Partially oxygenated sickled cells: Sickle-shaped red cells found in circulating blood of patients with sickle cell disease


*Division of Hematology, The Children's Hospital of Philadelphia, and Departments of Pediatrics, Biochemistry and Biophysics, and Human Genetics, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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ABSTRACT A previously uncharacterized type of sickled cell was found in venous blood of patients with sickle cell disease when blood was collected without exposure to air and fixed immediately with 1% glutaraldehyde solution equilibrated with 5% oxygen. These cells were either elongated, resembling irreversibly sickled cells (ISCs), or nonelongated, with a raisin-like shape. Both types assumed a normal discoidal shape upon full oxygenation. Since these cells exist only under partially oxygenated conditions, they are described as partially oxygenated sickled cells (POSCs). POSCs are morphologically distinct from partially deoxygenated sickled cells formed during deoxygenation by having rounded edges, while the latter have sharp edges. Transmission electron microscopy of POSCs revealed various amounts of misaligned Hb S polymers. Investigations in vitro demonstrated the formation of POSC-like cells by partial oxygenation of deoxygenated cells. Since POSCs contain intracellular fibers and sickle readily upon deoxygenation, they may have clinical and pathological significance.

Sickle cell disease was so named because of the abnormal morphology of red blood cells (RBCs) observed by microscopy of fixed and stained blood smears obtained from affected patients (1). Subsequently, relationships were found between oxygen and RBC sickness (2) and between intracellular polymerization of abnormal hemoglobin and cell deformation (3–5). Demonstration of sickness of RBCs from patients with sickle cell disease (SS cells) in vitro is highly dependent upon the method of deoxygenation. For example, classic sickle- or crescent-shaped cells are formed after slow deoxygenation (6–9), whereas SS cells with a granular or mosaic appearance result from rapid deoxygenation with nitrogen (6) or sodium dithionite (7). These differences may be explained by the number and size of domains of polymerized Hb S formed in the cells (10). During morphologic studies of SS cells, we noticed that the number and shape of sickled cells in venous blood were altered by the oxygenation of blood during and after blood collection. To avoid postcollection artifacts, we developed a method to collect blood under venous oxygen pressure without exposure to air. This process enabled us to discover two additional types of reversibly sickled cells, elongated sickled cells with a shape like irreversibly sickled cells (ISCs) and shrunken cells with a raisin-like appearance. Since these cells exist only under partially oxygenated conditions, they are described as partially oxygenated sickled cells (POSCs) (11). We report our investigation of the morphologic properties and analysis by electron microscopy of intracellular polymers of POSCs found in venous blood of patients with sickle cell disease.

MATERIALS AND METHODS

Preparation of Blood Samples. To minimize sickness during blood drawing (12), blood was drawn from the antecubital vein after release of the tourniquet. A conventional 5-ml plastic disposable syringe was used by the technique similar to that used for collection of samples for blood gas analysis. Since the initial portion of blood entering the syringe may have been exposed to the small amount of air that existed in the syringe, we drew excess blood for the experiments and were careful to use only the middle fraction of blood drawn (1.5–2 ml). This was immediately injected into a 2-ml EDTA vacutainer previously equilibrated with 5% oxygen (5% O₂/95% N₂). A 0.2-ml aliquot of the 2 ml sample was transferred into another vacutainer containing 1 ml of a 1% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) also previously equilibrated with 5% oxygen. This fixed sample was used for morphologic analysis of SS cells and for electron microscopic examination of intracellular polymers. To confirm that the initial blood sample had not been exposed to air, we drew blood into a specially designed plastic syringe with a side arm. The side arm was attached to the barrel of the syringe near its base, and a small hole was made through the side arm into the syringe barrel so that the movement of the plunger was not impeded. Gas can be introduced only when the syringe plunger is pulled back beyond the side arm. The gas flows through the syringe and out an attached needle so that the syringe is filled only with desired gas. After equilibration, the plunger can be pushed forward and the syringe used for the transfer of samples at desired atmospheric conditions. Data on morphologic analysis as well as partial oxygen pressure of these reference samples were indistinguishable from those of samples prepared by our standard method described above. To avoid subsequent contamination with air, blood was transferred from the EDTA vacutainer using the syringe with a side arm.

Collection of Red Cells at Various Po2. To demonstrate the formation of POSC-like cells in vitro, morphologic studies of SS cells at various Po2 were carried out using a Hemox analyzer (TCS Medical Products, Southhampton, PA) (13). Before each experiment, 3 ml of buffer containing inosine (8) was placed in the cuvette and equilibrated with 25% oxygen at 37°C. A small volume (10 μl) of 10% silicone solution (SAG 10; Union Carbide) was added to prevent foaming. The buffer was then deoxygenated slowly with nitrogen gas. Blood (0.6 ml) collected under venous oxygen pressure was introduced into the cuvette using the syringe with a side arm when the Po2 in the cuvette reached 40 mmHg. This red cell suspension was then slowly deoxygenated by flushing with nitrogen gas. The suspension was subjected to one cycle of oxygenation and deoxygenation. To study the morphology of SS cells, aliquots (10 μl) of suspensions of SS cells in the cuvette were collected with a 100-μl Hamilton syringe containing 30 μl of 1% glutaraldehyde solution, at Po2 = 40, 0, 40, 90, 180, 90.

Abbreviations: RBC, red blood cell; SS cell, RBC from patient with sickle cell disease; ISC, irreversibly sickled cell; POSC, partially oxygenated sickled cell; CSF, circular shape factor; ESF, elliptical shape factor; PDSC, partially deoxygenated sickled cell.

†To whom reprint requests should be sent at the address.

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40, and 0 mmHg (Fig. 1). The red cell suspension and the glutaraldehyde solution were mixed in the syringe and used for morphologic analysis.

**Morphologic Studies of Red Cells at Various Oxygen Tensions.** Morphologic analysis of fixed and stained red cells was performed in a microslide (7), with the use of a computer-assisted image analysis apparatus (14). Two parameters, circular shape factor (CSF) and elliptical shape factor (ESF), were determined to evaluate the shape differences of red cells (14). From each sample, ~200 cells from at least eight different fields were analyzed. Distribution of shape factors was analyzed by a CSF-ESF scattergram (14). Electron micrography was performed with a Philips 300 electron microscope. Specimens were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After washing in 0.1 M cacodylate buffer (pH 7.4) three times, the specimens were fixed in 1% OsO₄ for 30 min at room temperature. Samples were dehydrated in graded alcohol and embedded in Epon-EM-Bed-812. Thin sections were cut using an LKB Ultratome III microtome.

**RESULTS**

**Morphology of Red Cells Collected and Fixed Under Venous Oxygen Pressure.** If freshly drawn blood is fixed immediately with 1% glutaraldehyde solution without exposure to air, the morphology of red cells can be considered to reflect that of circulating SS cells. Fig. 2 shows morphologies of SS cells obtained from three patients. In patient A (Fig. 2A) almost all cells are discoidal. In patient B (Fig. 2B) there are large morphologic variations, including elongated sickle-shaped cells, star-shaped cells, and discoidal cells, as well as collapsed cells resembling raisins. Most elongated sickle cells lack sharp protrusions and resemble ISCs. Patient C has mainly elongated and discoidal cells (Fig. 2C). The percentages of ISC-like cells in patients A, B, and C were 1%, 20%, and 27%, respectively. We found that most ISC-like and raisin-like cells reverted to a normal discoidal shape upon bubbling with 25% oxygen or carbon monoxide (Fig. 2D). The percentages of true ISC in the fully liganded samples were <3%. Thus, most ISC-like and raisin-like cells are elongated and nonelongated POSCs, respectively. Transmission electron microscopy of POSCs demonstrates the presence of fibers (Fig. 3). The fibers seen in these cells, however, do not have the ordered alignment usually seen in completely deoxygenated SS cells (Fig. 3B). Hb S polymers in POSCs vary widely in amount and structure; some cells have almost no distinct fibers (Fig. 3C), whereas others have short disordered fibers (Fig. 3D). Other cells contain polymer-like structures that appear to be remnants of larger aggregates as shown in Fig. 3F. These results suggest that ISC-like and raisin-like cells found in fresh venous blood of patients with sickle cell disease are POSCs containing various amounts of disordered Hb S polymers. Sharp protrusions have disappeared or were not formed due to the partially oxygenated environment of venous blood.

To study the morphologic variation of circulating red cells among patients and the percentage of POSCs, we analyzed SS cells with a computer-assisted image analysis apparatus (14). Fig. 4 shows nine representative CSF–ESF scattergraphs of SS cells from eight patients. Each point represents one cell. Since both CSF and ESF values are unity for discoidal cells, points for these cells fall in the top right area, while those for elongated and nonelongated POSCs fall in the bottom left and top left areas of the scattergraph, respectively. The CSF–ESF scattergraph shown in Fig. 4 clearly demonstrates a wide morphologic variation in SS cells among patients. Some patients have many discoidal cells (Fig. 4A and G), whereas others have an increased number of elongated cells (Fig. 4C and F) or star- or raisin-shaped cells (Fig. 4B, D, E, and H). The CSF–ESF scattergraphs of patients A, B, and C in Fig. 2 are shown in Fig. 4A, B, and C, respectively. It is quite interesting to point out that the CSF–ESF scattergraph of the same patient, whose blood was drawn at an interval of 2 weeks, revealed a similar pattern (Fig. 4C and F).

**Morphology of Red Cells at Different PO₂.** To study if POSCs can be produced in vitro, the morphologic changes of red cells at different oxygen pressures were studied by changing the PO₂ of suspensions of SS cells. As shown in Fig. 5A, complete deoxygenation of SS cells (PO₂ = 0 mmHg) resulted in the formation of many sickle cells with sharp

![Figure 1](image1.png)  
**Fig. 1.** Sampling of SS cells at various PO₂ levels. Fresh SS blood was first suspended in a buffer previously equilibrated at PO₂ = 40 mmHg. The suspension was deoxygenated by flushing with nitrogen, oxygenated with 25% oxygen, and deoxygenated again with nitrogen. Samples for morphologic examination were obtained at PO₂ = 40, 0, 40, 90, 180, 90, 40, and 0 mmHg, as indicated by the arrows, and fixed immediately with 1% glutaraldehyde solution previously equilibrated at the same PO₂. The time course of this experiment was about 2 hours per sample.

![Figure 2](image2.png)  
**Fig. 2.** Morphology of venous SS cells obtained from three patients with sickle cell disease. Venous blood (without exposure to air) collected from three patients (A–C) was fixed immediately in 1% glutaraldehyde solution. SS cell suspensions were transferred into microslides and observed directly by microscopy. The picture in D was taken after oxygenation of the sample in B with 25% oxygen. (×680.)
protrusions. Although pictures are not shown, the morphology of the sickled cells with sharp protrusions was unchanged between 0 and 20 mmHg. This result agrees with the previous work showing that sickling is complete below 20 mmHg (15–17). By further oxygenation to 40 mmHg, almost all sickled cells lose sharp edges and convert to POSCs with dull edges (Fig. 5B), which are indistinguishable from those seen in the blood specimen collected from patients and fixed without exposure to air. Upon further oxygenation, the number of POSCs started to decrease, and at about 120 mmHg they almost disappeared. Above 120 mmHg, most cells became discoidal except for a small number of ISCs (Fig. 5C). The oxygenated red cell suspensions were deoxygenated again. No new sickle cells were formed until Po2 reached 40 mmHg. Samples obtained at 40 mmHg during deoxygenation contained mainly star-shaped sickled cells, with most having sharp edges (Fig. 5D). To distinguish these cells from POSCs, we call them partially deoxygenated sickled cells (PDSCs). The number of elongated sickle cells was much less than that observed during the first deoxygenation and the following oxygenation processes. These results clearly demonstrate that POSC-like cells are formed in vitro when deoxygenated SS cells were partially oxygenated. The morphology of SS cells under partially oxygenated conditions undergoes a dynamic change, reflecting not only the Po2 but also whether the blood has been previously oxygenated or deoxygenated.

**DISCUSSION**

Of interest is the existence of ISC-like cells, which we have described as POSCs, in circulating blood of patients with sickle cell disease. Percentages of elongated POSCs ranged between 1% and 41% among 54 patients studied. Although their morphology is similar to ISCs, these cells are not ISCs, as they regain their discoidal shape upon oxygenation or bubbling with carbon monoxide gas. Electron microscopy of POSCs revealed the presence of fibers, which are more disordered than those in completely deoxygenated sickled cells. It appears that intracellular fibers are partially disso-
associated due to oxygen present in venous blood. These ISC-like cells are present in patients' circulating blood, as are another type of POSCs, which have a raisin-like morphology. Kinetic studies of polymer formation in vitro during deoxygenation and reoxygenation of SS cells by Hahn et al. (18) support our results. They found that during reoxygenation of completely deoxygenated sickled cells, reversibly sickled cells containing a loose meshwork of randomly oriented fibers were formed and that there was a great variation in the rate of depolymerization among cells.

High percentages of ISCs in some previous reports (19–21) may be due to the inclusion of what we describe here as POSCs. As detailed by Rodgers et al. (22), the accurate quantification of ISCs requires rigid methodological criteria—i.e., complete saturation of Hb S either with oxygen or carbon monoxide. They reported that the percentage of ISCs in the peripheral blood after incubation with carbon monoxide was 6.5% ± 3.5%. We confirmed this result.

Although the existence of reversibly sickled cells in both venous and arterial blood has been reported (16, 23, 24), no detailed characterization of the nature of those cells has been done. Sherman (6) reported that 5–20% of erythrocytes in arterial blood appeared in a form best described as "incompletely deoxygenated sickled cells." In our study incompletely deoxygenated cells have pointy filamentous ends and are clearly different from ISCs or POSCs. More recently, Zipursky et al. (25) developed a method for the detection of reversibly and irreversibly sickled cells. They reported that reversibly sickled cells are bizarre, irregularly shaped cells with multiple pointy projections. These cells are similar to PDSCs and differ from those found in venous blood as described in this paper.

We demonstrated the formation of POSCs in vitro by partial oxygenation of completely deoxygenated SS cells (Fig. 5B), the number of which depends on the number of sickled cells under completely deoxygenated conditions. Similar results were obtained by using sickled cells that were exposed to oxygen pressures below 20 mm Hg, the oxygen pressure under which sickling is complete (15–17).

The morphology of POSCs depends on the shape of sickled cells from which they form; elongated POSCs are formed from elongated sickle cells, while nonelongated POSCs are formed from star-shaped cells. As schematically shown in Fig. 6, we assume that POSCs are deformed cells in which sharp edges or needle-like protrusions were lost due to partial dissociation of deoxy-Hb S polymers by venous oxygen. Recent studies by Briehl and Guzman (26), who demonstrated that the Hb fibers melt from both ends rather than from the middle, support this hypothesis. As shown elsewhere (8), the number of elongated sickle cells increased during deoxygenation and oxygenation cycles; such conditions, as in the circulatory system, appear to promote the formation of elongated sickle cells. It is interesting to note that POSCs were not formed during deoxygenation (Fig. 5D). Under this condition, a significant number of PDSCs with pointy edges were formed. The existence of POSCs without pointy edges in venous blood suggests that these cells were formed by partial oxygenation of sickled cells that have...
passed thorough environments with oxygen pressures <20 mmHg.

POSCs can also be distinguished from ISCs by the presence of intracellular Hb S polymers. Transmission electron micrographs of the intracellular structure of POSCs demonstrated widely varying amounts of Hb S polymers, from almost undetectable to noticeable amounts of well-aligned polymers. These findings support the idea that POSCs are deformed due to the existence of partially dissociated Hb S polymers. Noguchi and Schechter (27) suggested the presence of varying amounts of polymers in SS cells. POSCs may be those cells that contain different amounts of disordered fibers. Our results suggest that the amount and the degree of alignment of polymers in such SS cells are different, depending on whether the cells were previously oxygenated or deoxygenated. Studies of SS blood at different oxygen pressures by Harris et al. (17) show a relatively high viscosity of SS cells at Po2 near 40 mmHg, which reflects the presence of intracellular polymers in sickled cells and POSCs.

Plots of CSF-ESF data can graphically illustrate morphologic differences in SS cells, such as discoidal, crescent, and star shapes, etc. The CSF-ESF scattergraphs of SS cells obtained from different patients vary greatly (Fig. 4). The patterns of CSF-ESF scattergraphs may be classified into at least three groups: (i) mainly discoidal red cells, (ii) discoidal and elongated red cells, and (iii) red cells with various morphologies including discoidal, elongated, and star-shaped cells. Two scattergraphs taken 2 weeks apart from the same patient were very similar. Although data are limited at present, it is interesting to speculate that the patterns may be specific to individual patients due to the environment of the vascular system. The pathological significance of POSCs is not known at present and must be further investigated in venous and arterial blood. Jensen et al. (19) and Serjeant et al. (21) collected venous blood from various sites using the catheterization method. Jensen et al. (19) found that the number of sickled cells was remarkably constant in the various venous sites of a given patient, despite moderately wide differences in oxygen saturation, whereas Serjeant et al. (21) reported an inverse relationship between oxygen saturation and the RBC sickling in vivo except in blood collected from the hepatic vein. Eaton and Hofrichter (10) reported that complete depolymerization may not occur before cells enter the microcirculation on their next trip, either because the total hemoglobin concentration is greater than the solubility of oxygen at arterial oxygen pressure or because the total hemoglobin concentration is so close to the solubility that depolymerization is slower than 1–2 sec spent in the arterial circulation. Cells entering the microcirculation with partially polymerized Hb S will undergo rapid and complete polymerization upon deoxygenation because the rate of heterogeneous nucleation is enormously increased or nucleation is complete. In these cells, there may be sufficient polymerized hemoglobin to initiate rapid sickling so that the deformed cells become lodged in the arterioles (10). POSCs may be able to cross from venous blood to arterial blood either when the Po2 of the alveoli is decreased or when there is a shunt.

Assuming that POSCs containing fragments of Hb S polymers enter the arterial circulation under hypoxic conditions, POSCs will sickle without a delay time. The relationship of the percentage of POSCs to mean cell volume, mean corpuscular hemoglobin concentration, α-gene number, β-globin haplotype, and Hb F level needs to be studied with respect to patients’ clinical course. It should also be interesting to determine changes in the percentage of POSCs before, during, and after vasoocclusive events.

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