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Ability of sickle cells to scavenge endothelium-derived nitric oxide is reduced

Leopoldo OLMOS, Jean-Vivien MOMBOULI[1], Nathan WASSERSTRUM, Paul M VANHOUTTE[2,3]

Center for Experimental Therapeutics, Baylor College of Medicine, Houston, TX 77030, USA ;
[1]University of Marien Ngouabi, Brazzaville, Republic of Congo ;
[2]Institut de Recherches Internationales Servier, 92415 Courbevoie Cedex, France

KEY WORDS sickle cell anemia; endothelium; vascular smooth muscle; erythrocytes; microcirculation; nitric oxide

ABSTRACT

AIM: To assess the ability of sickle cells to interfere with the release or transfer of endothelium-derived relaxing factor (EDRF) in comparison to normal erythrocytes. **METHODS:** A perfusion-superfusion bioassay system was used a canine carotid artery with endothelium (donor of EDRF) and a ring of the same vessel without endothelium (detector) were separated by tubing resulting in a five second interval for transfer of EDRF from donor to detector. Changes in isometric tension were monitored in both the donor and the detector preparations. Release of EDRF, as determined by sustained relaxations during the contractions to phenylephrine, was induced by infusing acetylcholine through the donor artery. **RESULTS:** Superfusion with normal and sickle erythrocytes caused impairment of the endothelium-dependent relaxations in both detector and donor tissues. When infused through the transfer line, sickle cells were less potent than normal erythrocytes in inhibiting relaxation in the detector tissues. In contrast, infusion of either normal erythrocytes or sickle cell through the donor artery caused similar degrees of inhibition in donor and detector arteries. Hemolysates from both types of erythrocytes were equieffective at either site of infusion. **CONCLUSION:** These results indicate that sickle cells are intrinsically less potent scavengers of EDRF than normal erythrocytes. However, exposure to the endothelium enhances the ability of sickle cells to inhibit lumenal release of endothelium-derived relaxing factor.

INTRODUCTION

Microvascular occlusions contribute to the morbidity and the mortality associated with sickle cell anemia[1-4]. In addition to the intracorpuscular changes in these red blood cells that lead to rheological dysfunctions, increased adherence to the endothelium constitues a critical step leading to hypoxic hemodynamic impairment[1,2,4]. Adherence is mediated in part by the unusual exposure of phosphatidylserine at the surface of sickle cells which bind to annexin V at the endothelial cell surface and to the matrix thrombospondin. Furthermore, several receptor-mediated pathways contribute to the enhanced adherence[5-13].

Nitric oxide (NO), a constitutive product of the endothelium, inhibits the adhesion of both normal and sickle erythrocytes[8]. NO maintains the arteries patent

[3] Correspondence to Paul M VANHOUTTE, MD, PhD.
Phn 33-1-5572-6123. Fax 33-1-5572-7276.
E-mail vanhoutt@servier.fr
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through its vasodilator and thrombostatic actions[14,15]. Sickle cells inhibit endothelium-dependent relaxations more potently than normal red blood cells in the rabbit aorta, a preparation where this response is fully accounted for by NO[16-18].

Red blood cells scavenge NO as a function of their content in oxyhemoglobin [19-21]. The content in oxyhemoglobin of sickle erythrocytes is lower, hence, one may anticipate a decreased ability of these erythrocytes to inactivate NO. The present study was designed to verify whether or not sickle cells affect endothelium-derived NO as do normal red blood cells.

MATERIALS AND METHODS

Blood samples were obtained in accordance with the provisions of the Declaration of Helsinki, from consenting pregnant women (four with homozygous hemoglobin S sickle cell anemia, and five without hemoglobinopathies, Tab 1). Twenty ml of blood were collected in tubes containing sodium citrate (0.32 mmol). The tubes were centrifuged (150×*g* for 10 min). The supernatant which contained platelet-rich plasma and the white buffy coat which contained mainly leukocytes were discarded. The lower layer of the packed erythrocytes (0.5 mL) was collected, washed four times in phosphate buffer (0.9 % NaCl, 5 mmol/L NaH_2PO_4, pH 7.4). Stock solutions of erythrocytes were prepared at a concentration of 1×10^{11} cells/L. The concentration of free hemoglobin was below 1 nmol/L using a determination assay (Sigma, ST Louis, MO, USA). The stock solution was diluted as needed to obtain nominal concentrations of ranging from 1×10^{9} to 1×10^{5} red blood cells/L in the perfusate. Hemolysates were prepared from the stock solutions of both types of red blood cells by adding an equal volume of sterile distilled water. The hemolysates were centrifuged at 3000×*g* for 10 min. The ghost-free supernatant, was collected to achieve a stock solution of hemoglobin of approximately 2×10^{-8} mol/L after injection into the cascade bioassay system.

Tab 1. Red blood cell characteristics in blood from donor women. Mean±SEM.

Parameters	Control (*n*=5)	Sickle cell anemia (*n*=4)
Haemoglobin (g/L)	107±26	86±1
Hematocrit (%)	33.4±2.4	25.7±1.1
Red cell count ($\times10^9$/L)	3.8±0.3	3.1±0.2
Mean corpuscular haemoglobin (pg)	31.7±0.2	30.4±0.8
Mean corpuscular haemoglobin concentration (mm^3)	35.2±0.9	34.7±1.1

All procedures were approved by the local animal Protocol Review Committee. Carotid arteries were removed from anesthetised (pentobarbital 3 g/L, iv), and exsanguinated mongrel dogs (15-30 kg). The arteries were placed immediately in cold modified Krebs-Ringer bicarbonate solution (control solution ; composition in mmol/L: NaCl 118.3; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 11.1; calcium sodium edetate 0.026, 37 °C; gassed with 95 % O_2 and 5 % CO_2; pH 7.4). The arteries were cleaned of connective tissue and cut into segments (5 cm length) and rings (4 mm width). In certain preparations, the endothelium was removed[22].

The experiments were performed using perfusion-superfusion bioassay technique[22] (Fig 1). The segments (donor tissues) were cannulated and suspended between two stirrups, one of which was anchored to the bottom of the organ chamber while the second was connected to a force transducer (Grass FTC103, Cleveland, OH, USA) to record isometric changes in tension. A ring of carotid artery without endothelium (bioassay or detector tissue) was suspended below the perfusion chamber and superfused with control solution passing either through a plastic tubing line (direct line) or perfusates from the cannulated segment. The ring of carotid artery was also suspended between stainless steal stirrups for the measurement of isometric force. The perfusion flow rate was adjusted at 5 mL/min using a peristaltic pump (Harvard Apparatus, Southnatick, Mass, USA). The organ chamber had injection ports for the applications of vasoactive substances just before (site 1) and immediately after (site 2) the cannulated artery. A plastic tubing (transfer line) allowed a spacing of 5 s between the perfused segment and the bioassay tissue. A third injection port (site 3) was positioned at the end of the plastic tubing immediately above the latter. Injections of prepared stock solutions were made at constant flow rate of 50 $\mu mol\cdot L^{-1}\cdot min^{-1}$.

The following drugs were used: acetylcholine hydrochloride, *L*-phenylephrine hydrochloride, and indomethacin (Sigma, St Louis, MO, USA). The compounds were dissolved in distilled water, except for indomethacin, which was prepared in Na_2CO_3 and soni-

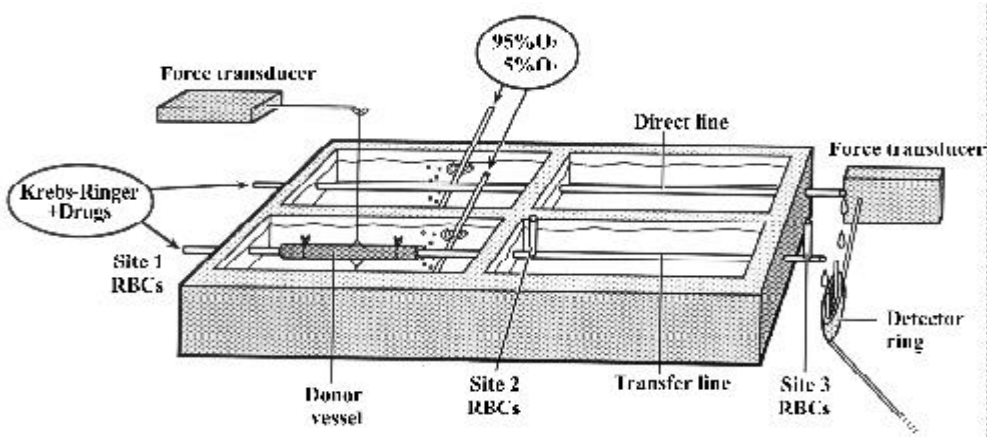

Fig 1. Schematic representation of the used perfusion-superfusion bioassay system.

cated for 10 min. All experiments were carried out in the presence of indomethacin (10 μmol/L; to prevent interference from endogenous prostanoids) added routinely to the control solution perfusing and bathing the tissues. In the bioassay system the segment of carotid artery (donor) served as a source of endothelium-derived NO, which was detected downstream by the ring without endothelium (detector). Both the donor and detector preparations were stretched progressively to the optimal point of their length-active tension relationships (donor: 14.2±1.5 g, n=40; detector: 12.9±1.6 g, n=40), as determined by their response to 60 mmol/L K^+ tested at each level of stretch. After an equilibration period 60 min, the experiment started with the application of phenylephrine to cause contractions in both the donor and detector. At the plateau of the contraction, acetylcholine (1 μmol/L) was applied to the detector ring through the direct line to assess the removal of the endothelium. The solutions of erythrocytes or hemolysates were also tested to determine whether they had direct actions on vascular smooth muscle. Afterwards, acetylcholine was applied through the donor artery to evoke a sustained dilatation and release of endothelium-derived NO, which provoked a relaxation in the detector ring as well. The actions of the solutions of erythrocytes and haemoglobin were tested by injection through the donor (site 1), the transfer line (site 2) or immediately above the detector (site 3) (Fig 1).

Contractions to phenylephrine are expressed in grams (g). The sustained relaxation evoked by acetylcholine and its reduction by red blood cells or hemolysates are given in percentage of the tension developed in the presence of phenylephrine. Results are presented as mean±SEM. Statistical comparisons were performed using one way ANOVA or Student's t-test for unpaired observations. Differences were considered to be significant when P was less than 0.05.

RESULTS

Application of phenylephrine (1 μmol/L; a submaximal concentration to obtain stable responses[22]) evoked sustained contractions in both donor (18.9±4.7 g) and detector (13.8±2.5 g) tissues (n=40) (Fig 2). In the detectors, acetylcholine (1 μmol/L), normal red blood cells or sickle cells produced no direct changes in the contraction evoked by phenylephrine. Acetylcholine infused through the donor artery, caused a near maxi-

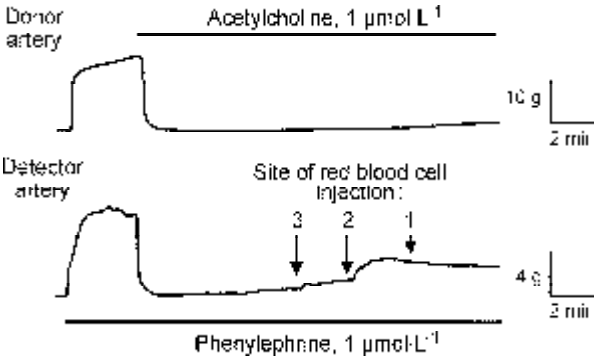

Fig 2. Recording of changes in isometric tension obtained simultaneously in both a donor (top trace) and a detector (bottom trace). Acetylcholine and phenylephrine were applied through the donor (site 1). The detector ring was placed under the donor artery immediately following the infusion of acetylcholine to obtain simultaneous relaxation in both donor and detector preparations. Numbers in brackets indicate sites of infusion of red blood cells in the bioassay system (Fig 1).

mal relaxation (donors: 94.5 %±2.0 % and detectors: 78.4 %±9.1 % of the plateau contraction to phenylephrine, n=40).

Infusion of both normal and sickle red cells through the donor (site 1) caused a time- and concentration-dependent inhibition of the relaxation to acetylcholine in both the donors and the detectors. The concentration-dependent inhibitory effect evoked of normal and sickle red cells on the relaxation to acetylcholine obtained in the detectors did not differ significantly. However, at the concentrations of erythrocytes used, the relaxation of the donor was not inhibited fully (Fig 3).

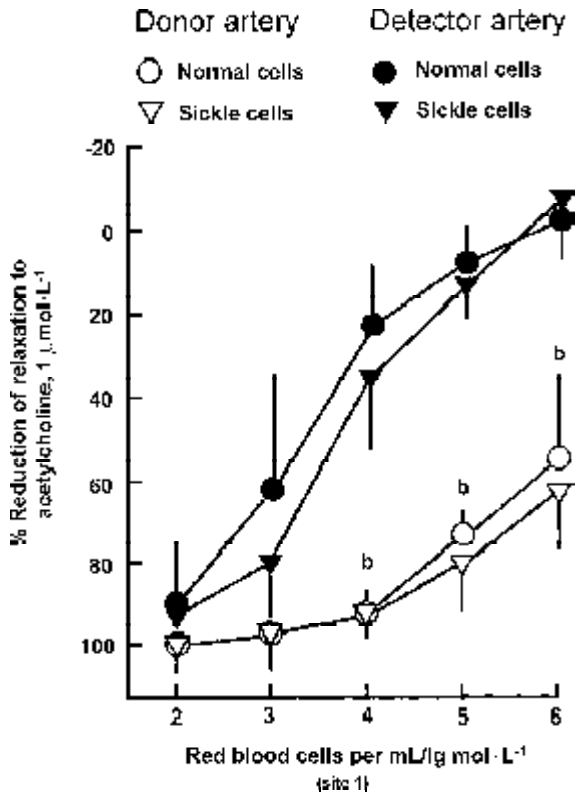

Fig 3. Concentration-inhibition curves to intact red blood cells during endothelium-dependent relaxations to acetylcholine (1 μmol/L) obtained simultaneously in the donors and the detectors during contractions to phenylephrine (1 μmol/L). The sustained level of relaxation induced by acetylcholine is taken as 100 %. Red blood cells in increasing numbers were applied through the donor (site 1, Fig 1). Data were obtained in donors (open symbols) or detectors (closed symbols) treated with either with normal (circles) or sickle (triangles) cells and shown as mean±SEM. ^bP< 0.05 *vs* detector artery.

When injected at site 2, immediately after the donor, both types of erythrocytes caused a concentration-dependent inhibition of the relaxation to acetylcholine in the detector. Normal cells evoked a full inhibition of the relaxation whereas the sickle cells failed to cause maximal inhibition at the highest concentrations used. The concentration-inhibition curve for sickle cells was to the right of that for normal cells (Fig 4).

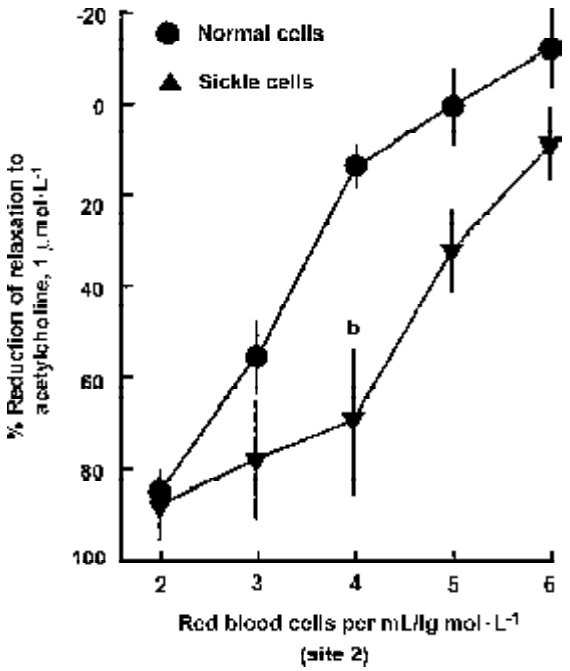

Fig 4. Concentration-inhibition curves to increasing numbers of normal (circles) or sickle (triangles) erythrocytes on the transfer of endothelium-derived NO released by acetylcholine (1 μmol/L) in down arteries superfusing detectors contracted with phenylephrine (1 μmol/L). The relaxation caused by acetylcholine is taken as 100 %. Red blood cells were applied distal to the donor artery (site 2, Fig 1). n=5. Mean±SEM. ^bP<0.05 *vs* normal cells.

The effects of hemolysates of both normal and sickle cells were compared with that of intact cells (1×10^7 cells/L). Both normal and sickle cells inhibited the relaxation when applied at site 3. The normal cells elicited a significantly greater reduction of the relaxation to acetylcholine (29.4 %±3.6 %, at site 2) than the sickle cells (9.8 %±2.1 %, Fig 5). Application at site 2 yielded further inhibition in both cases, but, the normal cells remained significantly more effective than sickle cells. Given at site 1, both cell types caused greater inhibition of the relaxation to acetylcholine than at site 2, but under these conditions, there was no longer a significant difference between normal and sickle cells. The hemolysates (2×10^{-8} mol/L hemoglobin) prepared from normal or sickle cells caused comparable inhibitions when applied at the different sites. The effect at

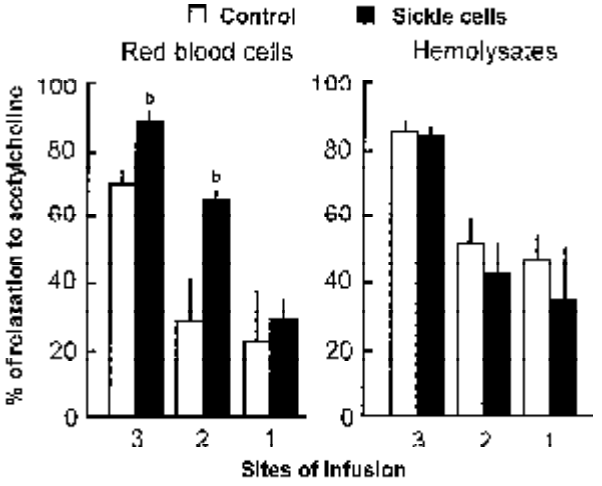

Fig 5. Comparison of the effects of normal (open bars) and sickle (closed bars) erythrocytes (1×10^7 cells/L) and their hemolysates on the action, the transfer or the generation of endothelium-derived NO. The relaxation was obtained in detectors surperfused from the donors during a contraction to phenylephrine (1 μmol/L). The sustained level of relaxation caused by acetylcholine (1 μmol/L) is taken as 100 %. The ordinate represents the percentage of the relaxation that remains after application of red blood cells or hemolysates at site 1, 2, or 3 (Fig 1). n=5. Mean±SEM. $^bP<0.05$ *vs* normal erythrocytes.

site 1 and 2 did not differ, but as significantly larger than at site 3 (Fig 5).

DISCUSSION

The present study demonstrates that sickle cells scavenge endothelium-derived NO to a lesser extent than normal red blood cells. However, an undetermined mechanism enables them to compensate for the deficit in NO scavenging potency after they have been in contact with endothelial cells. The present study also demonstrates that hemoglobins prepared from sickle and normally cells are equally effective in scavenging endothelium-derived NO. Direct application of erythrocytes or hemolysates to the detector tissue showed that, the vascular smooth muscle of the canine carotid artery does not contract in response to hemoglobin or sickle cells. Since leukocytes and platelets were separated from the red blood cells, all of the observed effects of intact red blood cells or hemolysates may be attributed to the inhibition of either the action, the half-life or the release of endothelium-derived NO.

In the first set of experiments, the erythrocytes were added through the donor artery, during the sustained stimulation of the release of endothelium-derived NO by acetylcholine. The concentration-inhibition curves obtained with sickle cells in both the donor and detector arteries overlapped with those obtained using normal red blood cells. This contradicts the claim that sickle cells are more potent inhibitors of endothelium-dependent relaxations[10]. However, the inhibition of the relaxation in the detector was more complete than that obtained in the donor artery. This reflects the inability of erythrocytes to cross the endothelial cell layer. *In vivo*, red blood cells have little effect on NO released abluminally in large arteries, because of their central position in the blood stream during laminar flow. However, taking into conjunction the absence of platelets and white blood cells, which occupy peripheral positions in the blood stream, and the low flow imposed, under the present experimental conditions the erythrocytes could interact intimately with the endothelial surface.

To cancel out the influence of membrane to membrane interactions, concentration-inhibition curves to the erythrocytes were also obtained by applying the cells immediately distal to the donor artery. In this case, sickle cells were less potent than normal red blood cells in scavenging NO. In comparison with effects obtained following infusion through the donors, sickle cells lost some of their effectiveness after application through the transfer line, whereas, normal red blood cells retained the same inhibitory potency. This suggests that membrane to membrane interactions contribute to the potency of sickle cells to inhibit endothelium-dependent vasodilatation. This is consistent with the observation that ghosts of sickle cells impair endothelium-dependent relaxation[16].

Infusion of the erythrocytes through the transfer line permits evaluation of either chemical inactivation of NO or blockade of its action on vascular smooth muscle. To distinguish between the two possibilities, experiments were carried out using a concentration of red blood cells that exerted only threshold actions on the vasodilatation obtained in the donor. Applications of red blood cells were first performed immediately above the detector, then across the transfer line, and through the donor artery. The small, but significant, inhibition caused by erythrocytes when applied immediately above the detector was amplified acutely after application of either type of red blood cells through the transfer line. Likewise, known scavengers of endothelium-derived NO are effective when given more time to interact with the endogenous nitrovasodilator[23]. Nor-

mal red blood cells did not cause further inhibition after passage from the transfer line to the donor which was consistent with the results obtained during the concentration-inhibition curves. By contrast, sickle cells caused a further inhibition to match the inhibitory action of normal red blood cells. However, the solutions of hemoglobin prepared from both normal or sickle red blood cells evoked small, but significant, inhibition that was enhanced after application through the transfer line. Passage of the hemolysates through the donor did not cause further inhibition. These findings are consistent with the ability of hemoglobin to scavenge NO, without interfering with the stimulation of the release of NO by endothelial cells. Oxyhemoglobin is the most potent NO scavenging form of hemoglobin[18]. Sickle cells have a lower content in oxyhemoglobin, due to the higher concentrations of 2,3-diphosphoglycerate and the intracorpuscular polymerisation of deoxyhemoglobin S. Accordingly, sickle cells are less potent scavengers of NO. However, in solution hemoglobin S and hemoglobin A have equal affinity for oxygen. This explains why in the oxygenated physiological saline the two types of hemolysates were equally potent scavengers of NO.

The additional inhibitory action of sickle cells results from the characteristics of the interactions between these cells and the endothelium. Hemoglobin A or S solutions had comparable effects. The molecular basis for the interactions of sickle cells with endothelium that permitted this enhancement was not investigated in the present study. However, a number of pathways exist that account for the stronger adherence of these erythrocytes in comparison with normal red blood cells[5-10,24]. Under laminar flow conditions red blood cells are not in frequent contact with endothelial cells. Hence, the phenomenon observed under the present experimental conditions may not be in effect in large arteries *in vivo*.

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