br J Haematol. Author manuscript; available in PMC 2014 January 02.

Published in final edited form as:
Br J Haematol. 2013 July ; 162(1): . doi:10.1111/bjh.12336.

Interplay between coagulation and vascular inflammation in sickle cell disease

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Abstract

Sickle cell disease is the most common inherited hematologic disorder that leads to the irreversible damage of multiple organs. Although sickling of red blood cells and vaso-occlusion are central to the pathophysiology of sickle cell disease the importance of hemolytic anemia and vasculopathy has been recently recognized. Hypercoagulation state is another prominent feature of sickle cell disease and is mediated by activation of both intrinsic and extrinsic coagulation pathways. Growing evidence demonstrates that coagulation may not only contribute to the thrombotic complications, but also to vascular inflammation associated with this disease. This article summarizes the role of vascular inflammation and coagulation activation, discusses potential mechanisms responsible for activation of coagulation and reviews recent data demonstrating the crosstalk between coagulation and vascular inflammation in sickle cell disease.

Keywords

sickle cell; hemolysis; coagulation; tissue factor; inflammation

Introduction

Sickle cell anemia is the most common and severe variant of sickle cell disease. It is a hematologic disorder caused by a single nucleotide mutation that substitutes glutamic acid with valine at the sixth position of the β-globin gene (Frenette and Atweh 2007, Hebbel, *et al* 2009, Rees, *et al* 2010). Homozygosity for this mutation leads to abnormal polymerization of hemoglobin tetramers within erythrocytes under hypoxic conditions. Aggregation of abnormally large hemoglobin polymers results in the formation of sickled red blood cells that are less flexible, adhere to the endothelium and are prone to hemolysis (Frenette and Atweh 2007, Hebbel, *et al* 2009, Rees, *et al* 2010). Clinical manifestations of sickle cell disease include recurrent painful crises, chronic hemolytic anemia, acute chest syndrome, pulmonary hypertension, stroke, kidney failure, priapism, leg ulcers, osteonecrosis and cardiac disease (Frenette and Atweh 2007, Hebbel, *et al* 2009, Rees, *et al* 2010). Sickling of red blood cells results in the two primary pathologic events in sickle cell disease: vaso-occlusion mediated ischemia-reperfusion injury and hemolytic anemia. Both of these are thought to lead to increased vascular inflammation and activation of coagulation (Frenette and Atweh 2007, Hebbel, *et al* 2009, Rees, *et al* 2010). The interactions between vascular inflammation and coagulation and the contribution of coagulation to the pathology of sickle cell disease are the primary focus of this review.

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In order to study the complex disease pathology, mouse models of SCD were developed. The first generation of mouse models of SCD were created by homozygous deletion of mouse β-globin and addition of the human sickle β-globin (β^S) gene; these HbS mice develop mild to moderate anemia with almost none of the clinical manifestations of sickle cell disease due to the lack of human α-globin and low β^S expression (Beuzard 2008). To address this issue, newer mouse models have been developed in which mouse α-globin and β-globin genes are deleted. These and other mouse models are described in detail in a recent review (Beuzard 2008).The Berkley (BERK) model are homozygous knockouts for α-globin and β-globin, and express a transgene containing human α-globin and β^S-globin, as well as $^G\gamma$- and $^A\gamma$-globin to prevent erythrocyte sickling during gestation and fetal death (Paszty, *et al* 1997). The Townes mouse model of SCD also involves homozygous deletion of mouse α-globin and β-globin gene, and knock-in of a transgene containing human α-globin, $^A\gamma$-globin, and either β^S-globin or healthy β^A-globin (Wu, *et al* 2006). Embryonic lethality is prevented because mice express fetal Hb (HbF), which persists until approximately one month of age when the switch to HbS is complete. The advantage to these mice is that healthy AA mice can be generated as littermate controls with SS mice, by breeding heterozygous β^A/β^S pairs. The BERK and Townes models of SCD have severe anemia, leukocytosis and multi-organ damage consistent with the human pathology (Paszty, *et al* 1997, Wu, *et al* 2006).

The pathology of sickle cell disease

Vaso-occlusion and ischemia reperfusion injury

The primary pathological event in sickle cell disease is the sickling of red blood cells, which adhere to both the vascular endothelium and white blood cells (Frenette and Atweh 2007, Rees, *et al* 2010). Healthy mature red blood cells have low to nonexistent expression of adhesion molecules on their surface. However, anemia stimulates the bone marrow to release immature red blood cells (reticulocytes). These reticulocytes express high levels of adhesion molecules such as α4β1 integrin and CD36, which facilitate interactions with adhesion molecules on endothelial cells and leukocytes (Joneckis, *et al* 1993). It has been reported that sickle red blood cells adhere to vascular cell adhesion molecule (VCAM) on endothelial cells via interaction with α4β1 integrin (Ataga, *et al* 2008, Gee and Platt 1995).

The vascular endothelium is also activated in sickle cell disease, and its role in the pathology of this disease has been extensively reviewed (Hebbel, *et al* 2004). There is up-regulation of adhesion molecules, such as VCAM, P-selectin, and E-selectin, which bind adhesion molecules on red blood cells and on polymorphonuclear lymphocytes (Hebbel, *et al* 2004). Biomarkers of endothelial activation, such as soluble (s)VCAM, sE-selectin, and sP-selectin are elevated in patients with sickle cell anemia compared to controls (Ataga, *et al* 2008, Setty, *et al* 2012). Endothelial P-selectin interacts with P-selectin glycoprotein ligand 1 (PSGL-1) expressed on almost all hematopoietic cells (Luo, *et al* 2012), and also contributes to the interaction of sickle red blood cells with the endothelium (Gutsaeva, *et al* 2011). Luo and colleagues recently demonstrated that inhibition of PSGL-1 in sickle cell mice reduced leukocyte adhesion and rolling, and markers of endothelial activation. PSGL-1 inhibition also reduced inflammation and liver injury (Luo, *et al* 2012). Endothelial E-selectin interacts with E-selectin ligand 1 (ESL-1) on activated leukocytes (Mohan, *et al* 2005) in sickle cell patients, as described below. Activated PMNs can bind to red blood cells via interactions between CD11b/CD18 with various receptors on red blood cells (Hofstra, *et al* 1996). Indeed, red blood cells isolated from sickle cell patients are reported to adhere to PMNs and activate the respiratory burst; dense red blood cells that had undergone repeated sickling events were more adherent (Hofstra, *et al* 1996). Adherence was due in part to interactions between integrins on red cells and PMNs (Hofstra, *et al* 1996). In the humanized Berkley (BERK) mouse model of sickle cell disease, intravital microscopy revealed enhanced

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leukocyte rolling in sickle cell mice compared to controls after an inflammatory stimulus. Interestingly, circulating sickle red blood cells adhered more frequently to leukocytes than the endothelium (Turhan, *et al* 2002). Inhibition of leukocyte adhesion to the endothelium, through the use of E-selectin and P-selectin knockout mice transplanted with sickle bone marrow also reduced sickle red blood cell adhesion (Turhan, *et al* 2002). Furthermore, the frequency of red blood cell-PMN interactions is highly elevated and blood flow in the cremaster microcirculation is decreased in sickle cell mice compared to control (Hidalgo, *et al* 2009). In E-selectin knockout mice that were transplanted with sickle cell bone marrow, the frequency of red blood cell-PMN interactions decreased and blood flow in the microcirculation was improved, suggesting that signaling from E-selectin to PMNs contributes to red blood cell-PMN interactions (Hidalgo, *et al* 2009). Indeed, E-selectin was demonstrated to promote increased expression and activity of CD11b/CD18 integrin on PMNs from sickle cell mice (Hidalgo, *et al* 2009). The complex interactions between sickle RBCs, leukocytes and endothelial cells involve multiple adhesion molecules (Figure 1). In essence, these interactions result in recurrent vaso-occlusive events in postcapillary venules that lead to cyclic ischemia-reperfusion injury in multiple organs (Frenette 2002, Hebbel, *et al* 2009, Rees, *et al* 2010).

Ischemia-reperfusion injury activates xanthine oxidase leading to the production of free radicals (O_2^- and H_2O_2), which incite oxidative stress in endothelial cells (Carden and Granger 2000). Ischemia-reperfusion injury also enhances the expression of adhesion molecules, such as VCAM, intercellular adhesion molecule (ICAM), P-selectin, and E-selectin on endothelial cells. Moreover, oxidative stress stimulates the release of platelet activating factor (PAF) and leukotriene B4 (LTB4) from endothelial cells and these in turn stimulate expression of CD11b/CD18 and CD62L (L-selectin) on PMNs (Carden and Granger 2000). Finally, activated PMNs and monocytes also contribute to oxidative stress in sickle cell disease by generating reactive oxygen species during the respiratory burst (Carden and Granger 2000, Hofstra, *et al* 1996).

Vascular inflammation

Patients with sickle cell anemia have chronic leukocytosis, particularly an increase in monocytes and polymorphonuclear neutrophils (PMNs) (Qari, *et al* 2012, Vichinsky, *et al* 1997). Monocytes from sickle cell patients are highly activated, and express more interleukin (IL)-1β and tumor necrosis factor (TNF)-α compared to monocytes from healthy control subjects (Belcher, *et al* 2000, Wun, *et al* 2002). Interestingly, monocytes from sickle cell patients can induce E-selectin, ICAM and VCAM expression on human endothelial cells in culture (Belcher, *et al* 2000), indicating that endothelial cell-monocyte interactions are highly interdependent. PMNs from sickle cell patients are also activated compared to control subjects, marked by decreased surface CD62L expression and shedding of CD62L into the plasma (Lard, *et al* 1999). Plasma levels of lactoferrin and elastase are also higher in sickle cell patients, indicating degranulation of specific and azurophilic granules in PMNs, respectively (Lard, *et al* 1999).

In addition to an activated endothelium and leukocytosis, the inflammatory phenotype of sickle cell disease is also characterized by high levels of acute phase proteins and cytokines. Sickle cell patients have higher levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, interferon (IFN)-γ, C-reactive protein (CRP), macrophage inflammatory protein (MIP)-1α and monocyte chemotactic protein (MCP)-1 than healthy controls (Pathare, *et al* 2004, Qari, *et al* 2012). These inflammatory markers are chronically elevated in sickle patients at "steady state", and are often increased further after acute painful crises (Pathare, *et al* 2004).

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Mouse models of sickle disease share this inflammatory phenotype. Belcher and colleagues (Belcher, *et al* 2003) reported elevated levels of various inflammatory markers in four transgenic mouse models of sickle cell disease (NY-S, Berkley-$S^{Antilles}$, NY-S/$S^{Antilles}$, and Berkley- mice). All strains of sickle mice had leukocytosis, predominantly of neutrophils, monocytes and lymphocytes. Furthermore, plasma levels of serum amyloid protein (SAP, the murine analog of human CRP) and IL-6 were elevated. Additionally, there was an enhanced expression of VCAM-1, ICAM-1 and platelet endothelial cell adhesion molecule (PECAM) in the lungs of sickle mice compared to non-sickle controls. The expression of these adhesion molecules was dependent on the transcription factor NF-κB (Belcher, *et al* 2003). We have recently confirmed these data in the Berkley mice and showed a similar pro-inflammatory phenotype in the Townes mouse model of sickle cell disease. In addition, an enhanced accumulation of neutrophils in the lung and liver, and increased expression of the chemokines MCP-1 and KC was observed in sickle cell mice in both models (Chantrathammachart, *et al* 2012a).

Hemolytic anemia and NO depletion

Other important components in sickle cell disease pathology are hemolytic anemia and decreased nitric oxide (NO) bioavailability (Kato, *et al* 2007). Intravascular hemolysis results in release of cell-free hemoglobin at a rate of up to 30 g per day, which then liberates heme into the plasma. Plasma heme levels average 4.3 ± 1.1 μM in sickle cell patients, whereas it is undetectable in healthy controls (Reiter, *et al* 2002). This excess amount of hemoglobin and heme saturate their respective scavenging molecules, haptoglobin and hemopexin (Reiter, *et al* 2002), the levels of which are decreased in sickle cell patients (Muller-Eberhard, *et al* 1968). Hemoglobin and free heme consume NO to generate methemoglobin and nitrate (Kato, *et al* 2007). The production of NO is also depleted in sickle cell disease; sickle red blood cells release arginase, which degrades L-arginine, the substrate for NO production by endothelial nitric oxide synthase (eNOS) (Kato, *et al* 2007, Morris, *et al* 2005). Reactive oxygen species react with NO to produce peroxynitrite (ONOO −) which nitrosylates various proteins causing their dysfunction (Kato, *et al* 2007). The loss of NO in sickle cell disease impairs its homeostatic functions, such as vasodilation and inhibition of platelet activation. Furthermore, NO plays an important role in regulating endothelial cell activation and cytokine production (Reiter, *et al* 2002), both of which are major contributors to the pathogenesis of sickle cell disease.

Free heme liberated from hemoglobin during hemolysis is also pro-inflammatory by directly activating endothelial cells and macrophages. Heme induced TNF-α and keratinocyte chemoattractant (KC) expression in macrophages depends on toll-like receptor 4 (TLR4) signaling (Wagener, *et al* 2001). TLR-4 signaling has also been implicated in heme-mediated acute lung injury in a sickle cell mouse model (Ghosh, *et al* 2011). Heme also incites oxidative stress and heme oxygenase (HO)-1 expression in macrophages (Figueiredo, *et al* 2007). Further, free heme can also contribute to vascular inflammation via endothelial cell dysfunction and activation. Heme generates intracellular ROS and increased expression of VCAM, ICAM and E-selectin on endothelial cells *in vitro* (Wagener, *et al* 1997). Injection of heme also increases ICAM-1 expression in livers of mice, and leukocyte migration into various tissues. In addition to upregulation of adhesion molecules and chemokine production, heme can stimulate PMN migration by directly activating chemokine receptors *in vitro* and *in vivo (Porto*, *et al 2007*). Furthermore, free heme activates respiratory burst in PMNs (Porto, *et al* 2007).

Hypercoagulation and thrombosis in sickle cell disease

The coagulation cascade can be divided into the extrinsic, intrinsic and common pathways (Mackman 2012, Pawlinski, *et al* 2004). The extrinsic pathway consists of the

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transmembrane receptor tissue factor (TF) and plasma factor VII/VIIa (FVII/FVIIa). TF binds FVII/FVIIa and forms a TF:FVIIa complex that activates FX to FXa (Mackman 2012, Pawlinski, *et al* 2004). The intrinsic pathway provides an alternative way to activate coagulation via FXIIa and FXIa. It also amplifies the generation of FXa via the FVIIIa/FIXa intrinsic tenase complex. Coagulation activation results in thrombin generation, fibrin deposition and activation of platelets (Mackman 2012, Pawlinski, *et al* 2004).

Activation of coagulation in sickle cell disease has been well documented (Ataga 2009, Ataga and Key 2007, De Franceschi, *et al* 2011). Excessive thrombin generation in sickle cell patients is indicated by increased plasma levels of prothrombin fragment 1.2 (F1.2) and thrombin anti-thrombin (TAT) complexes (Ataga, *et al* 2008, Peters, *et al* 1994, Stuart and Setty 2001, van Beers, *et al* 2008). Higher thrombin peak height, higher rates of thrombin formation, and higher endogenous thrombin potential has been reported in platelet-poor plasma of sickle cell patients compared to age-matched controls using calibrated automated thrombogram method of measuring thrombin generation (Noubouossie, *et al* 2011). In addition, increased plasma levels of D-dimers, fibrinopeptide A, fibrin-fibrinogen peptide E and plasmin-antiplasmin complexes indicate ongoing thrombin-dependent fibrinogen cleavage, clot formation and subsequent fibrinolysis (Adam, *et al* 2009, Ataga, *et al* 2008, Leslie, *et al* 1975, Tomer, *et al* 2001a, Tomer, *et al* 2001b, van Beers, *et al* 2008, Westerman, *et al* 1999). Moreover, the levels of protein C and protein S are decreased in patients with sickle cell disease, presumably due to increased consumption of these natural anticoagulants (el-Hazmi, *et al* 1993, Francis 1988, Green and Scott 1986, Tam 1997, Westerman, *et al* 1999). In contrast, plasma levels of tissue factor pathway inhibitor, a natural inhibitor of TF, were not changed in sickle cell patients (Key, *et al* 1998). Finally, patients with sickle cell disease have a propensity to develop thrombotic complications. In situ thrombosis of small pulmonary vessels is a common finding during autopsy studies (Adedeji, *et al* 2001, Kirkpatrick and Haynes 1994, Oppenheimer and Esterly 1971). The incidence of inpatient pulmonary embolism is higher in the sickle cell disease population than in the non- sickle cell disease population (Novelli, *et al* 2012). It has been shown that sickle cell patients have a higher prevalence of pulmonary thrombosis but not deep vein thrombosis compared to age- and race-matched controls, suggesting that pulmonary thrombosis occurs as a primary rather than secondary embolic event in this disease (Stein, *et al* 2006). However, a recent cross-sectional study demonstrated that sickle cell patients had equally high rates of both deep venous thrombosis and pulmonary embolism. Importantly, non catheter-related venous thromboembolism was an independent risk factor for death in this cohort of patients. Mekontso Dessap and coworkers demonstrated pulmonary artery thrombosis in a sickle cell patient during acute chest syndrome (Mekontso Dessap, *et al* 2011). Sickle cell disease is also a significant risk factor for pregnancy-related venous thromboembolism (James, *et al* 2006). Furthermore, thrombosis has been reported within the vena cava and hepatic vein of sickle cell patients (Ng and Ashari 2003, Singh, *et al* 2010). Clinical evidence also points to the role of thrombosis in triggering stroke (Hillery and Panepinto 2004, Prengler, *et al* 2002). Ischemic stroke commonly occurs in sickle cell patients, with a risk of 0.5 – 1% per year (Adams 2007); strokes are often precipitated by painful crises, when markers of coagulation and inflammation are elevated (Prengler, *et al* 2002). Consistent with these clinical observations, we and others have shown that plasma levels of thrombin-antithrombin complexes are also increased in mouse models of sickle cell disease (Chantrathammachart, *et al* 2012a, Guo, *et al* 2008). Furthermore, microthrombi and increased fibrin deposition were observed in multiple organs in sickle mice including lung, liver, kidneys and brain (Guo, *et al* 2008, Trudel, *et al* 1994). Exposing sickle cell mice to hypoxic conditions resulted in further increases in plasma TAT levels and thrombosis within the pulmonary vasculature (de Franceschi, *et al* 2003, Guo, *et al* 2008). Interestingly, it was recently reported that biomarkers of coagulation activation (TAT and D-dimer) correlated with a history of stroke, retinopathy and acute chest syndrome in a cohort of 52 sickle cell

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disease patients (Ataga, *et al* 2012), indicating that coagulation might contribute to these pathologies.

Activation of coagulation in sickle cell disease

The hypercoagulable state and thrombotic complications of sickle cell disease are well documented (Ataga and Key 2007, De Franceschi, *et al* 2011, Singer and Ataga 2008), but the stimulus for coagulation activation is yet to be determined. Potential causes of coagulation activation in sickle cell disease are discussed below.

Increased TF expression

TF is the primary activator of the extrinsic coagulation pathway (Bach 1988, Edgington, *et al* 1991). It is constitutively expressed by perivascular cells such as vascular smooth muscle cells, adventitial fibroblasts, and pericytes. TF expressed by these cells is normally sequestered from blood, and it forms a hemostatic envelope to reduce blood loss upon vascular injury (Drake, *et al* 1989, Fleck, *et al* 1990, Mackman, *et al* 2007). In addition, inducible TF expression is observed in vascular cells during many pathologic conditions, including sickle cell disease (Mackman, *et al* 2007).

Sickle cell patients have increased TF expression on monocytes (Setty, *et al* 2012) with elevated whole blood TF procoagulant activity (Key, *et al* 1998). Furthermore, circulating endothelial cells isolated from sickle cell patients showed increased levels of TF mRNA, antigen, and activity (Solovey, *et al* 1998). TF expression in whole blood and circulating endothelial cells was similarly increased in patients in pain crisis and those with steady-state disease (Key, *et al* 1998, Solovey, *et al* 1998). TF expression was also increased in circulating monocytes and in the endothelium of the lung microvasculature in mouse models of sickle cell disease (Solovey, *et al* 2004). Hypoxia/reoxygenation challenge further increases TF staining in both cell types (Solovey, *et al* 2004). We have recently shown that TF expression was also increased in neutrophils isolated from sickle cell mice (Chantrathammachart, *et al* 2012a). It has been proposed that the interaction of TF-positive neutrophils with endothelial cells is a critical step for initiating thrombosis in the mouse cremaster arterioles in a laser injury model (Darbousset, *et al* 2012). Perivascular cells can also be a source of TF contributing to the activation of coagulation as shown in a mouse model of endotoxemia (Pawlinski, *et al* 2010). Endothelial cell injury and increased vascular permeability observed in sickle cell disease can make perivascular TF accessible to circulating clotting factors and activate coagulation (Ghosh, *et al* 2012, Polanowska-Grabowska, *et al* 2010, Wallace, *et al* 2009). Importantly, we have demonstrated that inhibition of TF in sickle mice (Berkley and Townes mice) reduces plasma levels of TAT to the baseline levels observed in control mice, indicating a critical role of TF in activation of coagulation (Chantrathammachart, *et al* 2012a). The relative contribution of different cellular sources of TF to the activation of coagulation is currently being investigated in our laboratory.

Sickle Red Blood Cells and Activation of Coagulation

In normal red blood cells, procoagulant anionic phospholipids, such as phosphatidylserine (PS), are almost completely restricted to the inner leaflet of the membrane (Connor and Schroit 1991). Repeated cycles of sickling result in increased exposure of PS on the surface of sickle red blood cells (Franck, *et al* 1985). PS exposure increases procoagulant activity by providing a negatively charged surface which facilitates assembly of the clotting cascade components. This process is mediated via an electrostatic interaction between negatively charged PS and positively charged γ-carboxyglutamic acid domains present in the clotting proteins, including FVII, FIX, FX and prothrombin (Owens and Mackman 2011). Analysis

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of blood from sickle cell patients demonstrated a positive correlation between PS positive red blood cells and prothrombin fragment 1.2, D-dimers, and plasmin-antiplasmin complex (Setty, *et al* 2000, Setty, *et al* 2001). Interestingly, no positive correlation has been found between PS positive platelets and these markers of coagulation activation, which suggests that sickle red blood cells, rather than platelets, contribute to the hypercoagulable state in sickle cell disease (Setty, *et al* 2001).

Circulating Microparticles

Microparticles (MPs) are small membrane vesicles released from activated or apoptotic cells. The procoagulant properties of MPs are due to the presence of PS and TF on their surface (Owens and Mackman 2011). Two recent papers demonstrated that MPs derived from different cellular sources contribute to thrombin and fibrin generation via different mechanisms (Aleman, *et al* 2011, Van Der Meijden, *et al* 2012). Platelet- and erythrocyte-derived MPs, which are TF negative, failed to induce thrombin generation in FXII-deficient plasma, and inhibition of FVII had no effect on thrombin generation (Van Der Meijden, *et al* 2012). The majority of circulating MPs in sickle cell patients originate from erythrocytes and platelets (Allan, *et al* 1982, Wun, *et al* 1998). These MPs are TF negative and demonstrate increased exposure of PS on their surface (Shet, *et al* 2003). Consistent with the observation made by Van Der Meijden and coworkers, it has been shown that erythrocyte-derived MPs isolated from the blood of sickle cell patients contribute to thrombin generation via a FXI dependent but not FVII dependent mechanism (van Beers, *et al* 2009). In contrast, thrombin generation induced by TF-positive monocyte-derived MPs requires FVII but not FXII (Van Der Meijden, *et al* 2012). A recent study demonstrated that thrombospondin-1 mediated release of erythrocyte MPs facilitates endothelial cell injury and vaso-occlusion in sickle cell mice (Camus, *et al* 2012). It has been shown that the blood of sickle cell patients does contain a small fraction of monocyte- and endothelial cell-derived MPs, which have been identified as TF positive (Shet, *et al* 2003). In contrast to this observation, a recent study reported no evidence for the presence of TF positive MPs in sickle cell patients (van Beers, *et al* 2009). Furthermore, Setty and coworkers demonstrated that MP-associated TF procoagulant activity was not elevated in the plasma of young children with sickle cell disease compared to age-matched controls, and MP-associated TF procoagulant activity did not correlate with markers of coagulation activation (Setty, *et al* 2012). A similar lack of correlation was observed in adult sickle cell patients (Ataga, *et al* 2012) and in our recent mouse study (Chantrathammachart, *et al* 2012a). Together, these data suggest that in sickle cell disease, MPs contribute to the activation of coagulation most likely via PS-dependent mechanism that requires activation of the intrinsic coagulation pathway.

Platelets

A moderate increase in platelet numbers is observed in older children and adults with SCD in steady state (Francis 1991). Circulating platelets in these patients are chronically activated and platelet aggregation is increased (Kenny, *et al* 1980, Westwick, *et al* 1983). This may be attributed to the increased numbers of young, metabolically active platelets or increased plasma levels of platelets agonists, such as epinephrine, adenosine diphosphate or thrombin, in the blood of sickle cell patients. Platelet levels of P-selectin and CD40 ligand are also increased in sickle cell patients compared to healthy control subjects (Garrido, *et al* 2012, Lee, *et al* 2006, Tomer, *et al* 2001a). An increased number of platelet-monocyte aggregates has been observed in both sickle cell patients and a mouse model of sickle cell disease (Polanowska-Grabowska, *et al* 2010). The formation of these aggregates was mediated via platelet P-selectin (Polanowska-Grabowska, *et al* 2010). Interestingly, platelet P-selectin and CD40 ligand have been shown to induce rapid expression of TF in monocytes (Lindmark, *et al* 2000). Furthermore, inhibition of CD40 ligand receptor reduced monocyte TF expression induced by the plasma from sickle cell patients (Lee, *et al* 2006). Platelets from sickle cell

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patients also express higher levels of the pro-inflammatory cytokine LIGHT (TNFSF14), which promotes endothelial activation and inflammation (Garrido, *et al* 2012) and demonstrated increased $\alpha_{IIb}\beta_3$-depenent adhesion to fibrinogen (Proenca-Ferreira, *et al* 2010). Therefore platelets might participate to vaso-occlusive events by binding at the site of inflammation with consequent release of inflammatory mediators.

Ultralarge von Willebrand factor (vWF) multimers

Sickle cell patients demonstrate increased levels of circulating ultralarge vWF multimers in their blood (Chen, *et al* 2011, Krishnan, *et al* 2008). This increase in ultralarge vWF multimers can result from the combination of increased secretion, defective clearance, and impaired cleavage by ADAMTS13. Recent studies demonstrate that impaired processing of ultralarge vWF multimers by ADAMTS13 in sickle cell patients is not the result of ADAMTS13 deficiency, but might reflect resistance of vWF to proteolysis. The resistance could result from either the oxidation of Met1606 at the ADAMTS13 cleavage site, or the binding of free hemoglobin to the A2-domain of vWF (Chen, *et al* 2010, Zhou, *et al* 2009). Since a portion of ultralarge vWF multimers remains anchored to the endothelium upon activation of endothelial cells (Dong, *et al* 2002), it is likely that the increase observed in circulation reflects a similar increase in the endothelium-bound ultralarge vWF multimers in sickle cell patients. It has been shown that ultralarge vWF multimers mediate the adhesion of sickle red blood cells to the endothelium (Wick, *et al* 1987) and platelets aggregations (Ruggeri 2007). Therefore, the endothelium-bound ultralarge vWF multimers may contribute to both vaso-occlusion and generation of thrombogenic surface on the endothelium in SCD.

Regulation of TF expression and activation of coagulation in sickle cell disease

Several mouse studies have examined the mechanism and regulation of TF expression in sickle cell disease. Using a bone marrow transplantation approach, Kollander and colleagues demonstrated that endothelial TF expression is highly dependent on NF-κB(p50) in peripheral blood mononuclear cells, but not in the vessel wall, of sickle cell mice (Kollander, *et al* 2010). These data suggest that peripheral blood mononuclear cells indirectly promote endothelial TF expression via a NF-κB(p50)-dependent mechanism.

An in vitro study demonstrated that heme, a product of intravascular hemolysis, induces the expression of functionally active TF on both micro- and macro-vascular endothelial cells (Setty, *et al* 2008). Consistent with these results, multiple regression analyses identified an association between markers of hemolysis and markers of coagulation activation in sickle cell patients (Setty, *et al* 2012). Recently, we have shown that heme can induce activation of coagulation in mice in a TF-dependent manner (Sparkenbaugh, *et al* 2012). Endothelial nitric oxide synthase (eNOS) negatively regulates endothelial cell TF expression via generation of NO (Solovey, *et al* 2010). Consistent with that observation, inhalation of NO attenuated increased endothelial TF expression and reduced the number of thrombi in small lung vessels in sickle mice subjected to hypoxia/reoxygenation injury (de Franceschi, *et al* 2003, Solovey, *et al* 2010). Furthermore, lovastatin treatment also reduced the increased TF staining observed in pulmonary endothelial cells of sickle cell mice (Solovey, *et al* 2004). Interestingly, lovastatin treatment in sickle cell mice did not attenuate inducible TF expression in monocytes or constitutive TF expression in perivascular cells (Solovey, *et al* 2004). However, a short term use of simvastatin had only a modest effect on plasma levels of TF antigen in sickle cell patients (Hoppe, *et al* 2011). In addition, the histone deacetylase inhibitors trichostatin A and suberoylanilide hydroxamic acid also reduced endothelial TF expression in Berkley mice after a hypoxia/reoxygenation challenge (Hebbel, *et al* 2010).

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The reduction may result from either direct inhibition of TF expression (Wang, *et al* 2007) or could be an indirect result of the increased expression of fetal hemoglobin and overall attenuation of disease severity (Bradner, *et al* 2010, Sangerman, *et al* 2006).

Finally, hydroxyurea treatment has been shown to reduce many markers of coagulation activation. A recent study by Colella and colleagues showed that sickle cell patients on hydroxyurea treatment had significant reduction in leukocyte TF mRNA expression, plasma levels of TF protein, as well as plasma levels of F1.2 fragments and TAT complexes (Colella, *et al* 2012). Treatment with hydroxyurea was also associated with a decrease in the formation of red blood cell derived PS-positive MPs and subsequent attenuation of thrombin generation (Gerotziafas, *et al* 2012). Coincidentally, hydroxyurea therapy is also associated with decreased mortality and morbidity, reduced painful crises and chest syndrome (Steinberg, *et al* 2003), and fewer vaso-occlusive events (Charache, *et al* 1995).

Contribution of coagulation to the pathology of sickle cell disease

Several clinical studies have investigated the effect of different antiplatelet and anticoagulant agents in sickle cell disease. The thromboxane inhibitor aspirin or the ADP receptor antagonist ticlopidine had either modest or no effects on the duration or frequency of acute pain crisis in sickle cell patients (Cabannes, *et al* 1984, Chaplin, *et al* 1980, Greenberg, *et al* 1983, Osamo, *et al* 1981, Semple, *et al* 1984, Zago, *et al* 1984). Recently, a small phase 1 study demonstrated that the platelet inhibitor prasugrel significantly reduced ex vivo platelet reactivity and was well tolerated in sickle cell patients (Jakubowski, *et al* 2012). The impact of anticoagulation on the frequency or severity of vaso-occlusive events has been investigated using warfarin, acenocoumarol or heparins (Chaplin, *et al* 1989, Salvaggio, *et al* 1963, Schnog, *et al* 2001, Wolters, *et al* 1995). Most of these studies were not randomized, and were performed on a small number of patients. They demonstrated modest effects at best. Notably, the only adequately powered and placebo-controlled study examining the effect of low molecular weight heparin in sickle cell disease showed a significant reduction in the duration of pain crisis and time of hospitalization (Qari, *et al* 2007). However it is difficult to say if the protective effects were mediated by anticoagulant properties of low molecular weight heparin, or resulted from the inhibition of P-selectin-mediated cellular interactions (Kutlar, *et al* 2012, Matsui, *et al* 2002). Since all of the clinical studies investigating the role of anticoagulation in sickle cell disease focused primarily on the frequency or severity of vaso-occlusive events, it is still unknown if activation of coagulation contributes to the pathology of sickle cell disease or is just a secondary event. Some insight into that question comes from the recent studies using mouse models of sickle cell disease. Guo and co-workers demonstrated that the treatment of Berkley mice with enoxaparin for two weeks not only reduced vascular congestion in the lung but also attenuated endothelial cell injury measured by plasma levels of sVCAM-1 (Guo, *et al* 2010). The same group showed that a genetic deficiency of TF in non-hematopoietic cells reduces vascular congestion in the livers of sickle cell mice (Hillery, *et al* 2004). Inhibition of TF or thrombin also attenuated the enhanced thrombosis in cerebral microvessels of mice expressing the sickle form of hemoglobin (Gavins, *et al* 2011). Importantly, we have demonstrated that TF not only promotes activation of coagulation but also contributes to vascular inflammation in two mouse models of sickle cell disease (Berkley and Townes mice) (Chantrathammachart, *et al* 2012a). Inhibition of TF reduced leukocytosis and attenuated plasma levels of IL-6, SAP and sVCAM-1. In addition, TF inhibition also reduced expression of MPO and chemokines MCP-1 and KC in the lungs of Berkley mice. Furthermore, TF inhibition had no effect on plasma intravascular hemolysis, indicating that TF contributes to the vascular inflammation downstream of intravascular hemolysis (Chantrathammachart, *et al* 2012a) (Figure 2). It is still unclear if the TF:FVIIa complex promotes inflammation in sickle cell disease directly or via generation of

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downstream coagulation proteases. We have shown that endothelial cell-specific deletion of the TF gene reduced plasma levels of IL-6 but not TAT (Chantrathammachart, *et al* 2012a). This intriguing observation strongly suggests that endothelial cell TF contributes to the expression of IL-6 independent of thrombin generation, most likely via FVIIa– and/or FXa–dependent activation of protease-activated receptor-2 (PAR-2) (Camerer, *et al* 2000, Rao and Pendurthi 2005). TF/FVIIa–dependent activation of PAR-2 has been shown to promote inflammation in various mouse models (Badeanlou, *et al* 2011, Redecha, *et al* 2008). Furthermore, we have observed attenuation of vascular inflammation, including reduction of plasma levels of IL-6, in sickle cell mice lacking PAR-2 in all non-hematopoietic cells (Chantrathammachart, *et al* 2012b).

Little is known about the contribution of downstream coagulation proteases and fibrinogen to the pathology of sickle cell disease. Inhibition of thrombin abrogated microvascular thrombosis in mice expressing the sickle form of human β-globin. In addition, platelets isolated from these mice exhibited enhanced aggregation after stimulation with thrombin but not ADP (Gavins, *et al* 2011). PAR-1 is the main thrombin receptor. Injection of a PAR-1 agonist peptide into sickle cell mice increased the expression of endothelial P-selectin and reduced microvascular blood flow by enhancing the adhesion of red blood cells to the endothelium (Embury, *et al* 2004). Surprisingly, a complete deficiency in fibrinogen was associated with increased mortality in the milder mouse model of SCD (SAD mice) (Roszell, *et al* 2007).The authors did not investigate the mechanism underlying this observation. However, the pathologic effects of fibrinogen deficiency observed in sickle cell mice may be due to an exacerbation of EC injury caused by increased thrombin signaling. Supporting this hypothesis, sickle cell mice lacking fibrinogen demonstrated increased plasma levels of TAT and sVCAM-1 (Hillery, *et al* 2004). Increased levels of circulating thrombin are also observed in patients with afibrinogenemia (Dupuy, *et al* 2001). Further studies are needed to investigate the mechanism by which TF and downstream coagulation proteases, including FXa and thrombin, contribute to vascular inflammation. Possible mechanisms may involve coagulation protease-dependent activation of PARs as well as thrombin-dependent fibrin generation which contributes to local tissue inflammation via activation of leukocytes and promoting infiltration of these cells into inflamed tissues (Figure 3) (Flick, *et al* 2004a, Flick, *et al* 2004b, Petzelbauer, *et al* 2005).

Conclusions

Complex interactions between sickle red cells, leukocytes and endothelial cells lead to vaso-occlusive crises, recurring episodes of ischemia-reperfusion injury and chronic hemolysis. It is evident that these processes are highly interdependent, can influence one another and lead to multiple pathologic manifestations of sickle cell disease, including vascular inflammation and activation of coagulation (Belcher, *et al* 2000, Hidalgo, *et al* 2009, Wun, *et al* 2002). Hypercoagulation is a prominent feature of sickle cell disease and leads to the prothrombotic complications in sickle cell patients. Activation of both extrinsic and intrinsic coagulation pathways contributes to the procoagulant state associated with this disease and is mediated by increased TF expression and increased PS exposure. Given the chronic activation of coagulation and our recent data demonstrating cross-talk between coagulation and inflammation in mouse models of sickle cell disease, further and more definitive evaluation of the potential therapeutic benefit of anticoagulants in sickle cell patients is warranted. More studies are necessary to determine the optimal target(s) in the coagulation and PAR-dependent signaling pathways, defined as that which will maximally inhibit coagulation and vascular inflammation while minimally predisposing patients to complications, such as bleeding. These studies should pave the way to the design of future trials examining the effect of anti-coagulant treatment on clinically relevant endpoints in SCD, such as vasoocclusive crisis, venous thromboembolism and pulmonary hypertension. The new oral

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anticoagulants targeting FXa or thrombin, which have been recently approved for clinical use (Eikelboom and Weitz 2010), provide a potential starting point for these studies.

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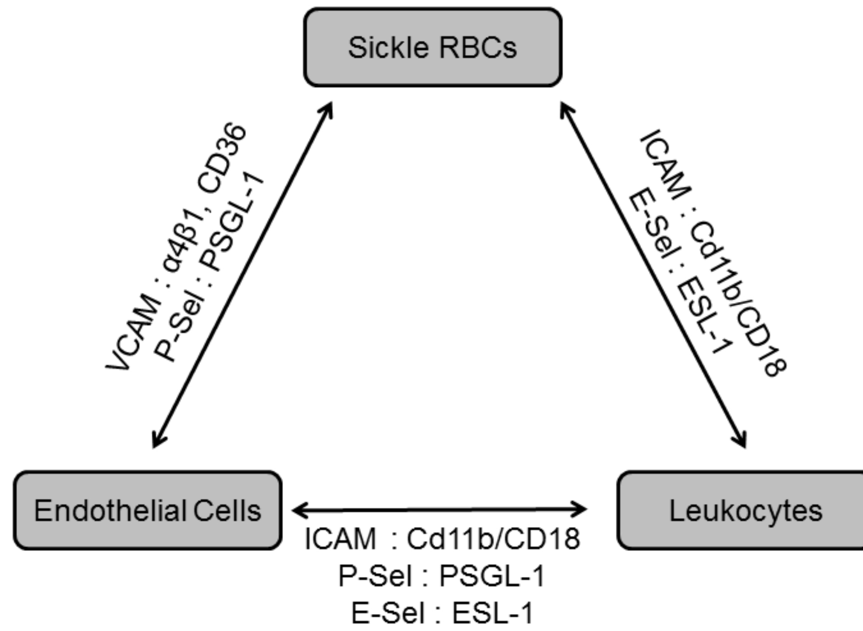

Figure 1.
Interactions between sickle RBCs, leukocytes and endothelial cells are mediated by multiple adhesion molecules.

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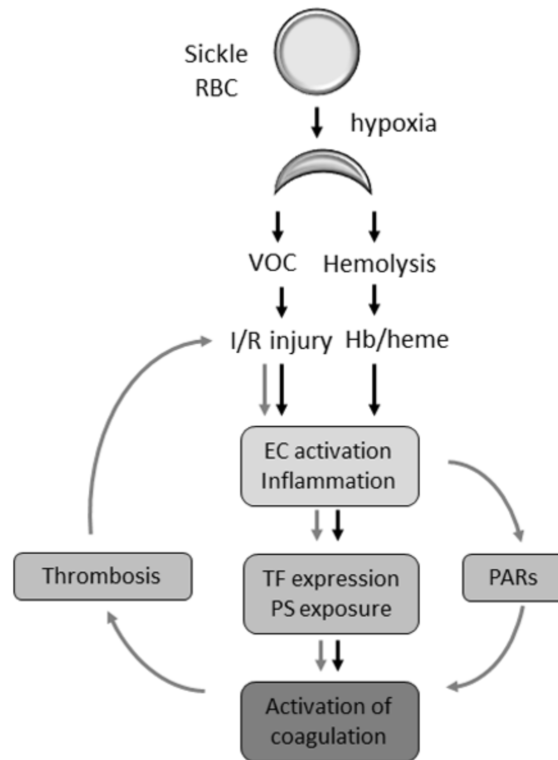

Figure 2. Cross-talk between coagulation and vascular inflammation in SCD

Vaso-occlusion (VOC) and hemolysis-dependent endothelial cell (EC) activation and inflammation lead to the increased expression of tissue factor (TF) by various cell types, phosphatidylserine exposure and subsequent activation of coagulation. We propose that activation of coagulation creates a positive feed-back loop that further enhances VOC, EC activation and inflammation via protease activated receptors (PARs) and thrombosis dependent mechanisms.

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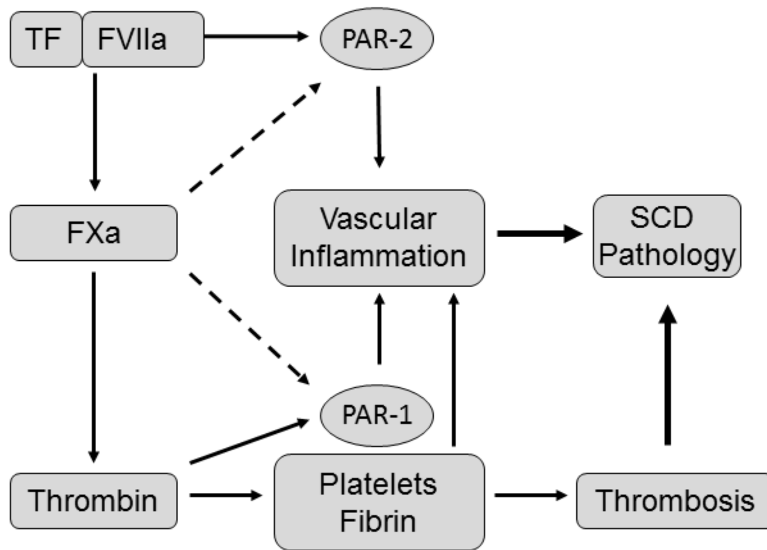

Figure 3.
Proposed mechanism by which TF, FXa and thrombin contribute to the pathology of SCD.