Biologically Active CD40 Ligand Is Elevated in Sickle Cell Anemia
Potential Role for Platelet-Mediated Inflammation

Sheritha P. Lee, Kenneth I. Ataga, Eugene P. Orringer, David R. Phillips; Leslie V. Parise

Objective—After activation, platelets expose CD40 ligand (CD40L) on their surface, then subsequently release the inflammatory mediator as a soluble fragment (sCD40L). Because sickle cell anemia (SCA) is noted for both platelet activation and chronic inflammation, we asked whether platelet-released CD40L potentially plays a role in SCA.

Methods and Results—ELISAs demonstrate that SCA patient plasma contains 30-fold more sCD40L than control plasma. Correspondingly, platelets from these patients contain less than half the CD40L found in control platelets. Platelets from patients in painful crises are further depleted of CD40L, with even higher plasma levels, suggesting a correlation to the patient’s clinical state. In addition, elevated sCD40L correlates with increased tissue factor in SCA plasma. Blockage of the CD40L receptor CD40 reduces SCA plasma-induced production of tissue factor and endothelial intercellular adhesion molecule-1 (ICAM-1). Finally, sCD40L activity in SCA plasma is confirmed by its induction of B-cell proliferation.

Conclusions—Platelet-derived sCD40L is elevated in SCA, further elevated in crises, and biologically active. The participation of sCD40L in SCA plasma-induced production of B cells, tissue factor, and ICAM-1 suggests that CD40L may contribute to the chronic inflammation and increased thrombotic activity known to occur in SCA. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: CD40L ■ sickle cell anemia ■ platelets ■ inflammation ■ coagulation

Chronic inflammation is one of the many manifestations of sickle cell anemia (SCA).1 Patients with SCA are prone to recurrent infections and increased leukocyte counts2 even during the steady state. Painful crises, the major complication experienced by SCA patients, are the result of vascular occlusion3 attributable to interactions of activated blood and endothelial cells.4,11 The polymerization of mutated hemoglobin S leads to red blood cell (RBC) sickling5 and membrane anomalies6,7 that make sickle RBC abnormally adhesive.3,8–10 Painful vasoocclusive crises may partly result from these adhesive RBCs interacting with adhesion receptors on inflamed endothelia.11 Indeed, RBC adhesiveness12 and higher white blood cell (WBC) counts13 correlate with clinically severe SCA. However, inflammatory mediators leading to these abnormalities in SCA are not well understood.

CD40 ligand (CD40L) is a tumor necrosis factor family member that potentially mediates inflammation in SCA. Classically known as the T-cell membrane protein that induces B-cell differentiation and immunoglobulin class-switching,14 CD40L is now known to be expressed on a variety of cell types, including platelets.14,15 On activation, CD40L is exposed to the platelet surface,15 then cleaved to generate a soluble product16 that retains the ability to activate its widely expressed receptor CD40.14 The CD40:CD40L interaction is thought to contribute to inflammation in systemic lupus erythematosus (SLE),17 atherosclerosis,18 and chronic lymphocytic leukemia.19 The soluble form of CD40L (sCD40L) also mediates prothrombotic activity by binding to the platelet integrin glycoprotein (platelet glycoprotein) IIb-IIIa20 and promotes procoagulant activity through upregulation of tissue factor (TF).21–23 Chronic inflammation, increased thrombotic activity, and hypercoagulation are known aspects of SCA.24,25 However, the status of sCD40L in SCA is unknown. We therefore hypothesized that platelet-derived sCD40L may be elevated in SCA as it is in other disease states, and that recognition of CD40L by its receptor CD40 may contribute to SCA pathology.

Materials and Methods

Human Subjects
This study was approved by the UNC committee on the protection of the rights of human subjects. Informed consent was obtained in accordance with the Declaration of Helsinki. Study subjects were
hemoglobin S homozygotes (HbSS), functionally asplenic, 20 to 63 years of age. Patients in crisis were defined as those hospitalized for a painful vaso-occlusive event without respect to crisis phase. Steady-state patients had not experienced a pain episode requiring acute intervention within the previous 2 weeks. Non-HbSS anemia patients included hemoglobin SC (n = 3) and Sβ-thalassemia (n = 7) patients. Transfused patients or those experiencing symptoms not attributed to sickle cell within the previous 2 weeks were excluded, as were patients diagnosed with other inflammatory or malignant conditions. Controls were normal (hemoglobin AA [HbAA]) healthy adults 19 to 49 years of age, and included both black and white subjects.

Preparation of Human Plasma and Platelets
Peripheral blood samples were collected by venipuncture into either 0.13 mol/L sodium citrate or ACID. Blood samples were processed within 90 minutes of blood draw. To obtain platelet- and microparticle-free plasma (PFP), samples were centrifuged at 200g for 15 minutes to remove RBCs and WBCs, then once at 750g for 20 minutes to remove platelets and once at 16 000g for 20 minutes to remove microparticles. Platelet quiescence was maintained by rest- and centrifugation. Platelets were carefully washed 3 times for 20 minutes at 37°C before every centrifugation. Prostacyclin (5 ng/mL) was added to platelet-rich plasma before the second centrifugation and to platelet-poor plasma before the third centrifugation.

For samples used in ELISAs, PFP was defibrinated (1 U/mL thrombin) as recommended by sCD40L ELISA manufacturer instructions. Plasma defibrination did not further elevate CD40L levels. Samples were stored at −80°C per manufacturer instructions until use in sCD40L (Alexis Biochemicals) and TF (American Diagnostica) ELISAs.

Platelet CD40L Levels
To measure platelet CD40L levels, platelet pellets were collected after a 20-minute centrifugation at 750g. Platelets were carefully resuspended in a modified Tyrode’s buffer of 5 mmol/L HEPES, 135 mmol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L NaHCO3, 0.42 mmol/L NaH2PO4, 1 mmol/L MgCl2, 1 mg/mL dextrose, 1 mg/mL BSA, 50 U/mL heparin, and 10 μg/mL apyrase, pH 6.5. Platelets were standardized to 3 × 108 platelets/mL after counting in a Beckman Coulter counter, then lysed in 10 mmol/L CHAPS, 0.3% detergentolate, 137 mmol/L NaCl, 20 mmol/L HEPES, and a broad spectrum protease inhibitor cocktail (CalBiochem). Concentrations of solubilized CD40L in the resulting lysates were then determined by ELISA as above.

B-Cell Proliferation
Ramos B cells were maintained in RPMI-1640 + 10% FBS at 37°C in 5% CO2. For proliferation experiments, B cells were incubated for 72 hours in RPMI-1640 +10% human plasma prepared as indicated above, without defibrination. sCD40L was precipitated from samples by incubating plasma with glutathione sepharose beads (Amersham Biosciences) and either anti-CD40L or nonspecific control rabbit IgG overnight at 4°C. 3H-thymidine (1 U/mL of culture media) was added during the last 18 hours of culture.

Monocyte TF Production
THP-1 monocytic cells were grown in RPMI-1640 +HEPES +10% FBS at 37°C in 5% CO2. For TF induction experiments, THP-1 cells were incubated in 10% human plasma that was prepared as described above. Monocytes were incubated for 24 hours, then washed 3X with PBS before being lysed with 1% Nonidet P-40 in a Tris-based lysis buffer containing 150 mmol/L NaCl and 2 mmol/L EDTA, pH 7.5.

Endothelial Cell Isolation and Culture
Collagenase (1 mg/mL) was used to harvest human umbilical vein endothelial cells (HUVECs) from the cannulated vein of human umbilical cords as described. HUVECs were then cultured in M199 media with 20% FBS, heparin, nonessential amino acids, endothelial cell growth factor, penicillin, and streptomycin. HUVECs were used from passages 2 to 4.

Cell Surface ELISA
HUVECs were grown to more than confluence to induce quiescence in 12-well tissue culture dishes, then treated with 10% human plasma for 24 hours. Plasma was prepared as above but was not defibrinated. For inhibition experiments, rabbit anti-human CD40 polyclonal antibody (Research Diagnostics) or rabbit IgG was added 30 minutes before treatment with human plasma. Cells were washed 3X with PBS, fixed with 1% paraformaldehyde, then blocked with 10% nonfat dry milk. A monoclonal antibody against intercellular adhesion molecule-1 (ICAM-1; Serotec, Inc.) at 1 μg/mL was incubated overnight at 4°C, followed by an anti-mouse IgG:horseradish peroxidase conjugate, with 3X PBS +0.1% Tween-20 washes between each step. Reactions were developed in tetramethylbenzidine (Sigma) and stopped with 2N H2SO4. Absorbance was read at 450 nm.

Statistical Analyses
All data are presented as mean±SD. Levels of sCD40L in human plasma were compared using nonparametric Wilcoxon sum rank tests as computed by SAS Proc Npar1way, where average scores were used as ties. The Student t test was used to determine significance for all other data at a power of 0.05.

Results
CD40L Is Elevated in the Plasma of SCA Patients
To determine whether CD40L is elevated in SCA, sCD40L levels were measured by ELISA in platelet-free plasma and PPP. PPP was prepared by centrifugation of quiescent platelet-rich plasma under conditions determined to prevent platelet activation or contribution of microparticles, as described in the Methods. The average concentration of sCD40L in HbSS plasma (1.30 ± 1.74 ng/mL) was 30-fold higher than sCD40L found in HbAA plasma (0.04 ± 0.05 ng/mL; Figure 1). This value is several fold higher than in chronic lymphocytic leukemia19 and acute coronary syndromes,18 in which CD40L is thought to contribute to the disease process. CD40L levels in HbSS plasma varied greatly among patients, ranging from 0.02 ng/mL to 6.0 ng/mL. sCD40L was also elevated by an average of 17-fold in the plasma of patients with non-HbSS anemia (0.68 ± 0.69 ng/mL), with values ranging from 0.01 to 2.16 ng/mL (Figure 1). Furthermore, the average sCD40L of HbSS plasma in crisis was elevated compared with that in steady state (1.74 ng/mL, with range of 0.31 to 6.0 ng/mL, versus 1.14 ng/mL, range of 0.02 to 4.79 ng/mL; Figure 1), although there was considerable overlap between the 2 groups. The difference between these groups was not statistically significant (P=0.065).

Platelet CD40L Is Decreased in SCA and Further Depleted During Crises
Because >95% of circulating CD40L is contained within platelets27 and released after platelet activation,16 we examined the possibility that the chronically activated platelets believed to exist in SCA patients28-30 might be the source of sCD40L in HbSS plasma. Using the same ELISA assay, CD40L levels were measured from unstimulated platelet lysates of HbSS patients and HbAA control subjects. We found that platelets from HbSS patients contained less than half of the CD40L found in platelets from HbAA individuals.
(5.69±4.81 ng/3×10⁹ platelets versus 13.30±6.77 ng/3×10⁹ platelets; Figure 2A). This 2-fold difference is more than enough to account for the elevated CD40L measured in the plasma of HbSS patients. Furthermore, the unstimulated platelets of HbSS patients in crises contained less than half of the CD40L than platelets of patients in the steady state (2.97±2.81 ng/3×10⁹ platelets versus 7.08±5.26 ng/3×10⁹ platelets; Figure 2B). These results provide evidence that platelets are a major source of elevated CD40L in SCA and suggest an association between decreased platelet CD40L, increased plasma CD40L, and painful crises in HbSS patients.

TF Levels Are Elevated in SCA and Correlate With Elevated CD40L

Because CD40L mediates expression of the coagulation initiator TF, we speculated that elevated CD40L may contribute to hypercoaguability in HbSS plasma by upregulating TF production. Therefore, we compared the levels of TF in HbSS and HbAA plasma (713.11 pg/mL ± 420.84 pg/mL, respectively).
pg/mL versus 6.34 pg/mL ± 12.34 pg/mL; Figure 3A). HbSS plasma contained >100-fold greater TF than the plasma from HbAA controls. Furthermore, elevations in TF correlated with elevations in sCD40L in matched HbSS plasma samples at a level of $R^2 = 0.600$ (Figure 3B).

**CD40:CD40L Interaction Augments HbSS Plasma-Induced TF Production by Monocytes**

Monocytes are known to respond to CD40L stimulation with increased TF production, thus promoting procoagulant activity.²² We therefore asked whether HbSS plasma increases TF production by monocytes, and if so, whether this increase is dependent on the CD40:CD40L interaction. Lysates of monocytic THP-1 cells were assayed for TF after incubation with plasma from either HbSS or HbAA individuals. HbSS plasma induced a significant increase in TF production relative to plasma from HbAA individuals or media alone. Optical density readings indicating relative TF expression were 2-fold greater in lysates from HbSS plasma-treated THP-1 cells than from HbAA plasma-treated THP-1 cells (Figure 4A). The CD40:CD40L interaction was not solely responsible for the elevation in TF production but appeared to promote coagulation in some cases because preincubation of the THP-1 cells with a function blocking anti-CD40 antibody prevented increased TF expression in 5 of 8 individual cases (Figure 4B).

**HbSS Plasma-Induced Expression of ICAM-1**

RBCs from HbSS patients are known to induce endothelial cell expression of adhesion molecules.³³ We found that HbSS plasma can also induce surface expression of endothelial ICAM-1. Relative to HbAA plasma, optical density readings corresponding to surface expression of endothelial ICAM-1 were 3-fold greater as a result of HbSS plasma treatment (Figure 5A). Similar to the results obtained with TF production, CD40 blockade significantly reduced HbSS plasma-induced ICAM-1 expression by HUVECs (Figure 5B), suggesting that the CD40:CD40L interaction can contribute to the adhesive state of the endothelium by inducing ICAM-1 expression on these cells.

**CD40L in SCA Plasma Induces B-Cell Proliferation**

A B-cell proliferation assay was used to further confirm the biological activity of sCD40L in HbSS plasma. B-cell proliferation was measured by $^3$H-thymidine incorporation after the culture of Ramos B cells in media alone, plasma from HbSS patients, or plasma from HbAA volunteers. We found that B-cell incorporation of $^3$H-thymidine was 31-fold greater in the presence of HbSS versus HbAA plasma (2.49×10⁻³ cpm ± 1.12×10⁻³ cpm versus 0.079×10⁻³ cpm ± 0.028×10⁻³ cpm; Figure 6A). Because ligation of CD40 with anti-CD40 antibodies was found to activate Ramos B cells, the role of the

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**Figure 3.** TF is elevated 100-fold in SCA plasma and correlates with higher CD40L. A, Quantitative ELISA results of TF in HbSS (n=12) vs HbAA plasma; $P=0.039$. B, Levels of TF in HbSS plasma plotted against corresponding sCD40L levels in the same samples. $R^2=0.60$ for sCD40L levels <2.5 ng/mL.
CD40:CD40L interaction was investigated by immunoprecipitation of sCD40L from HbSS plasma. Ramos B cells treated with sCD40L-cleared plasma exhibited significantly less proliferation than HbAA control plasma, with an \( \approx 75\% \) reduction in \(^3\)H-thymidine uptake. Proliferation was not reduced by control IgG (Figure 6B), indicating that the increased proliferation of B cells by HbSS plasma is CD40L dependent and that the sCD40L circulating in HbSS patients is biologically active.

**Discussion**

This study provides new insights into potential mechanisms contributing to inflammatory processes in SCA. CD40L has emerged as a potent mediator of inflammation, with elevated sCD40L levels being observed in a variety of diseases involving vascular inflammation.\(^{17-19}\) We now find that sCD40L is elevated in SCA as well. Indeed, our findings indicate an average 30-fold elevation of sCD40L in HbSS versus HbAA plasma, with corresponding decreases in the amount of CD40L stored in the platelets of HbSS patients. The elevated sCD40L in HbSS plasma positively correlates with increased TF and participates in the induction of TF and ICAM-1 expression via its interaction with CD40. The biological activity of sCD40L in HbSS plasma is further confirmed by its induction of B-cell proliferation. Together, these data identify sCD40L as potentially important for both inflammation and coagulation in SCA and suggest a previously unrealized participation of platelets in SCA pathogenesis.

The magnitude of sCD40L elevation in SCA (\( \approx 30\)-fold) can be compared with the increased levels of sCD40L found in other chronic inflammatory conditions such as SLE (\( > 20\)-fold),\(^{17}\) chronic lymphocytic leukemia (2.7-fold),\(^{19}\) and unstable angina (3-fold).\(^{18}\) In all of these latter conditions, CD40L is thought to contribute to the disease state. The 30-fold elevation of sCD40L reported here places CD40L among the inflammatory cytokines potentially sharing a causative role in vascular occlusion in SCA.

Plasma CD40L levels did not correlate with age, platelet counts, or WBC counts of HbSS patients (data not shown); however, because platelets contain \( > 95\% \) of the circulating CD40L\(^{27}\) and these stores are reduced by \( \approx 57\% \) in SCA (Figure 2), platelets are likely to be the major source of sCD40L in HbSS plasma. The relocation of CD40L from platelets to plasma in HbSS patients is consistent with reports that other platelet-released proteins are also elevated in SCA.\(^{28,29}\) Indeed, HbSS patients average 5.7 ng of CD40L per \( 3 \times 10^8 \) platelets compared with 13.3 ng of CD40L found in HbAA platelets (Figure 2). Assuming a platelet count of \( 3 \times 10^8 \) platelets/mL of plasma, 7.6 ng of CD40L relocated from platelets to the plasma would correspond to a nearly 200-fold increase over circulating levels of 0.04 ng CD40L/mL in HbAA plasma, suggesting that the amount depleted from HbSS platelets could more than account for the...
30-fold elevation found in HbSS plasma (Figure 1). Given that elevated sCD40L levels return to baseline within 2 hours of cardiopulmonary bypass and that the amount of sCD40L at sites of thrombosis would presumably be more concentrated, the potential exists for sCD40L to reach far greater levels than reported here. The further depletion of platelet CD40L during crises suggests a correlation to worsened clinical status of HbSS patients and leads us to propose that platelets contribute to the chronic inflammation in SCA by releasing CD40L into the plasma.

Increased in vivo platelet activation in SCA results in increased CD40L exposure to the platelet surface. Once exposed, the cleavage of CD40L from platelets results in the release of sCD40L to the plasma and a loss of platelet CD40L. Therefore, SCA platelets have more surface-exposed CD40L but less total CD40L stored, as measured here in platelet lysates (Figure 2).

CD40L may link chronic inflammation and hypercoagulation in SCA. We confirm here that the coagulation cascade initiator TF is abnormally elevated in HbSS plasma (Figure 3A) and is likely further elevated during episodes of crisis or hypoxic stress. Furthermore, elevated TF in HbSS plasma correlates to increased CD40L (Figure 3B). Because monocyte TF production can be induced by HbSS plasma and reduced by CD40 blockage, our results suggest that the CD40:CD40L contributes to hypercoagulation in SCA, particularly when taken in context with other studies showing that CD40L upregulates TF production. Platelets may therefore contribute to the hypercoagulation in SCA via CD40L exposure and release.

Notably, our study indicates that plasma from HbSS patients is itself inflammatory. HbSS plasma was sufficient to increase endothelial ICAM-1 expression 3-fold (Figure 5A) and Ramos B cell proliferation 31-fold (Figure 6A). Therefore, therapeutic approaches targeted to inflammatory plasma components such as CD40L may also be beneficial to SCA patients.

Recent evidence that CD40L provides a novel mechanism of platelet activation suggests a potential positive feedback loop whereby CD40L participation in SCA may be perpetuated. CD40L is thought to cause α- and dense-granule release, potentially maintaining the activation profile already characterized by SCA platelet studies. CD40L-induced P-selectin exposure may lead to further procoagulant activity and strongly suggests that targeting CD40L release or activity may be therapeutically beneficial to SCA patients.

Inhibitors of platelet glycoprotein IIb-IIIa have shown promising results in acute coronary syndromes and anti-CD40L treatment reportedly improves the clinical profiles of SLE patients. SCA patients may be candidates for either of these therapies. Future studies will be necessary to clarify the relative importance of CD40L in context with the other therapies.
inflammatory mediators in SCA plasma as well as to determine whether the elevated sCD40L levels in SCA are as predictive of inflammatory and thrombotic activity in SCA as they are in acute coronary syndromes.

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References


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