Increased levels of soluble P-selectin correlate with iron overload in sickle cell disease

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Accepted: 21 August 2007

ABSTRACT

Homozygous sickle cell disease (SCD) is characterised by increased soluble P-selectin (sP-selectin), suggesting increased platelet activation, and high non-transferrin-bound iron (NTBI), reflecting iron overload, possibly due to blood transfusion. Hypothesising a relationship between these processes, we measured both markers in 40 SCD patients and 40 age/gender/race-matched controls, finding increased levels of each marker in the patients (both $P<0.001$), but more pertinently a significant NTBI/sP-selectin correlation ($r=0.52$, $P<0.001$). Both indices were increased in the blood of 15 recently-transfused patients compared with 25 three-month transfusion-free patients ($P<0.001$), but only sP-selectin was higher in present sickle crisis ($P<0.001$). We suggest that increased NTBI associated with blood transfusion iron overload in SCD may promote platelet activation.

KEY WORDS: Platelets. Non-transferrin-bound iron. Sickle cell disease. Soluble P-selectin.

Introduction

The pathogenesis of sickle cell disease (SCD) is characterised by recurrent microvascular thrombotic occlusions that may be due to adverse changes in endothelial and platelet function.[1] Evidence in favour of this hypothesis includes excessive platelet and coagulation activation, possibly contributing to a prothrombotic state.[2–5] The adhesion molecule P-selectin (CD62P) is a membrane component of both the platelet α-granule and the endothelial cell Weibel-Palade body.[6] When the platelet is activated (e.g., by thrombin), P-selectin translocates to the external cell surface and is subsequently cleaved at a point close to the cell membrane. Hence, soluble P-selectin (sP-selectin) is widely considered to be a surrogate for platelet activation,[7,8] and can been found in the plasma of healthy subjects, although increased levels are present in patients with atherosclerotic and other thrombotic disorders, and in SCD.[8–10]

The requirement for regular blood transfusion in severe symptomatic SCD[11] generally leads to iron overload[12] and thus the presence of excess free iron not bound to transferrin (i.e., non-transferrin-bound iron [NTBI]). Iron has the potential to be a promoter of hydroxyl radical formation and other reactive oxygen species, and to be an inhibitor of endothelial nitric oxide production in plasma.[13,14] In this way, circulating NTBI (which is undetectable in health) may participate in numerous pathobiochemical pathways, one of which may promote endothelial and platelet damage, and an increase in erythrocyte fragility.[15] As a consequence of reduced red blood cell half-life, and thus anaemia, symptomatic SCD patients are often transfused,[11] leading to iron overload[12] and thus high serum NTBI.[16,17] The consequences of this NTBI are unclear but are believed to be deleterious, generally demanding iron chelation therapy.[11,12]

We hypothesise a positive relationship between sP-selectin and NBTI, reasoning that multiple transfusion would provide excess iron that in turn may promote cytotoxic reactive oxygen species,[13–15,17] leading to increased levels of the soluble adhesion molecule that reflects platelet activation. We test this primary hypothesis in patients with SCD, comparing them to demographically matched controls. We also record: a) each patient's three-month transfusion history; and b) their clinical status (i.e., in sickle cell crisis or stable) to test the additional hypothesis that these interventions influence our two research indices.

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Patients and methods

Forty consecutive patients with SCD (mean/SD age: 35/8 years; 23 males) were recruited from those attending our haemoglobinopathy service. Diagnoses were confirmed by Kontron high-performance liquid chromatography (HPLC). They were classified at the time of venepuncture as being in steady state (currently free of vaso-occlusive complications and a transfusion-free interval of greater than three months; $n=25$) or in crisis (acute bone pain requiring opiate analgesia and/or a vaso-occlusive event such as acute chest syndrome; $n=15$). Transfusion history was also recorded, focusing on the previous three months. Forty race-, age- and gender-matched subjects with a normal haematology profile and negative sickle screen test, and who had not been transfused for three months, were recruited from those attending out-patient clinics. Local Ethical Committee approval was obtained, as was informed consent of all participants.

Citrated and serum blood specimens were centrifuged within one hour at 3000 rpm (1000 x*g*) for 20 min and the plasma and serum stored at –40°C. Soluble P-selectin was assayed by a standard enzyme-linked immunosorbent assay (ELISA) technique (R&D Systems, Abingdon, UK) in citrated

plasma, and NTBI levels were determined in the serum sample by a bleomycin-based assay.[18] The principle of the latter, briefly, relies on bleomycin breaking one of the double strands of a sample of DNA to which free iron in the serum sample binds. This in turn catalyses the production of malonyldialdehyde by hydrogen peroxide and ascorbate, which can be detected in complex with thiobarbituric acid at a wavelength of 405 nm.

The relationship between sP-selectin and NTBI concentration, and transfusion status, was assessed using the Mann-Whitney U test and Spearman's Rank correlation method. With a non-normal distribution (Anderson-Darling test), data are presented as median and inter-quartile ranges. The power calculation for our primary hypothesis was based on the expectation of a correlation coefficient of at least 0.4, which we take to be meaningful. Accordingly, our power calculation was that 37 data points are needed, as these provide the one-sided alpha at 0.05 and 1-beta at 0.8.[19] Thus, we recruited slightly in excess for additional confidence.

For our secondary hypothesis (that transfusion history and/or clinical status of crisis/no crisis influences the research indices), a sample size of $n=30$ and $n=40$ provides the 1-beta = 0.8 and $P<0.05$ power to detect a 33% and a 30% difference, respectively, between the research indices. Analyses were performed on Minitab 13 (Minitab Inc, Progress Drive, State College, PA, USA).

Results

As expected,[9,16] sP-selectin and NTBI were significantly higher in SCD patients compared to the controls (Table 1). With respect to our primary hypothesis, there was a significant correlation between NTBI and sP-selectin (r=0.52, $P<0.001$) (Fig. 1). Soluble P-selectin also showed a significant positive correlation (r=0.59, $P<0.001$) with the number of red cells units transfused.

With respect to our secondary hypothesis, we found a further increase in sP-selectin during sickle crisis. In addition, sP-selectin was significantly higher in the recently-transfused patients compared to those free of recent transfusion (over three months). This finding paralleled the significantly raised NTBI in transfused patients. All differences are within the limits of our power calculation, indicating minimum risk of types 1 and 2 statistical error.

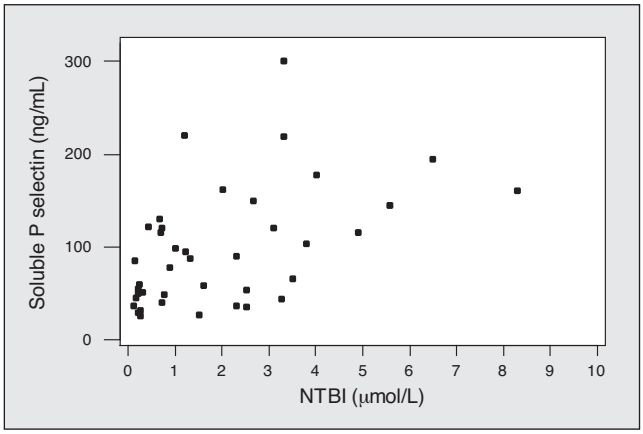

Fig. 1. Correlation between sP-selectin and NTBI.

Discussion

This study confirms the presence of raised plasma levels of sP-selectin[9,16,20,21] in SCD. Despite the widely held view that sP-selectin arises almost exclusively from platelets,[7] we acknowledge the possibility that some may arise from an activated endothelium, although some of this evidence is from transgenic sickle mice.[22] The increased level of sP-selectin (indicating platelet disturbances) during crisis suggests that it is a transient feature associated with acute events. Notably, platelet expression of P-selectin is raised in the veno-occlusive crisis of SCD,[21] and this supports our

Table 1. Levels of sP-selectin, NTBI and number of blood units received in study subjects.

Patients and controls			
	Patients ($n=40$)	**Controls ($n=40$)**	***P* value**
sP-selectin (ng/mlL)	87 (46–128)	66 (48–104)	<0.001
NTBI (μmol/L)	1.25 (0.25–3.2)	Not detected	<0.001
Steady-state disease compared to crisis			
	Steady state ($n=20$)	**Crisis ($n=20$)**	***P* value**
Units of packed RBCs received in two years	20 (14–26)	45 (20–70)	<0.001
sP-selectin (ng/mL)	72 (20–127)	102 (38–300)	<0.001
NTBI (μmol/L)	1.01 (0.4–3.2)	1.29 (0.2–3.0)	0.162
According to transfusion status			
	Non-transfused ($n=25$)	**Transfused ($n=15$)**	***P* value**
Mean units of packed RBCs received in the past three months	0	10.25 (4.75–15.75)	<0.001
sP-selectin (ng/mL)	68 (20–99)	106 (51–300)	<0.001
NTBI (μmol/L)	0.89 (0.2–1.6)	1.65 (0.5–3.2)	<0.001

Data presented as median and range; *P* value by the Mann-Whitney U test.

finding of raised soluble levels in our patients in crisis. However, in our study, as the increase in NTBI during crisis was not significant, the iron abnormality seems unlikely to contribute, in an acute setting, to raised sP-selectin alone.

Patients with a three-month transfusion history had raised sP-selectin. This may be associated with increased availability of iron (due to saturation of iron binding capacity and hence raised free iron as NTBI) from transfused and/or a patient's own blood, thus providing a mechanism for attack on platelets (which may respond by shedding membrane P-selectin). Increased cell surface expression of cell adhesion molecules may have several biochemical and clinical consequences, such as increased adhesion of sickle erythrocytes, platelets and leucocytes to endothelial cells.[23]

We suggest that NTBI associated with more frequent blood transfusion may potentiate the activity of free radicals on membrane components. This may contribute to changes in platelet physiology, with damage and activation, and thus the promotion of vaso-occlusive complications. Regrettably, due to small numbers, we are unable to comment on the relevant and plausible hypothesis that patients with a strong history of transfusion are more likely to be in crisis. As a result of excessive oxidative flux in the sickle cells, the presence of NTBI may further compound the oxidative damage to endothelial cells, platelets and sickle cells. The abnormal and damaged cellular components may further increase the risk of thrombosis and vascular occlusion. In addition, it has been demonstrated that haemoglobin released from damaged sickle cells induces platelet aggregation through free radicals generated by the redox cycling of haem iron.[24]

We acknowledge that a weakness of our study is that it is entirely observational and that correlation does not imply causation. Another weakness is the possible influence of the ligand (i.e., PSGL-1) that may bind some P-selectin.[25] Nonetheless, we suggest that the risk factors contributing to vascular occlusion may be further promoted by damaged platelets associated with excess transfusion-raised NTBI in SCD. Thus, although transfusion clearly ameliorates symptoms and raises haemoglobin, our data underline additional potential dangers in repetitive blood transfusion therapy. □

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