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

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Citation: Canalli AA, Franco-Penteado CF, Saad STO, Conran N, and Costa FF. Increased adhesive properties of neutrophils in sickle cell disease may be reversed by pharmacological nitric oxide donation. Haematologica 2008 Apr; 93 (4):xxxx-xxxx.doi: 10.3324/haematol.12119

Materials and Methods

Materials

Recombinant human ICAM-1 (10 µg/mL) was purchased from R&D Systems (Minneapolis, MN). Fibronectin (FN) was from Invitrogen (California, USA). Diethylamine NONOate (DEANO) was from Cayman Chemical (Ann Arbor, MI, USA), sodium nitroprusside (SNP) and ODQ were from Sigma Co. (St Louis, MO, USA) and BAY 41-2272 was kindly provided by Dr. Edson Antunes, University of Campinas, Brazil. Alexa Fluor anti-CD11b (Clone ICRF44), anti-CD11a FITC (Clone G43-25B), and anti-CD49d PE (Clone HUTS-21) were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). All other products were bought from Sigma Co. (St Louis, MO, USA) unless otherwise stated.

Isolation of human neutrophils from peripheral blood

Neutrophils were isolated from peripheral blood. Briefly, whole blood was placed over two layers of Ficoll-Paque of densities of 1.077 and 1.119 g/L respectively. After separation of monocytes and granulocytes by centrifugation at 700g for 30 mins., the granulocyte layer was washed once in PBS (phosphate buffered saline, pH 7.4) before lysing contaminating red cells (10 min, 4°C, lysis buffer; 155 mM NH_4Cl, 10 mM $KHCO_3$). Cells were washed once again in RPMI medium before resuspending in RPMI medium. Cells were counted using the Advia Hematology System (Bayer, Tarrytown, NY), cytospinned onto slides and a cell differentiation count performed. Neutrophil suspensions with purity greater than 92% were used immediately in assays; contaminating cells were mainly lymphocytes and eosinophils.

Neutrophil adhesion assays

Ninety six-well plates were prepared by coating individual wells overnight at 4°C with 60 µl of fibronectin solution (20 µg/ml in PBS) or recombinant ICAM-1 (10 µg/mL in PBS). Wells were washed twice with PBS before blocking non-coated sites with 0.5% (w/v) BSA for 90 mins. at 37°C. Wells were washed twice again with PBS before allowing plates to dry. Neutrophils (50 µl of $2x10^6$ cells/mL) were seeded onto the plate and cells were allowed to adhere to FN or ICAM-1 for 30 mins. at 37°C, 5% CO_2. Following incubation, non-adhered cells were discarded and wells washed three times with PBS. RPMI (50µl) was added to each well and varying concentrations of the original cell suspension (0-100 %) were added to empty wells to form a standard curve. Percentage cell adhesion was calculated by measuring the myeloperoxidase content[1] of each well and comparing it with the standard curve for each individual study subject. For NO-donor/drug co-incubation, cells were co-incubated during the adhesion assay.

Total nitric oxide metabolite (NOx) measurement

Neutrophils ($5x10^6$/mL) were lysed in UltraPure water and stored until the time of assay (-20°C). For assay, cell extracts were ultrafiltered through 10 kDa molecular weight cut-off filters (Millipore Corp., Bedford, MA, USA). Following reduction of nitrate to nitrite with nitrate reductase, total NOx (nitrite plus nitrate) in samples were measured using a nitrate/nitrite colorimetric assay kit (Cayman, Ann Arbor, MI, USA).

Supplementary References

1. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 1982;78:206-9.

Measurement of intracellular cGMP

For the measurement of intracellular cGMP, isolated neutrophils ($1x10^7$ cells/ml PBS) were incubated with the phosphodiesterase inhibitor, 3-isobutyl-l-methylxanthine (IBMX, 2mM; 30 mins., RT). Perchloric acid was then added to samples (4 % v/v final concentration) and, following centrifugation (6 000 g, 20 mins.), supernatants were retained and the pH neutralized by 8 M KOH addition. Samples were stored at –20°C and assayed for cGMP using commercially available ELISA kits (Cayman Laboratories, Ann Arbor, MI, USA) following the manufacturer's instructions.

Flow Cytometry

For flow cytometry, isolated neutrophils ($5x10^6$ 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). After washing in PBS, cells were fixed in 1% paraformaldehyde/ PBS and then analyzed (10,000) at 488nm on a Becton-Dickinson FACScalibur. SSC/FSC (side scatter/forward scatter) dot plots were used to gate the neutrophil population. Results are expressed as geometric mean fluorescence intensity values compared to that of an isotype control.

Supplementary Table 1 Clinical details of healthy controls, steady-state SCD patients and steady-state SCD patients on hydroxyurea therapy.

Parameter	*Healthy Controls*	*SCD*	*P* compared to Controls	*SCDHU*	*P* compared to SCD
n	24	33		19	
Males	12	13		8	
Age (yrs.)	36.8 ± 9.6	35.2 ± 8.8	N/S	34.7 ± 9.6	N/S
Red blood cell no. (10^{12}/L)	N/D	2.42 ± 0.66		2.56 ± 0.38	N/S
Hematocrit (%)	42.5 ± 3.6	23.0 ± 4.8	<0.0001	26.0 ± 3.4	<0.01
Hemoglobin (g/dL)	N/D	7.8 ± 1.7		8.8 ± 1.2	<0.01
Mean corpuscular volume (fl)	N/D	96.6 ± 9.5		102.9 ± 15.9	N/S
Mean corpuscular hemoglobin (pg)	N/D	32.8 ± 4.2		34.7 ± 5.4	N/S
WBC (10^9/L)	N/D	10.4 ± 2.4		8.3 ± 2.7	<0.01
HbF (%)	N/D	6.7 ± 4.2		13.6 ± 6.9	<0.001

SCD, steady-state SCD patients; SCDHU, steady-state SCD patients on hydroxyurea therapy; WBC, white blood cells; HbF, foetal hemoglobin; N/D, not determined; N/S, not significant. Data presented are mean ± S.D. (except n and number of males). Statistical comparisons were made by the Mann-Whitney test.