Characterization and Comparison of the Red Blood Cell Membrane Damage in Severe Human α- and β-Thalassemia


The aim of the present work was to understand the pathophysiology of the severe human thalassemias as represented by β-thalassemia intermedia and hemoglobin (Hb) H (α-thalassemia) disease. We have previously shown that the material properties of the red blood cell (RBC) and its membrane differ in severe α- and β-thalassemia, and we now show that this difference is probably caused by accumulation of α-globin chains at the cytoskeleton in β-thalassemia, whereas β-globin chains are associated with the cytoskeleton in α-thalassemia. In both α- and β-thalassemia, some of these globin chains have become oxidized as evidenced by loss of free thiols. Furthermore, there is similar evidence of oxidation of protein 4.1 in β-thalassemia, whereas β-spectrin appears to be subject to oxidation in α-thalassemia. These observations support the idea that the association of partly oxidized globin chains with the cytoskeleton results in oxidation of adjacent skeletal proteins. The abnormality of protein 4.1 in β-thalassemia is consistent with a prior observation, and is also in accord with the known importance of protein 4.1 in maintenance of membrane stability, a property that is abnormal in β-thalassemic membranes.

THE THALASSEMIAS constitute a group of heterogeneous disorders that cause significant worldwide morbidity and even mortality. The abnormalities within red blood cells (RBCs) of patients with the more severe forms of thalassemia result from accumulation of the unmatched globin chain that is present in excess. The pathophysiology relates primarily to the degree of anemia that is caused by both intramedullary hemolysis and RBC destruction in the peripheral blood. While the molecular biology has been studied effectively, the cellular derangements that lead to the premature removal of abnormal RBCs in the marrow and from the peripheral circulation are not clearly understood. Several abnormal cellular properties have been identified. Severely affected α- and β-thalassemic RBCs and their isolated membranes are both much more rigid than normal. However, the β-thalassemic membranes are much less stable and fragment easily, whereas the α-thalassemic membranes are more stable than normal. In addition to these differences in material membrane properties, RBCs from patients with hemoglobin (Hb) H disease are uniformly well hydrated, whereas there are populations of very dehydrated RBCs in patients with severe β-thalassemia.

In searching for an explanation of these abnormal and differing cellular properties, our attention focused on the observation that severely affected thalassemic RBCs are known to have increased membrane-associated globin. We have shown that much of the excess globin was associated with the cytoskeleton, and hypothesized that this skeletal-associated globin resulted in altered membrane function by producing oxidative damage to adjacent skeletal proteins mediated by the globin-associated heme, hemichromes, or iron. According to this proposal, the deposition of either α- or β-globin chains would produce substantially different biological alterations, thereby explaining the differing RBC properties in severe β- and α-thalassemia, respectively.

To test this hypothesis, it was first necessary to determine if there was evidence of membrane protein oxidation. Therefore, we analyzed thalassemic RBC membranes and skeletons in order to identify and quantify the specific globin chains present and then searched for evidence of oxidative attack on membranes using thiol-disulfide exchange chromatography to identify membrane proteins that had lost their free thiol groups presumably as a consequence of oxidative attack.

MATERIALS AND METHODS

Sodium dodecyl sulfate (SDS) was obtained from BHD (Poole, UK) and is a specially pure form of SDS that is required for thiol-disulfide chromatography (see below). Dithiothreitol disulfide (DTP), leupeptin, pepstatin, phenyl-methyl-sulfonyl fluoride (PMSF), deferoxamine, and Triton X-100 were obtained from Sigma Chemical (St Louis, MO). Beta-mercaptoethanol and urea were obtained from the BioRad Laboratories (Richmond, CA), and Thiol-Activated Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). A rabbit polyclonal antiprotein 4.1 antibody was a generous gift from Dr Joel Chassis of the Lawrence Berkeley Laboratories (Berkeley, CA), and was affinity-purified against purified human RBC protein 4.1. The antispectrin antibody used was an affinity-purified rabbit polyclonal antibody. All other reagents used were of the best grade available.

Methods. All blood samples were drawn according to protocols approved by Stanford University Institutional Review Board, the Hadassah Hospital Institutional Review Board, and the San Diego Naval Hospital Institutional Review Board. Samples of venous blood were collected in acid citrate dextrose (ACD) from spleenectomized (n = 4) and nonsplenectomized (n = 2) patients with severe β-thalassemia intermedia and from six nonsplenectomized patients with α-thalassemia (Hb H disease). The β-thalassemia samples were obtained from Jerusalem from patients who had been previously studied and reported. The α-thalassemia samples were all from patients of Filipino ancestry and were obtained from the San Diego Naval Hospital. Samples were always accompanied by a normal shipment control and arrived approximately 24 to 36


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hours after blood was drawn. The Hb values for the β-thalassemic patients ranged from 7.5 to 9 g/dL, and for the α-thalassemics from 8.5 to 10.5 g/dL. None of the patients had been transfused for over the preceding 4 months.

On arrival in the laboratory, the RBC were washed in phosphate-buffered saline (PBS), and the hematocrit was adjusted to 50% with PBS. Cells were then incubated with PBS, pH 7.6 (in a 1:1 ratio), to which 2 mmol/L DFP and 10 μg/mL of pepstatin A and of leupeptin were added with gentle shaking to inhibit proteolysis as previously described. Ghosts were prepared by hypotonic lysis exactly as previously described using 5 mmol/L phosphate buffer, pH 8, containing 100 mg/mL of PMSF and 0.1 mmol/L EDTA. Cytoskeletons were prepared by Triton extraction of ghosts as previously described using 1% Triton-X-100. Bromma, Sweden). The globin chain composition and content of ghosts and skeletons were determined by polyacrylamide gel electrophoresis (SDS-PAGE) using 6% to 18% gradient gels, which were stained with Coomassie blue. Quantification on these and all other gels was performed with an LKB Ultrascan XL laser densitometer (No. 2222-U20, Bromma, Sweden). The globin chain composition and content of ghosts and skeletons were determined using Triton acid urea gel electrophoresis.

Ghosts were examined for evidence of protein oxidation using thiol-activated sepharose chromatography. This method is based on the principle that proteins which contain reduced thiols will bind to the thiol-activated agarose gel. Those proteins appearing in the unbound fraction either have no free thiols or their thiols have been blocked or converted into disulfides, presumably by oxidative processes. Proteins with free thiols that were bound to the thiol-activated sepharose were eluted by addition of β-mercaptoethanol. PMSF (100 mg/mL) and 0.1 mmol/L EDTA were used as protease inhibitors during all steps of this procedure. Samples were subsequently dialyzed as described (exclusion limit, 1,000 d) to remove SDS and β-mercaptoethanol, concentrated by Minicons B 15 (Amicon, Beverly, MA), analyzed by SDS-PAGE, and stained with Coomassie blue. It is known that in normal human RBC membranes only two proteins, glycophorin A and protein 7, appear in the unbound fraction because these proteins natively have no free thiols. Because it is not always possible to process identical numbers of RBCs, the amount of each band of protein appearing in the unbound fraction was normalized to the protein 7 content as measured by laser densitometry of the Coomassie blue-stained gels. Western blots of the unbound fractions were performed as previously described. It was necessary to be sure that we had avoided oxidation of membrane proteins during the preparation of ghosts and subsequent analyses, particularly in thalassemic samples where there might have been an increase in iron, either bound to heme or directly bound to the membrane. Accordingly, we performed ghost preparation and thiol-disulfide exchange chromatography on one local severe nonsplenectomized β-thalassemic and two of our six patients with Hb H disease by the procedures described above, and then in parallel samples, 0.5 mmol/L deferoxamine was added to all steps to chelate iron. To evaluate the effect of shipment, the two Hb H samples from San Diego were divided into two aliquots, one of which was processed immediately and the other of which was incubated under sterile conditions at 37°C for an additional 16 hours and then processed. Ghosts from samples prepared with and without deferoxamine were compared on SDS-PAGE and no differences were identified (gels not shown). The unbound fractions (thiol free) of samples with and without 0.5 mmol/L deferoxamine in all steps of ghost preparation and thiol-disulfide exchange chromatography were also compared and there were no differences (data not shown). Overnight incubation of Hb H RBC, as anticipated, increased the amount of globin bound to membranes equally in samples processed with and without deferoxamine, and the amount of oxidized globin was identical irrespective of the presence or absence of deferoxamine.

RESULTS

Globin Chain Composition of Membranes and Skeletons

β-Thalassemia intermedia. The globin chain composition of ghosts and cytосkeletons from patients with severe β-thalassemia intermedia was analyzed by Triton acid urea gel electrophoresis (Fig 1) along with the Triton-solubilized integral proteins (supernatant). Thalassemic ghosts have increased amounts of globin consisting of 64% α-chains, 19% β-chains, and 17% γ-like chains. Following Triton extraction of thalassemic ghosts, the residual cytосkeletons contained 87% α-globin chains and 13% γ-like globin chains. The Triton-solubilized material called “supernatant” contains 54% α-globin and 46% β-globin.

Hb H disease. As relatively few analyses of ghosts and cytосkeletons in Hb H disease have been reported in literature, an SDS-PAGE analysis is shown in Fig 2. Both ghosts and cytосkeletons of patients show more globin than the control. Triton acid urea gel analyses of ghosts and cytосkeletons from patients with Hb H disease are shown in Fig 3. Depending on the patient, the ghosts contain varying mixtures of α- and β-globin chains. However, the cytосkeletons contain virtually only α-globin chains.

Thiol-Disulfide Exchange Chromatography

β-Thalassemia intermedia. Six analyses were performed on four patients with severe β-thalassemia (two splenectomized and two nonsplenectomized) with and without deferoxamine, and the amount of oxidized globin was determined as described above. The standard is cord blood hemolysate showing the migration of normal α-, β-, γA- and γG-globin chains.

Fig 1. Triton acid urea gel electrophoresis of ghosts and skeletons. The standard is cord blood hemolysate showing the migration of normal α-, β-, γA- and γG-globin chains.
mized and two nonsplenectomized). Ghosts from these patients were prepared and solubilized in SDS and then subjected to thiol-disulfide exchange chromatography. The unbound fractions were concentrated and analyzed by SDS-PAGE (Fig 4). Gel analyses of splenectomized β-thalassemic patients showed a protein with the electrophoretic mobility of globin in substantially increased amounts as compared with normal controls (Fig 4). One can normalize the amount of globin to the content of protein 7 present. The globin to protein 7 ratios are shown in Table 1.

A protein with the electrophoretic mobility of protein 4.1 also appeared in the unbound fraction of the splenectomized β-thalassemic samples (Fig 4), but not in the control and nonsplenectomized thalassemias. Because abnormality of the function of protein 4.1 has been described in severe β-thalassemia,19 we wished to determine if this was indeed protein 4.1. Therefore, Western blotting was performed using a monospecific polyclonal rabbit antiprotein 4.1 antibody, which confirmed the identification of this band (Fig 5). This also showed very faint traces of protein 4.1 in the control and nonsplenectomized samples (Fig 5).

α-Thalassemia. The unbound fractions from Hb H ghosts were concentrated and analyzed by SDS-PAGE and contained much more globin than normal (Table 1, Fig 6). In addition, all patient samples studied showed a faint band

| Table 1. Globin to Protein 7 Ratios in the Unbound Fraction of Thiol-Disulfide Chromatography |
|---------------------------------|-----------------|-----------------|
|                                 | Control         | α-Thalassemia   | β-Thalassemia Splenectomized |
| Mean                            | 0.13            | 4.37            | 4.21                      |
| Range                           | 0.06-0.198      | 3.45-5.98       | 3.56-4.86                 |
RBC MEMBRANE DAMAGE IN THALASSEMIA

Control

Protein 4.1

Ghost Unbound Fractions

Fig 5. Western blotting with antiprotein 4.1 antibody. The first lane contains normal ghosts as a reference point. The remaining lanes are of the unbound filtrate from a thiol-disulfide exchange column from two patients with β-thalassemia intermedia splenectomized, one patient with β-thalassemia intermedia nonsplenectomized, and two normal control samples.

with electrophoretic mobility of β-spectrin (Fig 6). To confirm this, we then performed Western blotting using an antispectrin antibody (Fig 7). This showed increased amounts of predominantly β-spectrin and faint amounts of α-spectrin in the unbound fraction of Hb H samples as compared with the normal control. These results were reproducible when one of these patients was reanalyzed at a later date.

DISCUSSION

In testing our hypothesis, we initially characterized the kinds of Hb and globin chains bound to ghosts and cytoskeletons in severe α- and β-thalassemia. Analysis of ghosts by SDS-PAGE showed increased globin in both β-thalassemia intermedia and Hb H disease (Fig 2). We then proceeded to analyze the type of globin present in the thalassemic ghosts and cytoskeletons. There are α-, β-, and γ-globin chains present in ghosts from patients with severe β-thalassemia intermedia (Fig 1). In contrast to hemoglobin, where the ratio of α- to β-globin chains is one, the ratio in the β-thalassemic ghosts differs (Fig 1), with an invariable predominance of α-globin chains. The cytoskeletons from these β-thalassemic RBCs contain an even greater preponderance of α-globin chains (~90%) (Fig 1), while the solubilized Triton extract consisting of integral proteins and lipids contains approximately equal amounts of α- and β-globin chains. We interpret these results as showing that ghosts from patients with β-thalassemia intermedia have globin that is bound in at least two forms: (1) α- and β-dimers or tetramers of hemoglobin are present, probably bound to protein 3. During Triton extraction, protein 3

Fig 6. Thiol-disulfide exchange chromatography of Hb H ghosts. In addition to increased amounts of globin, each of the patient samples shows a band with the electrophoretic mobility of β-spectrin.

Fig 7. Western blotting with antispectrin antibody. The first lane contains normal ghosts as a reference for the α-spectrin antibody. The next three lanes consist of the unbound filtrate from a thiol-disulfide exchange column from two patients with Hb H disease and a normal control sample.
and the attached hemoglobin are removed (Fig 1); and (2) Triton extraction leaves cytoskeletons, to which, in β-thalassemia intermedia, virtually only α-globin chains are bound. Other investigators have also shown free α-globin chains in β-thalassemic RBCs and insoluble α-globin chains in association with the RBC membrane. In Hb H disease, the converse is the case, with cytoskeletons containing virtually only β-globin chains (Fig 3).

To learn whether the membrane-associated globin was in its native state or had undergone oxidation, we used thiol-disulfide exchange chromatography, because the loss of free thiols is a common occurrence following an oxidative attack. Thiol-disulfide chromatography underestimates oxidative attack, because it identifies only loss of free sulfhydryls, primarily cysteine residues, whereas other amino acids, including tyrosine, methionine, and histidine, are not being measured, although they are also prone to oxidant stress. Furthermore, if only one of the two sulfhydryls of an α-globin molecule is oxidized, that α-globin will still adhere to the thiol-activated sepharose and not appear in the unbound fraction. Despite these methodologic limitations, the membrane-associated globin in both β-thalassemia intermedia and in Hb H disease showed evidence of oxidation by virtue of loss of the free thiols (Table 1, Figs 4 and 6).

In severe β-thalassemia intermedia, there is further evidence of oxidation of some protein 4.1 (Figs 4 and 5). As noted above, thiol-disulfide chromatography underestimates the extent of oxidation, because if six of the seven thiols of a protein 4.1 molecule are oxidized, it will still in theory bind to the thiol-activated sepharose.

The observation of oxidation of some protein 4.1 in severe β-thalassemia supports a prior observation showing that purified protein 4.1 from such patients is functionally abnormal, binds poorly to spectrin, and participates suboptimally in mediating the formation of the spectrin-actin-protein 4.1 tertiary complex. Protein 4.1 functions not only to form the spectrin-actin-protein 4.1 lattice of the membrane skeleton, but also as a possible anchoring point of the skeleton with transmembrane proteins by interaction with glycophorin C. RBC membranes deficient or defective in protein 4.1 are mechanically unstable and that membrane stability can be restored toward normal by rescaling normal protein 4.1 within such protein 4.1-deficient ghosts. The oxidized protein 4.1 demonstrated in β-thalassemic membranes could in part explain their membrane instability.

Results of thiol-disulfide exchange chromatography on RBC membranes from patients with Hb H were different. No protein 4.1 appeared in the unbound fraction; however, there was a β-spectrin band present, indicating loss of thiols of some of the β-spectrin. Apparently, this partial oxidation of Hb H β-spectrin did not affect its function as previously measured by spectrin self-association or binding to normal inside-out vesicles (IOV). In that study, it was noted that the IOVs in Hb H disease are abnormal, perhaps indicating an abnormality of protein 3 or ankryin. Neither protein 3 nor ankryin were detected in the unbound fraction of thiol-disulfide columns. However, as noted above, even the persistence of one thiol would result in binding to the thiol-activated sepharose. On the other hand, an abnormality in β-spectrin could alter skeletal protein associations and perhaps account for the membrane hyperstability seen in Hb H disease.

Therefore, in severe α- and β-thalassemia in humans, the unmatched excess globin chains are deposited in association with skeletal proteins—α-chains in β-thalassemia and β-chains in α-thalassemia (Figs 1 and 3). Some of these globin chains have undergone oxidation as evidenced by the loss of free thiols (Table 1, Figs 4 and 6). We hypothesized that the consequences of having accumulations of α- or β-globin chains at the cytoskeleton would be different, and this appears to be the case. In severe β-thalassemia, some protein 4.1 seems to be oxidized, whereas in Hb H disease, protein 4.1 is unaffected but some β-spectrin has been oxidized. Some of these protein alterations are consistent with what is known about the membrane properties of the thalassemias.

We have not proven that the abnormalities in membrane proteins and associated globin chains described here account for the previously reported abnormalities in membrane material properties or for the pathophysiology of the thalassemias. However, the results in human β-thalassemia are consistent with emerging information on murine β-thalassemia and a transgenic thalassemia-sickle variant that has allowed us to test hypotheses concerning specific membrane abnormalities and disease severity. In that murine model system, disease severity is directly correlated with membrane instability and with the amount of oxidized α-globin chains that become associated with the cytoskeleton and with the extent of oxidation of protein 4.1. If one can extrapolate from the murine disease to the human, then oxidation of membrane-associated α-globin chains and protein 4.1 may be important in the pathophysiology of human β-thalassemia. Similar correlations of the abnormalities in Hb H disease have not been made as yet.

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Characterization and comparison of the red blood cell membrane damage in severe human alpha- and beta-thalassemia

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