Association between Fibrinolysis and Sickle Cell Anaemia Vaso-Occlusive Crisis: A Cohort Study in a Tertiary Health Facility in Benin City

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ABSTRACT

Sickle cell anaemia (SCA) is associated with hypercoagulability. There is inconclusive evidence on the contribution of abnormal fibrinolytic activity to vaso-occlusive crisis (VOC) in SCA patients. The study aims to evaluate the association between SCA vaso-occlusive crisis and abnormal fibrinolysis using tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) and to correlate their levels with some haematological parameters of the SCA patients. This is a cohort study. Thirty SCA subjects recruited from the adult haematology clinic of the University of Benin Teaching Hospital Benin City in steady-state were followed up to the onset of a vaso-occlusive crisis. Thirty HbAA subjects were recruited from the donor clinic as controls. The t-PA and PAI-1 levels were estimated using enzyme-linked immunosorbent assays at recruitment and then following onset of VOC. Haematological parameters were determined with haematology auto-analyzer. Data were analyzed using SPSS 21. Statistical significance was set at 0.05. The median t-PA levels did not differ significantly between SCA VOC and steady-state (1.36 vs. 0.12ng/ml; p = 0.796). Similarly, median PAI-1 did not differ significantly between SCA VOC and steady-state (11.93 vs. 11.30ng/ml; p = 0.197). There was a negative correlation between t-PA and haematocrit during VOC (r = 0.922, p = 0.001). There was a positive correlation between tPA and PAI-1 but no significant correlation between tPA and PAI-1 with white blood cell count and platelet count. There were no correlations between tPA, PAI-1, white blood cell counts and platelet counts (p>0.05). Vaso-occlusive crisis is not associated with abnormalities in fibrinolytic proteins in SCA patients. T-PA correlated negatively with haematocrit level in VOC state.

Keywords: Sickle Cell, Anaemia, Vaso-occlusive, Crisis, Fibrinolysis

INTRODUCTION

Vaso-occlusive crisis is a cause of significant morbidity in sickle cell disease patients. It accounts for a significant proportion of hospital admissions, correlates with disease severity and contributes significantly to impaired quality of life in SCA patients.[1,2] Hypercoagulability has been implicated in the pathogenesis of VOC in SCA.[3] Significant alterations of haemostatic activities have been reported to contribute to the hypercoagulability in patients with SCA. Depletion of natural anticoagulant proteins (proteins C, protein S, antithrombin), elevated activated coagulation markers (fibrinogen and factor VIII), altered fibrinolytic activity, presence of lupus anticoagulant among others have been reported in patients with SCA.[3,4]

Fibrinolysis is a component of haemostasis and constitutes the enzymatic cascade which ultimately leads to the degradation of fibrin formed during haemostasis.[5] Fibrinolysis is important in moderating blood clot formation following endothelial injury to prevent occlusion of the blood vessels during haemostasis. Altered fibrinolysis can result in significant blood vessel occlusion and damage to body tissues and organs. Tissue hypoxia resulting from the occluded blood vessel may thus provoke episode of VOC. An association between fibrinolysis and VOC may have therapeutic implications. It may provide potential targets for the treatment of vaso-occlusive crisis such as the use of fibrinolytic agents in the management of VOC.

Fibrinolysis is mediated by several plasma proteins including plasminogen, plasminogen activator and plasminogen activator inhibitors (PAI-1). Plasminogen is the proenzyme form of plasmin which is the main enzyme of the fibrinolytic system responsible for the degradation of fibrin into soluble degradation products.[5] Plasminogen is activated by plasminogen activator. The

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activator exists in two forms; tissue-plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) in urine.[5]

Tissue type plasminogen activator is a serine protease, a polypeptide containing 527 amino acid residues with a molecular mass of 72kDa, secreted mainly by the endothelial cells.[6] It cleaves a single peptide bond in plasminogen to generate plasmin which dissolves clots in the vasculature. Fibrinolysis provides an extremely efficient pathway for vascular thrombolysis and its high affinity for fibrin in conjunction with a fibrin dependent stimulation of t-PA activity ensures that activation of plasminogen by t-PA is localized to the fibrin thrombus and reduces systemic plasminogen activation.[7]

Plasminogen activator inhibitors (PAIs) are the primary physiologic inhibitors of plasminogen activators in blood. There are two families of PAI. The serpins (plasminogen activator inhibitor-1 (PAI-1), plasminogen activator inhibitor-2 (PAI-2), and alpha 2- antiplasmin) and non serpins (Thrombin activated fibrinolysis inhibitor (TAFI), alpha2-macroglobulin, C1 esterase inhibitor).[8,9] PAI-1 members of the serpin is widely associated with disease processes.[5] PAI-1 is a serine protease with single-chain glycoprotein of 379 amino acids and it is secreted into blood from the granule of platelets and megakaryocytes.[10] Plasma concentration ranges from 15- 80µg/L.[11]

Available studies on the association between SCA vaso-occlusive crisis and abnormal fibrinolysis have yielded conflicting outcome. Some studies have reported a significant association between abnormal fibrinolysis and SCA; however others did not find any association.[12-15] Fibrinolytic activities in SCA vaso-occlusive crisis has not been adequately investigated in Nigeria. This study aims to investigate the association of sickle cell anaemia VOC with altered fibrinolysis using t-PA and PAI-1 as markers of fibrinolysis, and to correlate plasma t-PA and PAI-1 levels of SCA patients in VOC with their haematological parameters. This outcome may reveal potential targets for therapeutic interventions in SCA patients in VOC.

MATERIALS AND METHODS

This is a cohort study conducted at the University of Benin Teaching Hospital (UBTH) Benin City between January and July, 2019. Thirty consenting adult SCA patients were recruited in steady-state and followed up to the onset of a vaso-occlusive crisis. SCA patients were recruited during a routine clinic visit at the Consultant Outpatient Department. When in crisis, they were requested to present to the emergency room for treatment and re-evaluation of their fibrinolytic state. VOC was defined as acute onset of excruciating bone pain in a sickle cell disease patient.[1] Steady-state is a state characterized by relatively stable disease state in which the patient is symptom-free and not on any active medical treatment lasting for at least 2 weeks.[16] Thirty apparently healthy subjects (HbAA) were recruited as controls from the blood donor clinic.

Inclusion criteria for the SCA patients include a haemoglobin phenotype of SS (Sickle cell anaemia) and at least 18 years of age. Excluded were patients on antiplatelet (aspirin), antifibrinolytic agents, hormonal contraceptives, pregnant women, patients on hydroxyurea, and non-consenting subjects.

The sample size was estimated with the formula for comparing two means using a study power of 90% and 95% confidence interval.[17] The mean difference and standard deviation of a t-PA in a study by Colombatti *et al.* were used to calculated sample size.[18] A sample size of 27 was reached. Based on an attrition rate of 10%, 30 subjects were finally recruited.

After adequate counseling, socio-demographic and medical history was documented on a proforma at recruitment. At recruitment and during VOC, 7mls of venous blood each was collected from the antecubital vein. Four and a half millilitre of the blood was dispensed in a plain sample bottle containing 0.5ml of 0.109M sodium citrate (3.2%). The citrated plasma was separated immediately after centrifugation at 3000g for 15minutes. The supernatant platelet-poor plasma was transferred to a

clean plastic tube and stored at (-80^{0}c) until completion of study for estimation of t-PA and PAI-1 levels using enzyme-linked immunosorbent assay kits (ELISA) by Bioassay Technology Laboratory Shangai China. The t-PA kit has a batch no: E3707Hu; Lot no: 201905009; manufacture date: 15/05/2019 and expiry date: 15/05/2020. The PAI-1 has a batch no: E1159; Lot no: 201905009; manufacture date: 15/05/2019, expiry date: 15/05/2020).

The remaining 2.5ml of blood was dispensed into an ethylenediamine tetra-acetic acid sample bottle for estimation of complete blood count using an automated blood count analyzer at the haematology laboratory.

The study was approved by the institutional ethical review board of UBTH, Benin City. Participants were adequately counseled and participation was voluntary. Data collected were de-anonymized to minimize risk to participants and they were treated as confidential.

Data were analyzed using Statistical Package for Social Sciences (SPSS) version 21. Normally distributed outcome variables (blood count parameters) were summarized as mean and standard deviation while skewed variables (t-PA, PAI-1) were summarized as median and interquartile range. The difference in mean of blood count between VOC and steady-state was compared using analysis of variance test (ANOVA) while the difference in median of t-PA and PAI-1 were compared using independent sample median test. Pearson's correlation test was used to correlate t-PA, PAI-1 and blood counts. Statistical significance was set at 0.05.

RESULTS

Socio-demographics

The ages of the SCA patients and Controls ranged from 20-51 years and 20-43 years respectively. There was no significant difference in their mean ages (26.5 vs. 27.0 years; p = 0.698 respectively). The SCA subjects include 15(50%) males and 15(50.0%) females, while the Controls comprised 18(60.0%) males and 12(40.0%) females.

Fibrinolytic Markers

The difference in median t-PA across the groups (SCA in VOC, SCA in steady state and controls) was not statistically significant (p = 0.052) (Table 1). The median (interquartile range) t-PA of SCA patient was 1.36 (0.21-8.44ng/ml) during vaso-occlusive crisis and 1.24 (0.67-3.77ng/ml) in the steady-state while in the controls, it was 7.22 (5.31-18.71ng/ml). The difference in median t-PA was not statistically significant (p = 0.052). The median (IQR) of PAI of the SCA subjects during VOC and steady-state was 11.93 (7.97-16.86ng/ml) vs. 11.30 (7.97-18.61) respectively while the controls had 6.31 (1.14-10.71ng/ml). The difference in median PAI-1 was statistically significant (p = 0.005). Median PAI-1 between SCA subjects in VOC and during steady states did not differ significantly (p = 0.197) (Table 1).

Haematological Parameters

The mean WBC count was significantly increased in the VOC state (16.4± 0.6 x10^{9}cells/L) compared to steady-state (8.8 ± 3.2 x10^{9}cells/L) and controls (6.6 ± 1.7 x10^{9}cells/L), p<0.001. The mean haemoglobin concentration of the SCA subjects was significantly lower during VOC compared to the steady-state and controls (7.8± 1.2g/dl vs. 8.6 ± 1.3g/dl vs. 13.5 ± 1.4g/dl; p < 0.001). Similarly, the mean haematocrit was lower during VOC than in steady-state and Controls (23.6 ± 3.5 vs. 25.8 ± 3.8% vs. 40.7 ± 4.2%; p<0.001). The mean platelet count was significantly higher in the SCA groups (VOC and steady state) compared to the controls (341.4 ± 177.9 × 10^9/L vs. 305,484.1± 15,761.0 × 10^9/L vs. 220.6 ± 67.1 x10^9/L respectively; p = 0.008 (Table 2). However platelet count did not differ significantly between VOC and steady-state, p = 0.271).

Relationship between haematological parameters and fibrinolytic markers

During VOC, t-PA had a moderate negative correlation with haemoglobin concentration and haematocrit. No significant correlation was found between

t-PA and other haematological parameters. Similarly, there was no significant correlation between PAI-1 and haematological parameters during VOC (Table 3). There was a strong positive correlation between t-PA and PAI-1 (r = 0.922, p = 0.001) during VOC.

In the steady-state, no significant correlation was found between t-PA, PAI-1 and haematological parameters (Table 4). There was a strong positive correlation between t-PA and PAI-1 (r = 0.922, p = 0.001) in steady-state (Table 4).

In the Controls, no significant correlation was found between t-PA, PAI-1 and haematological parameters. Moderately positive correlation was found between t-PA and PAI-1 (r = 0.562, p = 0.001) in the Controls (Table 5).

Table 1: Tissue Plasminogen Activator and Plasminogen Activator Inhibitor 1 levels in SCA VOC and Steady States

	SCA VOC Median (IQR)	SCA Steady state Median (IQR)	Controls Median (IQR)	P- value
tPA (ng/mL)	1.36(0.21 - 8.44)	0.67(0.12 -3.77)	7.22(5.31 -18.71)	0.052
PAI -1 (ng /mL)	11.93(7.97 -16.86)	11.30(7.97 -18.61)	6.31(1.14 -10.71)	0.005

Table 2A: Haematological Parameters in SCA VOC and Steady State

	SCA VOC Crisis n = 30 Mean ± SD	SCD Steady n = 30 Mean ± SD	Control n = 30 Mean±SD	P- value
WBC× 10^9/L	16.4 ± 0.6	8.8 ± 3.2	6.7 ± 1.7	<0.001
Hb (g/dL)	7.8 ± 1.2	8.6 ± 1.3	13.5 ± 1.4	<0.001
PCV (%)	23.6 ± 3.5	25.8 ± 3.8	40.7 ± 4.2	<0.001
Platelet× 10^9/L	341.4 ± 177.9	305.5±157.6	220.6 ± 67.1	0.008

Table 3: Correlation of tPA, PAI-1 and haematological parameters in SCA patients in VOC state

	tPA		PAI -1	
	r	P-value	r	P-value
WBC	0.152	0.424	0.086	0.651
Hb	-0.498	0.005	-0.270	0.149
PCV	-0.501	0.005	-0.269	0.150
Platelet	-0.248	0.186	-0.025	0.898
tPA	1.000		0.632	0.001
PAI -1	0.632	0.001	1.000	

WBC: white blood cell count, Hb: haemoglobin; HCT: haematocrit; t-PA: tissue Plasminogen activator; PAI-1: Plasminogen activator inhibitor 1.

Table 4: Correlation of t-PA, PAI-1 and Haematological Parameters in SCA Steady State

	t-PA		PAI -1	
	r	P-value	r	P-value
WBC	-0.320	0.091	-0.293	0.123
Hb	-0.290	0.127	-0.233	0.223
PCV	-0.288	0.130	-0.231	0.228
Platelet	-0.290	0.128	-0.331	0.080
tPA	1.000		0.922	0.001
PAI -1	0.922	0.001	1.000	

WBC: white blood cell count, Hb: haemoglobin; HCT: haematocrit; t-PA: tissue Plasminogen activator; PAI-1: Plasminogen activator inhibitor 1.

Table 5: Correlation of t-PA, PAI-1 and Haematological Parameters in Controls

	tPA		PAI -1	
	r	P -value	r	P -value
Age	0.063	0.746	-0.039	0.842
WBC	0.061	0.752	-0.046	0.811
Hb	-0.254	0.184	-0.119	0.538
PCV	-0.260	0.173	-0.121	0.531
Platelet	-0.095	0.626	-0.023	0.906
tPA	1.000		0.562	0.001
PAI -1	0.562	0.001	1.000	

WBC: white blood cell count, Hb: haemoglobin; HCT: haematocrit; t-PA: tissue Plasminogen activator; PAI-1: Plasminogen activator inhibitor 1.

DISCUSSION

Fibrinolysis is an important and integral part of the haemostatic system acting as a balance to blood coagulation by preventing thrombotic occlusion of blood vessels. Altered fibrinolysis has been reported in various disease conditions especially those associated with thromboembolism.[5] However, there are conflicting reports in the literature on the association of abnormal fibrinolysis with vaso-occlusive crisis in SCA patients.

Using plasma levels of t-PA and PAI-1 as markers of fibrinolysis, we found no significant difference in their levels between SCA vaso-occlusive crisis and steady states. The lack of a significant difference in the fibrinolytic proteins between steady state and VOC may suggest that abnormal fibrinolysis does not preclude vaso-occlusive crisis in SCA patients neither does VOC result in abnormal fibrinolysis in SCA patients. This finding is consistent with reports of Ekwere *et al.* who reported no significant difference in t-PA alongside other coagulation and fibrinolytic markers (D dimer, fibrinogen, plasminogen) between SCA steady-state and VOC.[12] Earlier experimental works by Gordon *et al* also showed that SCA patients in VOC do not have decreased fibrinolysis.[13] Francis also reported no difference in plasma t-PA antigen and activity in SCA patients in steady-state and VOC.[14] Some authors have reported conflicting findings.[15] Nisiri *et al.* reported normal t-PA levels in SCA patients but a significantly increased PAI-1 suggesting that there is an imbalance between procoagulation and fibrinolysis due to increased PAI-1 levels in the SCA subjects.[15] Famodu *et al.,* reported increased fibrinolysis in VOC.[19] The later reflects the capacity of the

haemostatic system to adjust to the physiologic demand arising from the crisis rather than being responsible for the crisis. The increased endothelial injury and activation of coagulation factors associated with VOC require commensurate fibrinolytic response to maintain haemostasis.[3]

The index study also demonstrated a positive correlation between t-PA and PAI-1 both in the SCA VOC crisis and steady-state. The correlation was stronger in the steady-state compared to during VOC. This reflects the balance between both fibrinolytic markers is maintained during VOC and steady-state; it also reflects the capacity of compensatory adjustment to maintain haemostasis in the SCA population.

The mean total white blood count of the SCA subjects during VOC was significantly increased compared to the steady-state. This is consistent with most reports in literature on white blood cell count during VOC events.[20, 21]Chronic leukocytosis in SCA subjects is intensified during VOC due to continuous activation of neutrophils and monocytes, and an increase in several proinflammatory mediators including TNF- , IL-6, and IL-1 .[22] Further study suggests that haem-linked iron is released during intravascular haemolysis in sickle cell anaemia patients, contributes to the inflammation and activation of monocytes through toll-like receptors signaling increment.[23] In some instances, the VOC is provoked by episodes of acute infections capable of stimulating leukocytosis.[24]

The mean haemoglobin and haematocrit concentrations of the SCA subjects in VOC were significantly lower compared to the steady-state values. Haemolysis of the red blood cells is accentuated during VOC. The sickled cells area damaged both intravascularly and extravascularly because of their rigidity during VOC and thus contributed to the reduced haematocrit and haemoglobin. Damaged red cells release haemoglobin scavenges nitric oxide, further increasing vasoconstriction and thus creating a vicious circle of sickling.[25] Organ sequestration and clearance of the rigid sickled cells by the reticuloendothelial system may also contribute to worsening anaemia during VOC.[25]

Platelet counts did not differ significantly between the steady-state and during VOC despite a high mean difference. Reports on platelet count differences between VOC and steady-state are variable.[13,26-29] Some have reported an increase in count during VOC and others otherwise. However, what is consistent is that there is increased activation of platelets during VOC.[27, 28] Platelet count is reported to decline transiently at the onset of VOC and increase during recovery.[26]Therefore, it is possible to document reduced or increased count during VOC depending on the phase of the event. Heterogeneity in the study population (different subjects in VOC and steady states) may contribute to variation in platelet counts reported in literature However the index study involved estimating parameters of the same subject at steady-state and during crisis and thus would be more likely to reflect the true relationship between platelet during VOC and steady states.

Tissue plasminogen activator was found to correlate negatively with haematocrit and haemoglobin levels in SCA subjects in VOC. There was no significant correlation with other haematological parameters. Similarly, no relationship was found between PAI-1 and haematological parameters. Increased coagulation activation in vaso-occlusive crisis provokes a compensatory increase in t-PA secretion for adequate fibrinolysis and red cells are consumed in the process resulting in a decline in haemoglobin and haematocrit levels. There is paucity of studies on the relationship between t-PA and haematological parameters.

The study has some strengths and limitations. This main strength of the study is that the SCA participants were recruited in steady-state and followed up to when they were in crisis for re-evaluation. This approach removes any confounder associated with the heterogeneity of the study

population between both states. The limitation of the study is that t-PA and PAI-1 antigens were evaluated. Assays involving the functional activity of the proteins may better reflect the fibrinolytic activity. However, studies involving antigen and functional activities of the fibrinolytic proteins showed a good correlation between the antigen levels and functional activity.[14] Another limitation is that t-PA and PAI-1 are regulators of fibrinolysis. Inclusion of a direct assay such as the euglobulin clot lysis test may better represent fibrinolysis. However, the levels of these markers correlate well with fibrinolysis.

In conclusion, there is no significant association between fibrinolysis and SCA vaso-occlusive crisis. Tissue plasminogen activator correlated negatively with haemoglobin and haematocrit levels. We recommend further studies to explore the association of SCA vaso-occlusive crisis and abnormal fibrinolysis.

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