Ligand State of Intraerythrocytic Circulating HbC Crystals in Homozygote CC Patients

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Whole blood and Stractan-Percoll fractions of blood from splenectomized patients with homozygous hemoglobin C (CC) disease were studied under aerobic and anaerobic conditions. Erythrocytes containing typical CC crystals are found in the densest fraction as documented by freeze-fracture electron microscopy. We report that the intraerythrocytic Hb C circulating crystals are in the oxygenated liganded state as demonstrated by melting upon deoxygenation and by absorption spectroscopy. Furthermore, crystals are more likely to form in cells with low concentration of Hb F. Changes of ligand state (which results in melting of the intraerythrocytic crystal) might be involved in the pathophysiology of this disease, removing the danger of vasoocclusive episodes.

SOME SPECIES normally have tetrameric hemoglobin that exhibit ligand-dependent polymerization (e.g., deoxy-dependent in tetrameric clam hemoglobins,3 oxy-dependent in deer red cells5). In humans, hemoglobin aggregation or polymerization occurs in individuals with defined mutations: Hb S (β 6 Glu → Val) in which the polymer forms after the molecule is deoxygenated,5 or Hb C (β 6 Glu → Lys) in which in vitro crystallization has been demonstrated in other ligand states but with some preference to the deoxy.4 Moreover, in vivo, crystals are found in circulating erythrocytes of splenectomized homozygous Hb C individuals with a mild chronic hemolytic anemia.3,5 Because in vitro studies show that Hb C crystallizes in different unit cell arrays3,6 according to its ligand state (oxy, deoxy, CO), it is appropriate to ask, to which ligand states do the in vivo crystals belong and what is the potential consequence of this phenomenon on the pathophysiology of CC disease.

With the use of photomicroscopy, and documented by freeze-fracture electron microscopy, absorption spectroscopy and electrophoresis, we show here that the in vivo Hb C crystals present in red cells of splenectomized CC patients are in the oxy state, although previous data had suggested that crystallization of Hb C is favored in the deoxy state.4 This finding opens the possibility that changes of ligand state of the crystal, with its consequent melting, might be involved in the pathophysiology of the disease.

MATERIALS AND METHODS

Blood was obtained from two splenectomized patients homozygous for Hb C, a female and a male. Our control was obtained from a normal homozygous AA male donor. Blood specimen collection and informed consent procedures were approved by the institution’s Human Experimentation Committee. The blood cells were fractionated on Stractan-Percoll gradients according to the method of Fabry et al.7 The most dense fraction was removed and washed in isotonic saline.

Crystal-containing cells harvested from the densest fraction of a Percoll-Stractan gradient separation were introduced into an anaerobic chamber with inlet-outlet ports for flushing. Solutions or standard microscope slides used for anaerobic purposes were prepared in a nitrogen glove bag and sealed with vacuum grease. A Panasonic video recorder was attached to the microscope to allow for direct observation and recording. All solutions were adjusted to 290 mosm using a Micro-osmometric osmometer (Precision Instruments, Sudsbury, Mass). All chemicals were reagent grade. The buffer for the suspension of red cells consisted of 10 mmol/L of Hepes, 60 mmol/L of NaCl, 80 mmol/L of KCl, and 1 mmol/L of MgCl2 at pH 7.55. The isosmolar sodium dithionite (a reducing agent capable of converting hemoglobin to the deoxy state) solution was prepared by adding 200 mg sodium dithionite (sigma) to 10 cc of distilled water, and 5 cc of this solution was brought to 20 cc with the aforementioned buffer used for red cell suspension. The sample for freeze-fracture electron microscopy was prepared as follows: cells were collected from the densest fraction of Percoll-Stractan gradients, washed in phosphate buffered saline with glucose, (PBSG: 136 mmol/L of NaCl, 8.1 mmol/L of Na2HPO4, 2.6 mmol/L of KCl, 1.9 mmol/L of KH2PO4, 0.2% glucose, pH 7.4), and were then incubated in PBSG, 20% glycerol for two hours at 4 °C at a 2% cell suspension. Cells were centrifuged at 1000 g for ten minutes and adjusted to approximately 50% cell suspension by removal of most of supernatant medium (PBS 20% glycerol) prior to freezing in liquid nitrogen-cooled Freon 22. The sample was fractured in a Balzers BAE808 using a double replica device and, without etching, was shadowed in platinum and carbon.

Corning agar gel electrophoresis and a Corning 720 Densitometer (Palo Alto, Calif) were used to determine the percentage of fetal hemoglobin. The Betke procedure48 for fetal hemoglobin staining was used to distinguish cells containing fetal hemoglobin. Absorption spectra were recorded on a Cary 17D spectrophotometer.

RESULTS

The first series of experiments was designed to observe the behavior of the CC cells containing crystals under oxy and deoxy conditions, with the expectation that a change of ligand state of the crystal would be accompanied by its dissolution. When aerated cells from the most dense Stractan-Percoll fraction were observed under the microscope in isotonic saline solution, 22% of these cells were found to contain crystals. The crystal structure of the most dense fraction of erythrocytes is revealed by freeze-fracture electron microscopy (Fig 1). When crystal-containing cells were exposed to the isosmolar sodium dithionite solution by addition of the solution to the coverslip edge or alternatively flushing the chamber, reducing agent capable of converting hemoglobin to the deoxy state), rapid melting ensued. No
crystals could be found after ten minutes, although shape changes began as early as ten seconds. Video and sequence photography showed the melting of the crystal with a corresponding change of shape of the erythrocyte toward the spherocyte (Fig 2). We observed cells in the same field, or alternatively, compared several fields before and after the addition of solution. Saturated CO in buffer had no effect upon the crystals, even after 30 minutes.

Visible spectroscopy of hemolysates of the most dense cell fraction showed the hemoglobin to be 100% oxy with no significant presence of met Hb, using the calculations of Benesch et al. In addition, the absorption spectrum of a suspension of cells was compatible with the oxy spectra.

Because the peripheral blood is well aerated during the procedure to separate the fractions on the Stractan-Percoll gradients, the possibility that our method selected only oxygenated crystals had to be excluded. Venous whole blood from the patient was drawn into Vacutainer tubes containing EDTA. One tube was immediately deoxygenated using a nitrogen-vacuum apparatus. Blood from another tube was withdrawn and exposed to air for the same time period that the former was deoxygenated (20 to 30 minutes). Each sample was then drawn into a syringe filled to the needle point with an isoosmotic solution of 10% formaldehyde in 0.8% NaCl, 0.15 mol/L of potassium phosphate, pH 7.4. Cell counts (1,000 or more cells) were made of these whole blood preparations. Our results show that 3.7% of the cells contain crystals in the oxygenated venous sample whereas a negligible number were observed in the deoxygenated venous sample. The sample of venous blood fixed immediately after extraction contained 3.5% of crystal-containing red cells. In a separate experiment, the number of crystal-containing cells was estimated simultaneously in arterial and venous samples, taken from the same patient, several months later. Twelve separate counts of 1,000 cells per count demonstrated that the mean number of red cell containing crystals was 1.6 ± 0.22 in arterial blood and 1.1 ± 0.23 in venous blood, a difference that is significant at the $P < .05$ level. The absolute number of circulating crystals varies in these patients over time.

The examination of Hb F content on the four Stractan-Percoll red cell fractions revealed that the lowest percentage of Hb F was associated with the densest fraction (F4) and that HbF content increased progressively toward fraction 1 (Table 1). Examination of F4 using the Betke procedure (which allows the detection of intracellular HbF), demonstrated that none of the stained (or HbF-rich) cells contained crystals. When aliquots of F4 of CC blood stained by the Betke procedure were compared with unstained specimens, the red cell crystal count (19% ± 22%) was within counting error (10%), suggesting that the methods do not interfere with the detection of intracellular crystals.

**DISCUSSION**

The experiments described here demonstrate that the circulating intraerythrocytic HbCC crystals are in the oxygenated liganded state. Based on the fact that oxy and deoxy HbCC crystals have a different unit cell array, the deoxy state of these crystals is excluded by the lack of effect of the carbon monoxide equilibration: if the crystal was of the deoxygenated type, it should have become ligated with CO and have melted as a consequence. Conversely, when the crystal-containing cells were exposed to a deoxygenerating agent (sodium dithionite), the crystals were observed melting and the cells acquired a spherocytic shape due, most likely, to the sudden release of osmotically active particles. A similar experiment with pure HbA crystals was performed in 1938 by Haurowitz, and is considered the first evidence that oxy...
and deoxy hemoglobins have different conformations and different crystal habits. Visible spectroscopy of red cell lysates and red cell suspensions of HbCC cells harvested from the densest fraction of a Stractan-Percoll isopycnic gradient, eliminated Met-hemoglobin as the crystal state and further corroborated our finding that the crystals were in the oxygenated state. These crystal-containing red cells are similar to those induced by hypertonic media by Lessin et al., in their parallel line structural array when studied by freeze-fracture techniques (Fig 1). In addition, our results suggest that crystals of HbC are more likely to appear in cells containing the least amount of HbF. The molecular basis of this finding must be explored further, particularly the possibility that HbF could interfere with the crystallization of HbC.

Although HbCC crystals in vitro can be readily induced in the deoxygenated state, as predicted by the low solubility of deoxy HbC, it is not surprising that in red cells they are of the oxy type, as erythrocytes spend considerably more time oxygenated than deoxygenated. Non-splenectomized HbCC patients have no detectable crystal-containing erythrocytes in their circulation. In contrast, splenectomized HbCC patients have a uniquely dense red cell fraction in their blood that contains a significant proportion of cells (about 20% to 30% of the cells in the bottom fraction) with morphologically detectable in erythrocytic crystals. Nevertheless, these patients do not exhibit vasoocclusive episodes and the experiments presented here might explain this discrepancy. When crystal-containing cells obstruct the microcirculation because of their reduced deformability (for example, at the precapillary sphincter), a progressive decrease in pO₂ and pH will follow, which should result in the melting of the intraerythrocytic crystal and spherocytic transformation according to the data presented here. This last event should not pose a problem to the microcirculation due to the small size of the HbCC cells and the fact that spherocytes are not obstructive, as demonstrated by the absence of vasoocclusion in hereditary spherocytosis.

These potential events are within the time frame and conditions of our experimental evidence. The lower percentage of crystal-containing cells in venous blood as compared with arterial blood is compatible with this interpretation. The series of events proposed here would be opposite to those found in sickle cell anemia (characterized by vasoocclusive events) in which red cells become more rigid as the occlusion progresses.

The absence of crystal-containing cells in non-splenectomized patients is also understandable from the data presented here. The slits in the sinusoids of the spleen are a rigorous quality-control step for red cells. Cells containing crystals as well as spherocytic cells (as in hereditary spherocytosis) are inexorably trapped, making the melting of the crystal irrelevant to this process, as both (crystal-containing cells and spherocytes) are detrimental to the survival of the cell in the splenic circulation.

We conclude from these studies that the intraerythrocytic HbC circulating crystals are in the oxygenated liganded state and appear in cells with low concentration of HbF. When CC crystal-containing cells are deoxygenated, the crystal melts and cells undergo a spherocytic transformation, explaining the absence of vasoocclusion and providing an explanation for the efficient spleen-induced removal of these cells.

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