Alteration of the Erythrocyte Membrane Skeletal Ultrastructure in Hereditary Spherocytosis, Hereditary Elliptocytosis, and Pyropoikilocytosis

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The membrane skeleton of normal erythrocytes is largely organized into a hexagonal lattice of junctional complexes (JC) crosslinked by spectrin tetramers, and occasional double tetramers and hexamers. To explore possible skeletal alterations in hereditary spherocytosis (HS), elliptocytosis (HE), and pyropoikilocytosis (HPP), we have studied the ultrastructure of the spread membrane skeletons from a subpopulation of HS patients with a partial spectrin deficiency ranging from 43% to 86% of normal levels, and in patients with HPP who, in addition to a mild spectrin deficiency, also carried a mutant spectrin that was dysfunctional, thus reducing the ability of spectrin dimers to assemble into tetramers. Membrane skeletons derived from Triton-treated erythrocyte ghosts were examined by negative staining electron microscopy. HS membrane skeletons contained structural elements, consisting of JC and spectrin filaments similar to the normal skeleton. However, less spectrin filaments interconnected the JC, and the decrease of spectrin filaments attached to JC appeared to correlate with the severity of spectrin deficiency. Only in severe HS associated with severe spectrin deficiency was the loss of spectrin sufficient enough to disrupt the overall skeletal architecture. In contrast, membrane skeletons prepared from red blood cells (RBCs) of subjects with HPP were strikingly different from HS RBCs with a comparable degree of spectrin deficiency. Although HPP RBCs were only mildly deficient in spectrin, their skeletal lattice was grossly disrupted, in contrast to only mild ultrastructural abnormalities of HS membrane skeletons with a nearly identical degree of spectrin deficiency. Skeletal alterations in HS are not always in concordance with spectrin deficiency. In HS, HE, and HPP, the severity of spectrin deficiency correlates with the severity of spectrin deficiency in HS and HPP but not with the severity of spectrin deficiency in HE. In this study, we use an ultrastructural approach to define alterations in the assembly of the intact membrane skeleton with the aim to compare the observed abnormalities with alterations in RBC shape and mechanical properties. Furthermore, we correlate the membrane skeletal disruption with the severity of the spectrin deficiency in HS and HPP and/or dysfunction of spectrin as characterized by a defective self-association of spectrin dimers into tetramers.

MATERIALS AND METHODS

Membrane skeleton preparations. Venous blood from normal individuals and patients with HS, HE, or HPP was collected into sterile tubes containing citrate-phosphate-dextrose or acid-citrate-dextrose as anticoagulant, stored at 4°C, and analyzed within a...
week. Ghost membranes were prepared by the method of Dodge et al. with 5 mmol/L NaPi (pH 7.4). Freshly prepared ghosts (1 mg protein/mL) were incubated on ice for 60 minutes in 4 vol of 5 mmol/L NaPi (pH 7.4) containing 0.1 mmol/L Triton X-100 (Sigma) to remove lipid and the bulk of integral proteins from the skeletal shells. The mixture was layered on top of a discontinuous gradient of 10% and 60% (wt/vol) sucrose containing 0.1 mmol/L NaPi (pH 7.0) and 0.5 mmol/L dithiothreitol, and centrifuged at 100,000g for 15 minutes in a swinging bucket rotor. The intact skeletons were collected from the 10%/60% sucrose interface and checked for morphology without staining in a phase contrast light microscope attached to a Newvicon camera (MTI Inc, Michigan City, IN).

Negative staining electron microscopy. The specimens for electron microscopy were prepared as previously described. Briefly, an aliquot of membrane skeletons in sucrose was diluted with 9 vol of 0.1 mmol/L NaPi (pH 7.0) buffer and applied to a thin-carbon coated grid. The grid was then rinsed with 0.1 mmol/L NaPi (pH 7.0) at 25°C for 10 minutes, and negatively stained with 1% uranyl acetate solution for 1 to 2 minutes. The excess solution was drawn into filter paper and grids were air-dried. Specimens were examined in a Jeol JEM-100S electron microscope with an accelerating voltage of 60 kV.

Spectrin content. Erythrocyte ghost proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on gels with a gradient of 3.5% to 17%. The amount of spectrin (bands 1 and 2) in the membrane, expressed as a ratio to the amount of band 3, was quantitated by densitometry of the stained gels at 540 nm and integration of the surface areas under the spectrin and band 3 peaks. In normal erythrocytes, the spectrin to band 3 ratio was estimated as 1.0 ± 0.1 (n = 60). In 14 patients with HS and 17 patients with HE/HPP, the ratios were in the range of 0.43 to 0.89 and 0.69 to 1.06, respectively. The partial spectrin deficiency in the above HS and HPP patients had been previously documented. The spectrin to band 3 ratio derived from individual patients are included in Tables 1 and 2. The contents of other major skeletal proteins, namely spectrin and protein 4.1, relative to band 3 were also measured and no abnormalities were detected. In patients with HS, the reduced spectrin content was confirmed by a spectrin radioimmunoassay as previously reported. In severe hemolytic HE and HPP, the ratios were in the range of 0.43 to 0.89 and 0.69 to 1.06, respectively. The partial spectrin deficiency in the above HS patients with mild spectrin deficiency (eg, 70% to 85% of normal), the integrity of the membrane skeletal network was near-normal or only slightly less intact than normal (Figs 1, C and D). In contrast, the skeletons derived from HS patients with severe spectrin deficiency (eg, 43% to 55% of normal) were grossly disrupted, as demonstrated by a marked reduction of spectrin crosslinks in the skeleton and a loss of the hexagonal lattice (Figs 1, E and F).

RESULTS

Ultrastructural alterations of membrane skeleton in HS, HE, and HPP. To visualize the possible ultrastructural alterations of membrane skeletons in HS, HE, and HPP erythrocytes, we prepared delipidated membrane skeletons by extracting ghosts with Triton X-100, and examined the negatively stained skeleton by electron microscopy. Figure 1, A and B, depict the ultrastructure of the spread normal erythrocyte skeleton. As previously reported, the individual filaments of spectrin stretch up to 200 nm long, connecting the JC of short F-actin. These JC are arranged primarily into a hexagonal array. Globular structures, presumably representing ankyrin (band 2.1) or ankyrin/band 3 complexes, are attached to most of the spectrin filaments. Most of the spectrin filaments are in the form of spectrin tetramers. By examining large areas of spread skeletons, we estimate that about 85% of spectrin crosslinks are preserved in a typical spread skeletal preparation. This estimate is based on the average number of crosslinking spectrin filaments (Sp) arising from one JC (Sp/JC) that interconnects with other JC in a spread skeleton as compared with the Sp/JC in the intact hexagon. This Sp/JC value was 5.0 to 5.5 (see Table 1 and Fig 3), instead of the anticipated Sp/JC value of 6 from an intact hexagonal lattice. In HS patients with mild spectrin deficiency (eg, 70% to 85% of normal), the integrity of the membrane skeletal network was near-normal or only slightly less intact than normal (Figs 1, C and D). In contrast, the skeletons derived from HS patients with severe spectrin deficiency (eg, 43% to 55% of normal) were grossly disrupted, as demonstrated by a marked reduction of spectrin crosslinks in the skeleton and a loss of the hexagonal lattice (Figs 1, E and F). The quantitative analysis of these images is shown (see Fig 3).

| Table 1. Skeletal Disruption and Spectrin Content of HS Erythrocytes |
|--------------------------|----------|----------|----------|
| Patient No. | Sp* JC | Sp† JC | n† |
| 1 | 0.86 | 5.0 | 106 |
| 2 | 0.85 | 4.7 | 60 |
| 3 | 0.84 | 4.9 | 50 |
| 4 | 0.82 | 5.1 | 48 |
| 5 | 0.80 | 4.7 | 64 |
| 6 | 0.74 | 3.9 | 83 |
| 7 | 0.71 | 4.3 | 110 |
| 8 | 0.68 | 3.8 | 32 |
| 9 | 0.65 | 4.6 | 67 |
| 10 | 0.55 | 3.7 | 71 |
| 11 | 0.54 | 3.3 | 56 |
| 12 | 0.52 | 3.4 | 44 |
| 13 | 0.43 | 3.3 | 24 |

Patients 1 through 5 had dominantly inherited HS. In patients 6 through 13, the inheritance was autosomal recessive.

Sp* to band 3 ratio as measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and densitometry of the Coomassie blue-stained gels.

†The average numbers of crosslinking spectrin filaments attached to each JC in the spread skeleton.

§The number of JC studied.

In HS patients with mild spectrin deficiency (eg, 70% to 85% of normal), the integrity of the membrane skeletal network was near-normal or only slightly less intact than normal (Figs 1, C and D). In contrast, the skeletons derived from HS patients with severe spectrin deficiency (eg, 43% to 55% of normal) were grossly disrupted, as demonstrated by a marked reduction of spectrin crosslinks in the skeleton and a loss of the hexagonal lattice (Figs 1, E and F). The quantitative analysis of these images is shown (see Fig 3).

In contrast to the skeletons derived from HS patients with a mild spectrin deficiency (Figs 1, C and D), HPP skeletons with a nearly identical degree of spectrin deficiency (70% to 80% of normal) exhibited a marked disruption of the skeletal lattice (Figs 2, A and B). Few spectrin crosslinks were identified in those skeletons. In addition, the remaining filamentous structures often appeared as a "string of beads" of spectrin filaments decorated by globular structures (Figs 2, C and D). Presumably, these structures represent either JC, ankyrin/band 3 complexes attached to the spectrin molecules, or possibly disconnected spectrin dimers attached...
spread skeletons from 4 normal individuals, 13 patients with HPP, 6 asymptomatic HPP carriers, 2 HPP patients, and 10 subjects with mild common HE (Figs 2, E and F) and subjects with mild spectrin deficiency (20% to 40% spectrin dimers) but normal or near-normal spectrin content, the skeletal lattice was likewise disrupted, but to a lesser extent than that of the patients with HPP.

In asymptomatic HPP carriers (Figs 2, E and F) and subjects with mild common HE (Figs 2, G and H), both of which exhibited a mild degree of spectrin dysfunction (20% to 40% spectrin dimers) but normal or near-normal spectrin content, the skeletal lattice was likewise disrupted, but to a lesser extent than that of the patients with HPP.

**Correlations of the degree of skeletal disruption to spectrin content and the percentage of SpD.** To obtain quantitative estimates of skeletal integrity, we counted the number of crosslinking Sp attached to each of the JC (Sp/JC). Figure 3 depicts the histograms of Sp/JC in the spread skeletons from 4 normal individuals, 13 patients with HS, 10 subjects with mild common HE, 2 patients with homozygous HE, 2 HPP patients, and 3 asymptomatic HPP carriers. In normal skeletons, most of the JC were interconnected by 5 or 6 spectrin filaments. An average value of 5.2 for Sp/JC was obtained in the normal skeleton, representing a preservation of 85% of the crosslinks of the intact hexagonal lattice during the specimen preparation. In skeletons from patients with mild spectrin deficiency the Sp/JC value was only slightly reduced.

The reduction of the number of interconnecting Sp was particularly striking in HPP RBCs, which were both spectrin-deficient and contained a mutant spectrin, while HE subjects carrying such spectrin mutants but having normal spectrin to band 3 ratio had only a moderately disrupted skeletal lattice. Furthermore, we have detected a positive correlation between the degree of membrane skeletal disruption (as reflected by the Sp/JC ratio) and spectrin content (spectrin to band 3 ratio) of the HS RBCs (Fig 4A). In striking contrast, the HE/HPP skeletons have Sp/JC values markedly lower than those of the HS skeletons with a comparable degree of spectrin deficiency (Fig 4A), reflecting the superimposed disruption of the skeleton due to a defective spectrin dimer-dimer association.

Because the skeletal integrity, as reflected by the Sp/JC value, depends both on the total spectrin content and the amount of unassembled spectrin in the cells (Figs 3 and 4A), we examined further the correlation among the net amount of spectrin tetramers, as calculated from the spectrin content and the percentage of spectrin tetramers in the membrane extracts (Table 2). Figure 4B shows a good correlation between the loss of the integrity of the skeletal network, as measured by the Sp/JC value, and the net amount of tetrameric spectrin crosslinks. Thus, both the reduction of spectrin content and the diminished self-association of spectrin dimers to tetramers contribute to the loss of structural integrity of the membrane skeleton in these disorders.

**DISCUSSION**

Previous electron microscopic studies of the skeletal network of the RBC membrane in situ showed a filamentous weblike structure containing a variety of poorly defined elements of varying sizes.4,26,27 Because of a high protein density in the native membrane skeleton, a detailed visualization of the individual structural subunits of the skeleton was...
Fig 1. Representative electron micrographs of uniformly spread membrane skeletons from normal RBCs and cells from patients with HS with varying degrees of spectrin deficiency. (A and B) Normal membrane skeletons. The spread skeleton shows a primarily hexagonal lattice of junctional complexes (JC), containing short F-actin and band 4.1, crosslinked primarily by spectrin (Sp) tetramers, hexamers, and double tetramers. Globular structures representing ankyrin (band 2.1) or ankyrin-containing complexes are attached to spectrin filaments at the ankyrin binding site, i.e., 80 nm from the distal end of spectrin. (C and D) Spread skeleton from HS RBCs with a mild spectrin deficiency (patients 5 and 7, respectively, Table 1). Spectrin to band 3 ratios (Sp/b3) is mildly reduced to 0.80 in (C) and 0.71 in (D) (normal range: 0.9 to 1.1). The membrane skeletal network appears only slightly less intact than in normal cells. (E and F) Spread skeletons from patients with severe spectrin-deficient HS (patients 12 and 11, respectively, Table 1). Sp/b3 ratio is markedly decreased to 0.52 in (E) and 0.54 in (F). The skeletal network is grossly disrupted, showing a loss of hexagonal lattice. The statistical analysis of the data is shown in Figs 3 and 4.
Fig 2. Representative samples of spread membrane skeletons from RBCs of patients with HPP, asymptomatic HPP carriers, or mild common HE. (A and B) HPP RBCs (patients 3 and 4, respectively, Table 2) are not only spectrin-deficient but they also contain dysfunctional spectrin, causing a defective self-association of spectrin dimers (SpD) into tetramers (SpT). (A) Sp/b3, 0.74: percentage of SpD of the total SpD and SpT pool, 50%, and (B) Sp/b3, 0.69; percentage of SpD content, 61% (normal range: 4% to 7%). Note a striking disruption of the skeleton with a complete loss of skeletal lattice. (C and D) Long filamentous structures in skeletons from patient with homozygous HE (patient 2, Table 2). This “string of beads” appearance presumably represents a string of SpT, interconnected by JC and/or decorated by ankyrin. (E and F) Spread skeletons from asymptomatic HPP carriers (patients 1 and 3, respectively, Table 2) with a normal spectrin content but increased fraction of SpD. (E) Sp/b3, 0.97; SpD, 7%, and (F) Sp/b3, 1.0; SpD, 32%. The integrity of these skeletons is slightly reduced as compared with normal skeletons. (G and H) Spread RBC membrane skeletons from patients with mild heterozygous HE (patients 1 and 6, respectively, Table 2), containing dysfunctional spectrin but having normal or near-normal spectrin content. (G) Sp/b3, 1.0; SpD, 41%, and (H) Sp/b3, 0.83; SpD, 53%. The integrity of the skeleton is moderately disrupted because of the reduced ability of SpD to self-associate into SpT. Statistical analysis of the data is shown in Figs 3 and 4. The magnification of all micrographs in Figs 1 and 2 is identical.

not possible. This problem was recently overcome by examinations of membrane skeletal fragments and artifactually extended membrane skeletons to improve the structural resolution. Such an approach showed that the intact uniformly extended RBC membrane skeleton is composed of a principally hexagonal lattice of JC containing oligomeric actin and protein 4.1, and crosslinked by spectrin tetramers and medium-sized oligomers. This uniform skeletal lattice is
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Fig 3. Histograms indicating the number of crosslinking spectrin filaments attached to each junctional complex (Sp/JC) in the spread membrane skeletons in HS, HE, and HPP. Spread membrane skeletons from RBCs of 4 normal individuals, 13 HS patients, 3 asymptomatic HPP carriers, 10 patients with mild common HE, 2 patients with HPP, and 2 HE homozygotes were examined by negative staining electron microscopy as in Figs 1 and 2. The average number of spectrin filaments crosslinking adjacent JC was counted separately to obtain their relative abundance, as indicated by the solid bar. The spectrin content in these patients and the percentage of spectrin dimers (SpD) in patients with HE/HPP are indicated. The shaded bars representing an average value of Sp/JC, (5.2), obtained from normal skeletons are shown as a reference value. Note that the number of crosslinking spectrin filaments (Sp/JC) is only mildly reduced in HS patients with mild spectrin deficiency (cases 1 through 5, 86% to 80% of Sp), while it is markedly reduced in RBCs severely deficient in Sp (cases 10 through 13, 55% to 43% of Sp). In contrast to HS, the value of Sp/JC is reduced in all HE and HPP subjects under study. This is particularly evident in patients with HPP and homozygous HE.

Fig 4. (A) Correlation between the degree of disruption of the membrane skeleton and spectrin content in patients with HS. The average number of interconnecting Sp filaments, represented by the values of Sp/JC from Table 1, were plotted against the Sp/b3 in four normal individuals (○) and 13 HS patients (●). The solid line was derived from the least square fit of the data. The data indicate a correlation between a decrease of skeletal integrity and a reduced spectrin content. For comparison, the graph also includes the plot of Sp/JC from patients or carriers with HE/HPP (Table 2) versus the spectrin content of these cells (□), HPP or homozygous HE; (△), mild-to-moderate common HE; (○), asymptomatic HPP carriers. Sp/JC values in HE/HPP skeletons are consistently lower than those of the HS skeleton with a comparable degree of spectrin deficiency, indicating a greater disruption of skeletal lattice. (B) Correlation between the integrity of the skeletal lattice and the net amount of spectrin tetramers (SpT) in the HS, HE, HPP patients. The net amount of SpT in HE/HPP skeletons was estimated on the basis of spectrin content and percentage of SpT (Table 2). The net amount of SpT in HS skeletons was reduced only slightly (4% to 7%) since their spectrin is present mostly in the form of tetramers (93% to 96%). Note that for HS, HE, and HPP, the loss of skeletal integrity correlates well with the net amount of SpT in the skeleton.
likely to account for two major mechanical features of the RBC membrane: (1) a high degree of structural integrity provided by this highly ordered relatively uniform hexagonal lattice, and (2) a high membrane deformability. The latter is likely related to the finding that in the native RBC membrane, in contrast to uniformly stretched membrane skeletons, the individual spectrin subunits are no longer extended; instead, spectrin undergoes considerable folding, and the distance between the individual JC is considerably shorter than that in the uniformly extended membrane skeleton. As a result, the RBC can undergo a large deformation without a disruption of the underlying skeletal network.

The differences in the severity of hemolysis and in RBC morphology among patients with HS, HE, and HPP parallels the marked heterogeneity of the underlying molecular defects. At this time, we do not have sufficient data to provide a clear molecular explanation of why a given molecular defect leads to a given clinical phenotype. To gain insight into the abnormalities in the subunit assembly of the intact RBC membrane skeleton and their possible relationship to alterations in RBC morphology and material properties, we have examined RBCs of patients with the above disorders who had one or two of the most common defects of membrane skeletons: (1) partial deficiency of the skeletal protein spectrin, and (2) the presence of mutant spectrin characterized by a defective assembly of spectrin αβ heterodimers into tetramers.

Our ultrastructural images of uniformly stretched membrane skeletons from patients with HPP, shown in Figs 2 and 3, show a striking disruption of the skeletal lattice. In all cases included in this study, the underlying defect involves the spectrin heterodimer contact, which is a horizontal stress supporting protein interaction that can be seen as being parallel to the plane of the membrane. A previously observed decrease in both skeletal and membrane stability is likely to represent the biomechanical counterpart of the above structural abnormalities.

Although the mechanical stretching of the skeleton to obtain uniformly extended areas for ultrastructural analysis may produce additional damage, our data on the normal skeleton suggest that such ultrastructural damage is relatively minimal. This is indicated by small differences between the predicted and the actual numbers of spectrin filaments interconnecting adjacent junctional complexes as determined by quantitative analysis of large areas of membrane skeleton. Furthermore, it is likely that this grossly disrupted mechanically unstable membrane skeleton accounts for a finding of striking RBC fragmentation and poikilocytosis that is commonly found in patients with severe hemolytic HE and HPP, since this mechanically weakened skeleton is unable to maintain the structural integrity of the RBC membrane.

In contrast to the above-mentioned gross skeletal disruption and enhanced membrane fragmentation in HPP associated with both a mild spectrin deficiency and a defect of spectrin heterodimer self-association, membrane lesions in HS associated with mild-to-moderate spectrin deficiency are likely to represent an entirely different process. In the majority of HS patients with a mild-to-moderate spectrin deficiency, ultrastructural analysis of the extended membrane skeletons (Figs 1 and 3) shows a near-normal skeletal lattice except for a reduced number of spectrin tetramers that crosslink the individual JC. These findings are in accordance with the absence of gross fragmentation on peripheral blood films of patients with mild HS and with the findings of near-normal mechanical stability of both RBC membranes and membrane skeletons in the majority of such patients. The principal cell lesion in HS involves a loss of RBC surface area, reflecting a loss of membrane lipids. It is believed that a partial deficiency in spectrin may result in an underlying membrane skeleton that may not adequately support all regions of the lipid bilayer. This might lead to a loss of small areas of unsupported lipid and untethered integral membrane proteins, as well as the alteration of membrane lipid properties, such as the asymmetric distribution across the bilayer, microdeformity, or transmembrane movement.

The near-normal skeletal ultrastructure of HS skeletons contrasts with the marked skeletal disruption of HPP, the latter skeletons, while having a nearly identical degree of spectrin deficiency, also contain a dysfunctional spectrin that fails to assemble into spectrin tetramers. These findings indicate that the spectrin self-association defect, rather than the spectrin deficiency, plays an important role in mechanical destabilization of the membrane skeleton. Only in cases of severe HS associated with a severe degree of spectrin deficiency was the RBC skeletal architecture grossly disrupted, a finding that possibly may account for additional alterations in RBC morphology, such as irregularities in cell contour and poikilocytosis.

The overall importance of spectrin deficiency and abnormalities in spectrin heterodimer contact in the destabilization of membrane skeletons is further illustrated by the data in Fig 4, indicating that at any given level of spectrin content in the RBCs the skeletal disruption, as reflected by the altered number of spectrin attached to JC, was considerably greater in patients carrying a superimposed mutation. Furthermore, we have calculated the total amount of tetrameric spectrin capable of crosslinking two adjacent JC, taking into account both the total spectrin content in the skeleton as well as the fraction of the assembled tetrameric spectrin. This net amount of tetrameric spectrin correlates well with the ultrastructural disruption of the skeleton as reflected by the altered number of spectrin attached to JC (Fig 4B). Thus, the structural integrity of the skeleton depends on the net amount of intact tetrameric spectrin that is capable of connecting two adjacent JC. As discussed above, the net amount of tetrameric spectrin is in turn determined by the absolute amount of spectrin in the membrane as well as the fraction of tetrameric spectrin (out of the total spectrin dimer and tetramer pool) in the membrane in situ.

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