Increased adhesive properties of neutrophils in sickle cell disease may be reversed by pharmacological nitric oxide donation

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ABSTRACT

Increased leukocyte adhesion to vascular endothelium contributes to vaso-occlusion in sickle cell disease. Since nitric oxide bioavailability is decreased in sickle cell disease and nitric oxide may inhibit leukocyte adhesion, we investigated whether stimulation of NO-signaling pathways can reduce the adhesive properties of neutrophils from sickle cell disease individuals (sickle cell diseaseneu). sickle cell diseaseneu presented greater adhesion *in vitro* to both fibronectin and ICAM-1 than control neutrophils. Co-incubation of sickle cell diseaseneu with the nitric oxide-donor agents, sodium nitroprusside and diethylamine NONOate (DEANO), and the guanylate cyclase stimulator, BAY41-2272, all significantly reduced the increased adhesion to fibronectin/ICAM-1. Oxadiazolo[4,3-a]quinoxalin-1-one, a guanylate cyclase inhibitor, reversed sodium nitroprusside/DEANO-diminished adhesion to fibronectin, implicating cGMP-dependent signaling in this mechanism. Interestingly, intracellular cGMP was significantly higher in neutrophils from sickle cell disease individuals on hydroxyurea (sickle cell diseaseHUneu). Accordingly, sickle cell diseaseHUneu adhesion to fibronectin/ICAM-1 was significantly lower than that of sickle cell diseaseneu. Agents that stimulate the nitric oxide/cGMP-dependent pathway may have beneficial effects on leukocyte function if used in these subjects.

Key words: adhesion, leukocyte, nitric oxide, sickle cell disease, vaso-occlusion.

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Introduction

Leukocytes play a central role in sickle cell disease (SCD) pathophysiology. Sickle cell crises are often associated with infection, neutrophil counts are higher in SCD individuals, and polymorphonuclear leukocytosis has been correlated with an increased rate of early death, acute chest syndrome and stroke.[1] There is growing evidence to suggest that leukocytes participate in the initiation and propagation of vaso-occlusive processes by which the recruitment of large, less deformable, adherent white cells to the vascular endothelium and their interaction with circulating erythrocytes may impair blood flow.[2]

Neutrophils of SCD individuals are more able to adhere to fibronectin and to endothelial monolayers than healthy individual neutrophils.[3] Neutrophils express a variety of adhesion molecules on their surface that are required for transendothelial migration. The L- and P- selectins mediate tethering and rolling on the endothelium, while firm adhesion is mediated by the β2 integrins Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18).[4] Mac-1 expression has been shown to be increased on stimulated SCD neutrophils.[5,6] Therefore, pharmacological approaches to inhibit increased leukocyte adhesive interactions may represent important strategies for the prevention of SCD vaso-occlusion.

Recent reports suggest that nitric oxide (NO) bioavailability may be decreased in SCD.[7] Vascular cell-free hemoglobin, released during hemolysis, can consume the NO produced by endothelial cells thus facilitating vasoconstriction and platelet activation.[7] NO is also known to affect adhesion mechanisms. inhibiting endothelial adhesion molecule expression[8] and reducing the adhesive properties of leukocytes.[9]

At present, hydroxyurea (HU) is the only widely-used therapy for the treatment of SCD, reducing the frequency of vaso-occlusive crisis, acute chest syndrome and transfusion, and increasing fetal hemoglobin (HbF) production in erythroid cells.[10] While it has been proposed that HU induces HbF production via tyrosyl radical activation on ribonucleotide reduc-

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tase,[11] there is evidence to suggest that HU may induce γ-globin gene expression in erythroleukemic cells and primary human erythroblasts via a NO-guanylate cyclase dependent pathway.[12,13] Furthermore, data suggest that HU may be oxidized by haem groups to produce NO *in vitro* and *in vivo*,[14] suggesting that HU may act as a donor of NO *in vivo*.

The aim of this study was to compare the adhesive properties of neutrophils from SCD individuals with those on HU therapy. The study also aimed to determine whether the altered NO bioavailability that is characteristic of SCD may cause changes in the intracellular levels of NO metabolites and the principal NO second messenger, cyclic guanosine monophosphate (cGMP, produced by the activation of the guanylate cyclase enzyme). Finally, we determined whether the adhesive properties of SCD neutrophils may be abrogated by pharmacological donation of NO *in vitro*.

Design and Methods

A total of 52 homozygous HbS/S patients were studied (19 of whom were on HU therapy). Six patients were also heterozygous for α-thalassaemia (2 patients on HU and 4 patients not on HU). Patients were not in crisis and had not received blood transfusions in the previous three months. Patients on HU therapy had been taking 20-30 mg/Kg for at least three months. Twenty-four healthy individuals were used as controls (age, 20-50). Informed consent was obtained from all patients and controls, and the study was approved by the local ethics committee. For clinical characteristics of all patients and controls, see *Online Supplementary Table 1*.

Neutrophils were isolated from peripheral blood[15] and static assays[15] were performed to determine their adhesion (2×10^6 neutrophils/mL RPMI medium) to immobilised fibronectin (20 μg/mL) or recombinant ICAM-1 (10 μg/mL) (30 mins., 37°C, 5% CO_2). The adhesion assay and materials used in this study are described in Supplementary Information. For NO-donor/drug co-incubation, cells were co-incubated during the adhesion assay.

Total NO metabolites (NOx; nitrite plus nitrate) were quantified in lysed neutrophil (5×10^6 cells/mL) ultrafiltrates using a nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI, USA) (see Supplementary Information). For intracellular cGMP determination, isolated neutrophils (1×10^7 cells/ml PBS) were incubated with a phosphodiesterase inhibitor and cyclic nucleotides were extracted with perchloric acid (see Supplementary Information) before quantifying cGMP using commercially available ELISA kits (Cayman Laboratories, Ann Arbor, MI, USA).

For flow cytometry, isolated neutrophils (5×10^6 cells/mL) were incubated with anti-CD11a FITC, anti-CD11b Alexa Fluor and/or anti-CD49d PE monoclonal antibody (20 mins., RT, in the dark) and processed as described in Supplementary Information. Results are expressed as geometric mean cell fluorescence intensity values compared to that of isotype controls.

Results are expressed as means ± S.E.M. Statistically significant differences between groups were determined using the Mann-Whitney non-parametric test. The Student's paired t-test was used to compare groups before and after treatment with specified drugs. Statistical significance was established as $p<0.05$.

Results and Discussion

Leukocyte adhesion to the endothelium of the microcirculation plays a key role in SCD vaso-occlusion.[2] Therefore, therapeutic approaches to inhibit this adhesion may be important for the prevention of vaso-occlusion. Figure 1 confirms the increased ability of neutrophils from SCD individuals (SCDneu) to adhere to fibronectin, an extracellular matrix component, and further demonstrates an increased ability of these cells to adhere to recombinant ICAM-1, an adhesion molecule expressed on activated endothelial cells. Heterozygosity for α-thalassemia did not significantly alter SCDneu adhesive properties (*data not shown*).

Decreased NO bioavailability may contribute to the pathophysiology of SCD.[7] Intracellular levels of NOx were significantly lower in SCDneu compared to levels in healthy control neutrophils (3.68±0.35 *vs.* 7.04±1.38 μM/1×10^6 cells; $p<0.05$; n=9, respectively), indicating that reduced NO bioavailability may result in decreased NO signaling in SCD leukocytes. Intracellular levels of the NO second messenger, cGMP, however, were not significantly different in SCD and control neutrophils (0.104±0.023 *vs.* 0.114±0.021 pMol/1×10^7 cells; $p>0.05$; n=19; n=11, respectively). Whether a reduction in intracellular NO-dependent signaling contributes to the increase in leukocyte adhesive properties in SCD must still be determined. There is, however, some evidence that the up regulation of cyclic adenosine monophosphate (cAMP)-dependent signaling may contribute to increase the adhesive properties of SCD neutrophils.[15,16] This could be due to augmented serum inflammatory factors in SCD.

Pharmacological stimulation of the NO-dependent pathway with the NO donors Diethylamine NONOate (DEANO; 1 μM) and sodium nitroprusside (SNP; 10 μM) significantly abrogated the adhesion of SCD, but not control neutrophils (CONneu), to both fibronectin and ICAM-1 (Figure 2). Furthermore, the NO-independent guanylate cyclase activator BAY 41-2272 (150 nM) also significantly decreased SCDneu, but not CONneu, adhesion to fibronectin and ICAM-1 (Figure 2). Co-incubation of SCDneu with the NO donors DEANO (1 μM) or SNP (10 μM), and with the inhibitor of guanylate cyclase 1H-[1,2,4] Oxadiazolo [4,3-

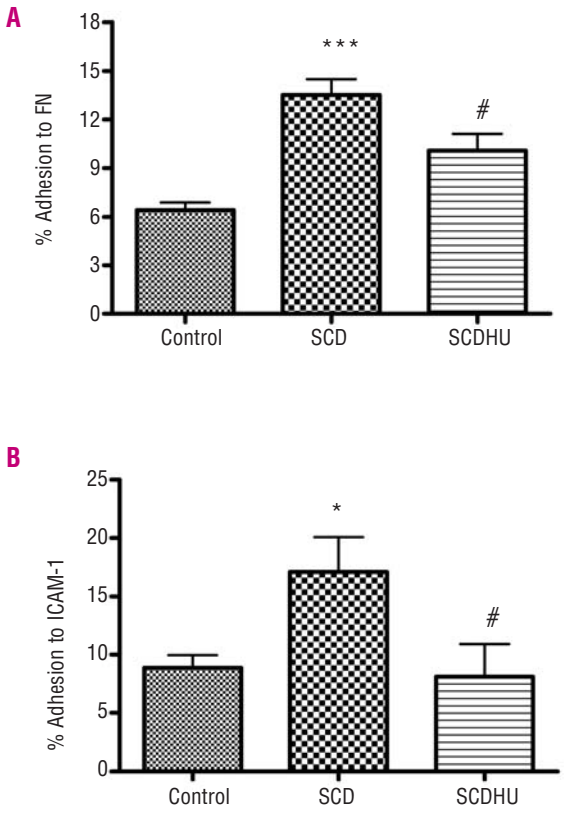

Figure 1. Adhesion of neutrophils isolated from control individuals, steady-state SCD individuals (SCD) and steady-state SCD individuals on hydroxyurea therapy (SCDHU; 20-30 mg/kg/day) to fibronectin (A) and recombinant ICAM-1 (B). Results are expressed as percentage of adherent cells ± S.E.M. N ≥ 16 and N ≥ 6, for (A) and (B) respectively. *, $p<0.05$; ***, $p<0.001$, compared to control adhesion; #, $p<0.05$, compared to SCD adhesion.

a]quinoxalin-1-one (ODQ; 10 μM), prevented the reduction in SCDneu adhesion to fibronectin that is induced by these NO donors (Figure 2c), suggesting that NO donors may reduce SCD neutrophil adhesion properties via a cGMP-dependent pathway.

The surface expressions of the integrin α-subunits, CD11a and CD11b, were determined on control and SCD neutrophils by flow cytometry (Table 1). Data suggest that the LFA-1 and Mac-1 integrin subunits, CD11a and CD11b, are not expressed at significantly different levels on the surface of SCDneu, compared with CONneu. Some studies have reported an increased CD11b/CD18 expression on unstimulated SCD neutrophils,[5] while others have detected significantly increased CD11b expression only after cell stimulation.[6] Integrins can mediate alterations in adhesive properties both following the mobilization of integrins to the cell surface and also as a consequence of changes in integrin affinity and avidity (mediated largely by integrin clustering).[17,18] The affinity of Mac-1 for its ligands, for example, is dynamically regulated by *inside-out* signaling, producing a form of the integrin that binds to its substrate with a much greater affinity.[17] It

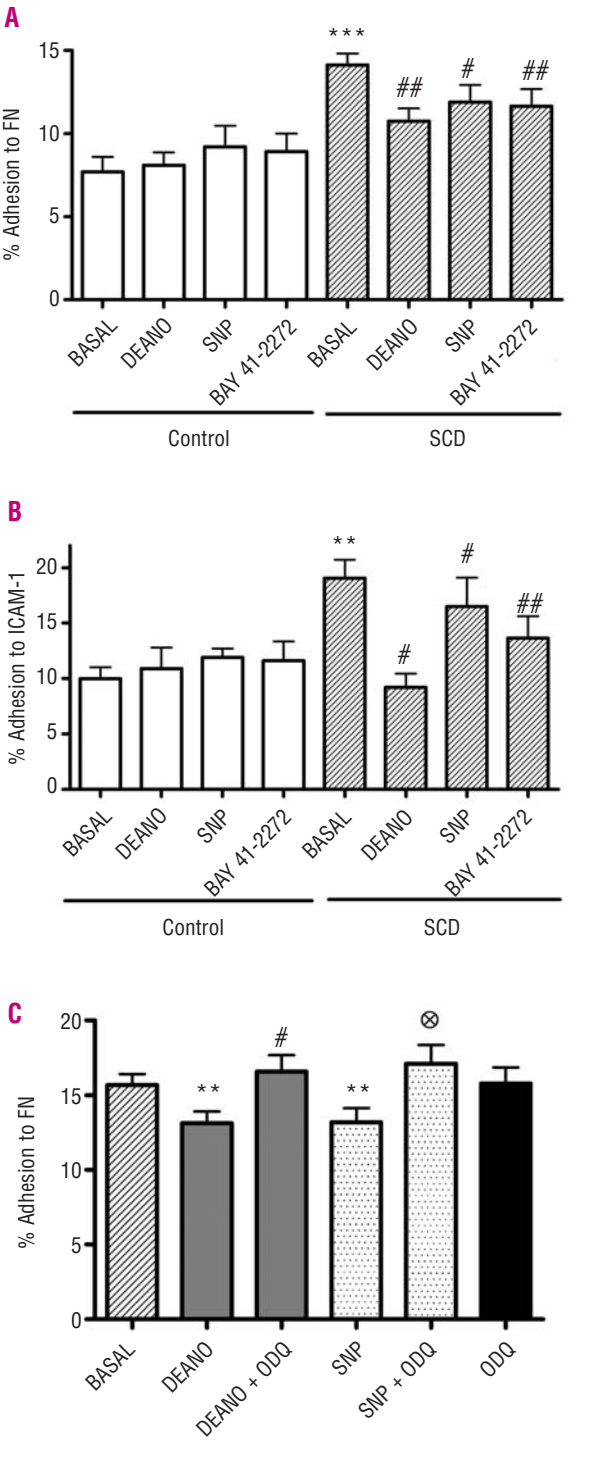

Figure 2. Adhesion of neutrophils isolated from control and SCD individuals to fibronectin (A) and ICAM-1 (B) after or without co-incubation with 1 μM DEANO, 10 μM SNP or 150 nM BAY 41-2272. (C) Basal adhesion of SCD neutrophils to fibronectin and adhesion following co-incubation with 1 μM DEANO, 10 μM SNP or 150 nM BAY 41-2272 in the presence or absence of the guanylate cyclase activator, ODQ (10 μM). Results are expressed as percentage of adherent cells ± S.E.M. N ≥ 5, N ≥ 3 and N ≥ 8 for (A), (B) and (C) respectively. (A) and (B): **, $p<0.01$; ***, $p<0.001$, compared to basal control neutrophil adhesion; #, $p<0.05$; ##, $p<0.01$, compared to basal SCD neutrophil adhesion. (C): **, $p<0.01$, compared to basal SCD neutrophil adhesion; #, $p<0.05$, compared to incubation with DEANO alone; ⊗, $p<0.05$, compared to incubation with SNP alone.

Table 1. Expressions of integrin a-subunits on the surface of neutrophils isolated from control individuals, steady-state SCD individuals (SCD) and steady-state SCD individuals on hydroxyurea therapy (SCDHU; 20-30 mg/kg/day).

Integrin α-subunit		Control		SCD		SCDHU	
		MFI (N=7)	p	MFI (N=8)	P	MFI (N=8)	p
CD11a	Basal	13.82±1.66		16.03±1.27		17.05±1.47	
	SNP	11.55±1.27	>0.05	11.66±0.77	0.0002	14.70±1.50	0.035
CD11b	Basal	162.9±47.8		151.23±32.4		154.0±46.9	
	SNP	191.37±41.8	>0.05	184.73±21.58	>0.05	207.96±33.04	>0.05
CD49d	Basal	7.73±0.72		9.81±0.80		10.53±1.22	
	SNP	7.28±0.59	>0.05	10.03±1.06	>0.05	10.43±1.34	>0.05

Neutrophil surface integrin expressions were determined under basal conditions (basal) and following incubation with 10 μM SNP (SNP; 30 min.; 37°C). Surface expression is reported as mean fluorescence intensity (MFI) on 10,000 cells and results are presented as the mean ± SEM of n individuals. P values compare SNP-incubated expression to basal expression (paired t-test). No significant differences were found between surface integrin expressions for the control, SCD and SCDHU groups (unpaired t-test).

is, therefore, possible that the increased adhesive properties of SCD neutrophils observed may be mediated by alterations in integrin affinity rather than expression. The surface expression of the VLA-4 integrin subunit, CD49d, was also determined on SCDneu, since the involvement of β1 integrins has been implicated in neutrophil recruitment during chronic inflammation[19] and NO signaling may have a role in controlling VLA-4 integrin expression on the neutrophil cell surface.[9] No significant alteration in CD49d expression was found, however, on the surface of SCDneu. Interestingly, following incubation with 10 μM SNP (30 mins., 37°C), the surface expression of the CD11a subunit was significantly decreased on both SCD and SCDHU neutrophils to levels similar to those of CONneu (Table 1). However, incubation with a NO donor did not decrease the surface expression of the major neutrophil integrin Mac-1, as demonstrated by the unaltered CD11b expression following incubation with SNP.

Data indicate that a reduction in adhesion molecule expression may not contribute significantly to the reduction in SCD neutrophil adhesion mediated by NO donors. Instead, these agents may abrogate SCD neutrophil adhesion via alterations in the function (via affinity or avidity alterations) of the adhesion molecules already expressed on the cell surface. Similarly, NO-dependent signaling has been shown to alter platelet GPIIb/IIIa integrin function, as well as neutrophil Mac-1 integrin affinity or avidity.[9,20]

Interestingly, the adhesion of neutrophils from SCD individuals on HU therapy (SCDHUneu) demonstrated a significantly lower adhesion to fibronectin and ICAM-1 that approached that of CONneu adhesion (Figure 1). NOx in these cells were not found to be significantly higher than in SCDneu (SCDHUneu; 4.22±0.92 μM/1×10^6 cells; p>0.05; n=11). However, intracellular cGMP levels were found to be approximately doubled in SCDHUneu compared with control and SCD neutrophils (0.241±0.034 pMol/1×10^7 cells; p<0.001; n=9).

The surface expressions of the CD11a, CD11b and CD49d integrin subunits were not found to be altered significantly on SCDHUneu, indicating that HU therapy may reduce neutrophil adhesion via alterations in integrin affinity / avidity, as also observed when SCD neutrophils were treated with NO donors. Alternatively, the possibility that SCD neutrophil adhesion may be mediated by receptors other than those studied here should not be ruled out. Since activation of cGMP-dependent signaling was found to reduce SCDneu adhesive properties, it is possible that the increased intracellular cGMP levels observed in SCDHUneu may contribute to the observed reduction in adhesion. These results provide further evidence to support the hypothesis that HU may exert some of its effects via up regulation of NO-cGMP signaling. Indeed, HU has been reported to induce NO-cGMP signaling in endothelial cells[14] and cGMP levels have been found to be increased in the plasma and red cells of SCD individuals on HU.[21,22] Furthermore, HU may increase NO bioavailability by decreasing hemolysis and, in turn, decreasing NO scavenging by cell-free plasma hemoglobin and arginine scavenging by cell-free arginase.[7] The correlation of SCD/SCDHU neutrophil adhesiveness to markers of the hemolytic rate, hemoglobin levels and reticulocyte counts did not, however, reveal any significant associations with hemolytic rate (*results not shown*). While HU may mediate a reduction in leukocyte adhesion by increasing intracellular cGMP levels, other mechanisms may also participate in the HU-mediated effect. In accordance with evidence that intracellular cAMP may participate in increased SCD neutrophil adhesive properties,[15] cAMP levels were found to be reduced in SCD patients on HU therapy[16] and it is possible that cross talk between the cAMP and cGMP pathways may result in alterations in the cyclic nucleotide balance with a conse-

quent reduction in SCD neutrophil adhesive function.

Our data suggest that although decreased NO bioavailability may not play a major role in the increased adhesive properties of SCD leukocytes, pharmacological stimulation of the cGMP-dependent pathway may be a potential approach for reducing neutrophil adhesion to the vascular endothelium. A number of NO-donating agents and drugs that stimulate cGMP accumulation show potential for use *in vivo*. Guanylate cyclase inhibitors, such as BAY 41-2272, have been successfully used to treat animal models of cardiovascular disease, pulmonary hypertension and inflammation.[23-25] Alternatively, drugs that have a more cell-specific effect, such as inhibitors of phosphodiesterases (PDE) may prove more effective in specifically reversing altered SCD neutrophil function. For example, sildenafil, a PDE5-specific inhibitor, is being tested clinically in SCD patients with pulmonary hypertension[26] and should it be found that this or any other PDE is expressed in leukocytes, such PDE inhibitors may also have the potential for abrogating altered SCD neutrophil adhesion.

Authorship and Disclosures

AAC and NC conceived the project, designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. CFP performed experiments. CFP and STOS assisted in data interpretation and approved the final manuscript. FFC contributed to project design, data interpretation and the final revision of the paper.

The authors report no potential conflicts of interest.

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