Blood Cells, Molecules and Diseases 55 (2015) 328–335

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Filterability of freshly-collected sickle erythrocytes under venous oxygen pressure without exposure to air

Siddharth Shah *, Rhonda Graves Acholonu, Kwaku Ohene-Frempong, Toshio Asakura

Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA, USA

ARTICLE INFO

Article history:
Submitted 16 February 2015
Accepted 22 June 2015
Available online 23 June 2015

Editor: Mohandas Narla

Keywords:
Sickle cell disease
Filterability
Partially oxygenated sickled cells
Partially deoxygenated sickled cells

ABSTRACT

We previously found that blood samples collected from steady-state patients with sickle cell disease (SCD) without exposure to air contain a new type of reversibly sickled cells (RSCs) with blunt edges at a level of as high as 78%. Since partial oxygenation of once-deoxygenated sickled cells with pointy edges to near venous oxygen pressure generates similar sickled cells with blunt edges *in vitro*, we named them as partially oxygenated sickled cells (POSCs). On the other hand, partial deoxygenation of once-oxygenated SS cells to venous oxygen pressure generates partially deoxygenated sickled cells (PDSCs) with pointy edges. In this study, we obtained blood samples from 6 steady-state patients with SCD under venous oxygen pressure without exposure to air, subjected them to various oxygenation/deoxygenation/reoxygenation cycles, and studied their filterability through a membrane filter with pore diameter of 3 μm, the theoretical minimum diameter of a capillary. Our results indicated that discocytes, POSCs with blunt edges, and irreversibly sickled cells could deform and pass through the filter, while PDSCs with pointy edges were rigid and could not. The filterability of SS cells seems to be related to the length and amount of deoxy-hemoglobin S fibers in the cells.

1. Introduction

A single glutamic acid-to-valine amino acid substitution at the 6th residue of the β-globin chain is responsible for almost all of the pathological problems seen in patients with sickle cell disease (SCD). Under hypoxic conditions, the deoxygenated form of sickle hemoglobin (deoxy-Hb S) in sickle erythrocytes (SS cells) polymerizes and rigid Hb S fibers subsequently distort the cell to assume a sickle shape that is characterized by having pointy edges [1–5]. Normal red blood cells (AA cells), which have a diameter of approximately 8 μm, can easily pass through the 3-μm theoretical minimum cylindrical diameter of the human circulatory system because AA cells are flexible [6]. Rigid sickled cells containing long, well-aligned deoxy-Hb S fibers cannot deform and therefore occlude capillaries and small vessels, causing various tissue and organ damages. Thus, the cause of SCD and the mechanism of sickling-dependent vaso-occlusion have been elucidated.

However, there has been an unanswered question about the existence of sickled cells in the blood of patients with SCD. As pointed out by Bunn and Forget [5], the results of *in vitro* studies on the PO_2-*vs.*-sickling relationship suggest that SS homozygotes would have a significant number of reversibly sickled cells (RSCs) at oxygen tensions normally encountered in the capillary and venous circulation. However, the measurement of the percentage of RSCs *in vivo* had been unexpectedly low. We found that the low reported percentage of sickled cells in the blood of patients with SCD is attributed to the oxygenation of RSCs to discocytes by the oxygen in the volume of air in the needle, syringe and blood collection tube during and after blood collection [7]. If venous blood is collected from steady-state patients with SCD under venous oxygen pressure without exposure to air, the blood samples contain as high as 78% sickled cells with blunt edges [7–11].

Studies *in vitro* showed that one of two types of RSCs is formed under venous oxygen pressure depending on whether SS cells are deoxygenated or oxygenated to venous oxygen pressure. When oxygenated SS cells are slowly deoxygenated to near venous oxygen pressure (PO2 = 40 mm Hg), classic sickled cells with pointy edges are newly formed. Upon further deoxygenation of the SS cells to 0 mm Hg and then re-oxygenation to near venous oxygen pressure (PO2 = 40 mm Hg), some deoxy-Hb S molecules are oxygenated and sickled cells with blunt edges are observed [7–9,11]. To distinguish these two types of RSCs that form under the same range of PO_2 near venous oxygen pressure, we named the former cells with pointy edges as partially deoxygenated sickled cells (PDSCs) and the latter cells with blunt edges as partially oxygenated sickled cells (POSCs) [7–9,11]. The RSCs found in the blood of steady-state patients with SCD are POSCs with blunt edges.

To answer the question of why steady-state patients with as high as 78% sickled cells with blunt edges reveal no sickling-dependent symptoms, we studied the filterability of POSCs through a 3-μm pore

Abbreviations: PDSC, partially deoxygenated sickled cell; POSC, partially oxygenated sickled cell; RSC, reversibly sickled cell; CSF, circular shape factor; ESF, elliptical shape factor; ISC, irreversibly sickled cell.

* Corresponding author at: Hospital of the University of Pennsylvania, Renal Division, 1 Founders Building, 3400 Spruce Street, Philadelphia, PA 19104.
E-mail address: siddharth.shah@uphs.upenn.edu (T. Asakura).

http://dx.doi.org/10.1016/j.bcmd.2015.06.012
1079-9796/

membrane filter (3-micron filter) under venous oxygen pressure. The results are compared with the filterability of SS cells that are fully oxygenated, SS cells that are fully deoxygenated, and PDSCs.

2. Material and methods

2.1. Collection of blood without exposure to air

Six patients with homozygous SCD who were in the steady-state and were not currently receiving any specific treatment or blood transfusion were selected from The Comprehensive Sickle Cell Center at The Children's Hospital of Philadelphia. Blood samples were collected from steady-state patients at the time of clinic visit for routine check-up. A blood sample was also obtained from one normal subject without SCD. Of the 2.5 ml of the blood sample that was collected for routine blood tests, approximately 0.5 ml of the blood sample was used for this study. Blood was drawn from the median antecubital vein without exposure to air by the method described elsewhere [7]. Aliquots were immediately transferred into three tubes. One drop of fresh blood was added to Tube-1 through the rubber stopper from the butterfly connected to the syringe containing the fresh blood. Tube-1 contained carbon monoxide (CO) gas and was used to determine the percentage of irreversibly sickled cells (ISCs). Tube-2 contained 0.5 ml of 2% glutaraldehyde solution (2% GAD) and was previously equilibrated with 5% O_2/95% N_2 gas mixture (hereafter referred to as 5% O_2) (PO_2 = ~40 mm Hg). One drop of fresh blood was added to Tube-2 through the rubber stopper without exposure to air to immediately fix the red blood cells (RBCs) and was used for morphological analysis of RBCs in venous blood. Tube-3 contained EDTA under 5% O_2/95% N_2. Approximately 0.5 ml of fresh blood was transferred to Tube-3 through the rubber stopper without exposure to air. This blood was used for the five filterability experiments that were performed at various oxygen pressures. This work was approved by the IRB Committee of the institution. Consent forms were signed by the blood donors or their guardians.

2.2. Determination of the percentages of discocytes and ISCs

2.2.1. Determination of the percentage of discocytes by computer-assisted image analysis

The percentage of discocytes in the pre-filtration, post-filtration, and CO aliquots was determined by computer-assisted image analysis as described by Horiuchi et al. [12]. Briefly, a suspension of fixed cells was introduced into microslides and multiple fields of RBC images were captured and saved to a computer. The elliptical shape factor (ESF; ESF = [short axis]/[long axis]) and circular shape factor (CSF; CSF = $4\pi \times$ [area] / [perimeter]^2) of over 200 cells from 5 or more fields were determined. Cells with ESF < 0.5 are designated as elongated sickled cells, cells with CSF < 0.8 and ESF > 0.5 as non-elongated sickled cells, and cells with CSF > 0.8 and ESF > 0.5 as discocytes. Data were organized into CSF/ESF bivariate scattergraphs.

2.2.2. Determination of the percentage of ISCs

Tube-1 (1 drop of blood in CO gas) was kept on ice for 2 h. Under these conditions, CO binds tightly with hemoglobin, causing all RSCs to convert to discocytes and leaving only ISCs as deformed cells [8]. The cells were then fixed with 2% GAD. An aliquot of the blood was placed on a microslide and subjected to computer-assisted image analysis to determine the percentage of ISCs.

2.3. Procedures of the filterability studies performed under various oxygen pressures and calculation of filterability

2.3.1. Filterability study of fresh RBCs performed under venous oxygen pressure without exposure to air

An aliquot (30–35 μl) of the whole blood in Tube-3 was transferred under venous oxygen pressure (5% O_2) to a tube containing 2.22 ml of Hemox buffer (pH 7.4). The buffer solution was previously equilibrated with 5% O_2. After the blood and buffer were gently mixed, half of the suspension was removed and used as the pre-filtration fraction. A small volume (~15 μl) of the pre-filtration fraction was removed and fixed with 2% GAD (previously equilibrated with 5% O2) for morphological analysis. The other half of the suspension was filtered through a 25 mm-diameter Isopore polycarbonate filter membrane with a pore size of 3 μm (Millipore, Billerica, MA) within a 25-mm stainless steel filter cassette that was kept at 5% O_2. Fig. 1 shows a diagram of the cell filtration apparatus. Each time the filter was used, a new filter membrane was placed in the filter cassette. Prior to use, the membrane was soaked in deionized water for more than 3 min and excess water was blotted off. The filter apparatus and all accessories were previously equilibrated with 5% O_2. The blood suspension was filtered by applying a mild vacuum and the contents that passed through the filter were collected in a test tube placed in the vacuum flask. A small volume (~15 μl) of the post-filtration fraction was removed from the bottom of the test tube and fixed with 2% GAD (previously equilibrated with 5% O_2) for morphologic analysis. The pre-filtration fraction and post-filtration fraction were each transferred to Eppendorf centrifuge tubes and centrifuged at 5000 rpm at room temperature for 1 min. The supernatants were transferred by pipette into 1 ml clear-glass cuvettes, and their Hb concentrations and solution volumes were determined. The RBC pellets were hemolyzed with 5 mM potassium phosphate buffer, pH 7.5, containing 0.5 mM EDTA [13]. The Hb absorption peak at $\lambda = 577$ nm (ABS@ 577 nm) was used to measure the Hb concentration in the supernatants and the Hb concentration in the hemolyzed RBC pellets with a Hitachi UV–VIS spectrophotometer (Tokyo, Japan). Total Hb concentrations and quantities (supernatant plus hemolyzed RBC pellet) were also determined. Finally, to study the morphology of cells trapped on the 3-μm-pore filter membrane, 2% GAD was passed through the filter under 5% O_2 to fix the cells. The filter cassette was then turned upside down and physiological saline was passed through the filter cassette to free the fixed trapped cells, which were collected in a test tube.

2.3.2. Calculation of filterability of RBCs through the 3-micron filter

Since it is difficult to accurately determine the number of RBCs in a small sample, we calculated the RBC mass (grams of RBCs) in the pre-filtration and post-filtration fractions from the Hb concentrations. Although the Hb concentrations vary from patient to patient, the following approximation for filterability is sufficient because we are only concerned with the relative amounts of Hb before and after filtration. One mole, or 32 g, of oxygen combines with 16,700 g of hemoglobin, and therefore the combining weight of hemoglobin is 16,700 g. Eq. (1) shows our method of conversion:

$$\text{Grams of RBCs} = \frac{(\text{ABS@577nm } mMol)}{(14.5)(L)} \text{X} \frac{(1\ Mol)}{(1000\ mMol)} \text{X} \frac{(16700\ grams)}{(1\ Mol)} \text{X} \frac{(1\ L)}{(1000\ ml)} \text{X } (\text{Y } ml) \quad (1)$$

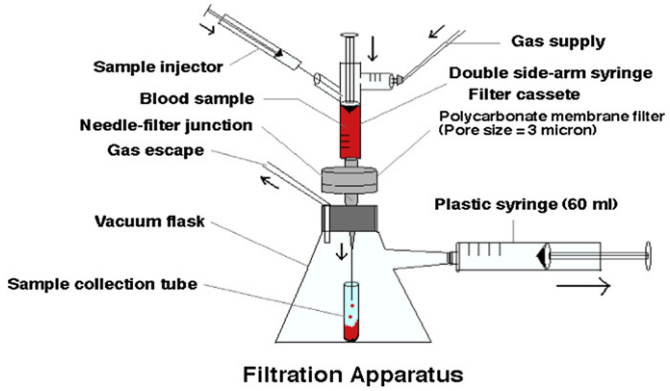

Fig. 1. Diagram of the filtration apparatus used in the filterability experiments.