Altered Assembly of Spectrin in Red Cell Membranes in Hereditary Pyropoikilocytosis

By Jiri Palek, Shin-Chun Liu, Pi-Yao Liu, Joseph Prchal, and Robert P. Castleberry

Red cells from hereditary pyropoikilocytosis (HPP) are unstable and more susceptible than normal to thermal damage in vitro. We have investigated whether or not the HPP instability results from alterations in spectrin assembly in the membrane. We identified the nearest damage in vitro. We have investigated whether or not the content presumably resulting from an increased leak characteristics, the disease has been designated hereditary pyropoikilocytosis (HPP). Subsequent studies revealed that such red cells have increased calcium membranes and modulate cell shape, membrane stability, deformability and a lateral mobility of spectrin. Presumably due to its aggregation in vivo membrane skeleton that provides a structural support to the network by oligomeric complexes of actin which bind to distal ends of the heterodimers. In normal red cells, spectrin together with actin and polypeptide 4.1 forms a two-dimensional submembrane skeleton that provides a structural support to the membrane and modulates cell shape, membrane instability and shape of alterations of HPP erythrocytes.

A UNIQUE, rare form of a congenital hemolytic anemia characterized by marked poikilocytosis and microspherocytes has been recently described by Zarkowsky et al. and others. Because the red cell morphology was reminiscent of heat treated cells, red cells from some of these patients were tested for their susceptibility to thermal injury. They were found to exhibit fragmentation and transform to microspherocytes and poikilocytes after in vitro exposure to 45°C, while normal red cells underwent similar shape changes at a temperature of 49°C. Because of these characteristics, the disease has been designated hereditary pyropoikilocytosis (HPP). Subsequent studies revealed that such red cells have increased calcium content presumably resulting from an increased leak of calcium from plasma into the cells and a decreased energy dependent extrusion of calcium from the cells. In contrast, HPP red cells did not exhibit identifiable abnormalities of hemoglobin, cytoplasmic enzymes or membrane phospholipid composition.

Recently, three laboratories reported abnormalities in chemical and physical properties of HPP spectrin, the major cytoskeletal protein of the red cell membrane. These abnormalities include decreased spectrin extractability from HPP red cell membranes, decreased endogenous phosphorylation associated with a normal spectrin kinase activity and an increased susceptibility of HPP spectrin to thermal denaturation as examined by circular dichroism.

In normal red cells, spectrin in assembly of spectrin in HPP red cell membranes in situ as revealed by crosslinking of the nearest membrane protein neighbors and ultrastructural examination of membrane skeletons. We further define the role of these alterations in membrane instability of HPP red cells.

MATERIALS AND METHODS

Patients

Two patients were studied. Both were black and had a history of hemolytic anemia with splenomegaly since birth. Their major clini-
Membrane Protein Analysis

Our technique of membrane protein analysis by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the quantification of the individual proteins by densitometric scanning have been described.13,20 The spectrin/band 3 ratio in SDS-PAGE was determined by cutting and weighing the surface areas under the densitometric tracings of bands 1+2, and 3, respectively.

Membrane Protein Crosslinking

Membrane proteins of fresh or heat treated red cells were crosslinked either by exposing intact cells to diamide (0.75 mM, 30 min, 37°C) or their ghosts to catalytic oxidants CuSO4 (10 μM) and o-phenanthroline (50 μM) (CuP; 0°C, 30 min). Some ghost samples were also crosslinked with 2 mM glutaraldehyde in 5 mM phosphate buffer, pH 7.4, at 25°C for 10 min. The details have been described elsewhere.26,27 Ghosts were solubilized in SDS and subjected to two-dimensional SDS-PAGE.28 In analyzing cleavable (disulfide) crosslinks, membrane proteins and the crosslinked protein complexes were first separated by electrophoresis in SDS agarose-polyacrylamide composite gels without DTT reduction. The composition of the crosslinked complex was subsequently identified by electrophoresis in the second dimension. DTT zone was positioned on the top of the second dimensional gel to cleave the intermolecular disulfide crosslinks.15,20

Other Determinations

Measurements of red cell ATP, GSH, and calcium influx using 45Ca have been previously described.13,14 Separation of red cells according to their density was done using a discontinuous stractan gradient.22

Incubation

Red cell susceptibility to thermal denaturation was tested by suspending washed red cells in 5 volumes of isotonic sodium chloride solution buffered with 10 mM glycyglycine containing 5 mM KCl, 2 mM MgCl2 and NaCl up to isotonicity. The suspensions were incubated in a water bath at temperatures ranging from 37°C to 50°C with shaking under a constant flow of prewashed humidified air, nitrogen, or oxygen. Some of the samples, subjected to prolonged incubation (at 37°C) contained adenine, inosine, glucose, and phosphate to maintain normal intracellular ATP levels.15

Electron Microscopy

Red cells were examined by scanning and transmission electron microscopy (SEM, TEM) as described.14 The ultrastructural examination of red cell membrane skeletons by TEM was performed by immobilizing red cell ghosts on carbon coated grids and extracting them with Triton X-10015 that removes most membrane lipids and integral proteins.15,16 Membrane skeletons were subsequently visualized by TEM. Some control and HPP red cell specimens were first immobilized on polylysine coated grids and subsequently directly extracted with 2% Triton X-100 and subjected to electron microscopic examination.

---

**Table 1. Clinical and Laboratory Features of the Two Probands With HPP**

<table>
<thead>
<tr>
<th></th>
<th>C.E.</th>
<th>D.B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, sex</td>
<td>8 yr., male</td>
<td>2 yr., female</td>
</tr>
<tr>
<td>Family history</td>
<td>Parents heterozygous</td>
<td>Negative</td>
</tr>
<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>7.1–9.9</td>
<td>7.9–9.0</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>5.1–23.0</td>
<td>15.0–24.0</td>
</tr>
<tr>
<td>MCV</td>
<td>51–63</td>
<td>54–66</td>
</tr>
<tr>
<td>MCHC+</td>
<td>34–35</td>
<td>32–34</td>
</tr>
<tr>
<td>Indirect bilirubin</td>
<td>2.8</td>
<td>1.9</td>
</tr>
<tr>
<td>(mg/100 ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red cell destruction†</td>
<td>4%/day</td>
<td>3.6%/day</td>
</tr>
</tbody>
</table>

*Calculated according to microhematocrits and hemoglobin concentration.† Measured according to reference 35.
RESULTS

Membrane Protein Composition

Figure 1 compares the SDS-PAGE of membrane proteins of fresh HPP red cells to normal erythrocytes. Densitometric tracings of such gels revealed that HPP red cells contained decreased amounts of spectrin relative to band 3 as indicated by a low spectrin to band 3 ratio (0.78) which in normal red cells or red cells of other hemolytic anemias was 0.93 and 0.94 ± 0.1 respectively (means ± SD of 12 determinations). When intact control and HPP red cells were solubilized directly and their proteins subjected to electrophoresis according to Fairbanks et al. similar decrease in spectrin to band 3 ratio was noted (data not shown). In addition, HPP red cells contained increased quantities of bands 2.1 and 4.5 (catalase—see references 25 and 28) and globin subunits. The latter abnormalities, however, were not specific for HPP. The increase in band 2.1 was noted in other hemolytic anemias presumably due to increase in reticulocytes (data not shown) while the increase in 4.5 and globin has been observed by us and others in red cells undergoing ATP depletion or calcium accumulation in vitro.25

Membrane Protein Crosslinking

Figure 2 depicts results of crosslinking of ghosts from fresh control and HPP erythrocytes and ghosts from red cells subjected to incubation at 46°C for 15 min. Oxidative crosslinking of ghosts from untreated control and HPP red cells produced several new complexes of mol wt of 260,000 and 450,000, respectively (arrows) that have been previously identified as heterodimers of spectrin 1 + 2 and a complex of 2 + 4.9.10,20 In contrast, ghosts from HPP red cells subjected to 46°C exhibited a major change in membrane protein crosslinking which was characterized by a formation of a high mol wt (>1 x 10⁶ Daltons) protein complex at the start of the gel. This complex was demonstrated both by intermolecular disulfide coupling with catalytic oxidants and a crosslinking of protein amino groups with glutaraldehyde. In the case of glutaraldehyde crosslinking (last two gels on Fig. 2), the high mol wt complex was quantified by densitometric scanning. The ratio of the high mol wt complex to band 3 in control and HPP ghosts was 0.13 and 0.43, respectively, indicating a threefold greater propensity of HPP cells to form the complex than the controls. HPP erythrocytes exposed to 46°C for 15 min have undergone morphological changes shown in Fig. 4, while control red cells maintained a normal morphology. During such treatment, red cell ATP was maintained in a near normal range and more than 80% of GSH remained in a reduced form.

The 1 x 10⁶ Dalton complex was nearly absent in similarly treated red cells of healthy volunteers or patients with sickle cell anemia (two patients), homozygous hemoglobin C disease, glucose-6-phosphate dehydrogenase deficiency associated with chronic hemolysis (1 patient) and hereditary spherocytosis (2 patients). In hereditary elliptocytosis, small amounts of such complex were formed at 47°C (data not shown).

The formation of the high mol wt protein complex was further detected after a direct crosslinking of intact heat treated HPP erythrocytes with diamide (0.75 mM, 30 min, 37°C) or incubation under 100% oxygen for 3 hr (Fig. 3). In normal red cells, such complex was produced at temperatures exceeding 49°C which are known to produce fragmentation and microspherocytosis in such cells.4,6 At this temperature, HPP erythrocytes exhibit considerably larger amounts of this complex and a concomitant depletion of bands 1 and 2 of spectrin. Furthermore, thermally treated HPP cells, and, to a lesser extent, control cells exhibited a retention of globin and a diffuse increase in staining intensities in the regions between bands 3 and 7, presumably resulting from an adherence of cytoplasmic proteins to the cell membrane. Both in normal
red cells and in HPP erythrocytes, the amounts of the $1 \times 10^6$ Dalton complex progressively increased when cells were exposed to increasing temperatures and this coincided with a transformation of cells from discoocytes to poikilocytes and spherocytes (Fig. 5).

Analysis of the Crosslinked Proteins by two Dimensional SDS-PAGE

In the case of disulfide crosslinking produced by catalytic oxidation of ghosts (Fig. 6) or exposure of intact cells to diamide (Fig. 7) membrane proteins and their crosslinked complexes were separated on cylindrical gels in the first dimension without a reducing agent. Subsequently, the crosslinked complexes were cleaved in the second dimension by passing them through a reductive zone containing DTT that was positioned on the top of the slab gel. After their reductive cleavage an electrophoresis in a second dimension, the individual protein constituents of the $1 \times 10^6$ Dalton complex are positioned in a vertical line under the original complex and can be identified according to their relative mobilities as compared to a control sample. The quantification of the individual proteins of the $1 \times 10^6$ Dalton complex by densitometric scanning revealed a marked enrichment of spectrin while other membrane proteins were present in relatively small amounts.

Formation of the Spectrin Enriched Complex Under Physiologic Conditions

Our demonstration of a spectrin rich complex in crosslinked HPP or normal red cells or their ghosts undergoing thermal fragmentation and poikilocyte formation in vitro contrasts with the absence of a similar complex in crosslinked fresh HPP erythrocytes which exhibit more than 60% poikilocytic forms (Fig. 4). Since such a complex could be present in a small subpopulation of HPP red cells only, we separated
HPP red cells on a discontinuous stractan density gradient and subsequently subjected the densest HPP cells (densities 1.128-1.135) to diamide or the ghosts from these cells to crosslinking with catalytic oxidants (CuSO4, o-phenanthroline). However, both treatments failed to produce the 1 x 10^6 dalton complex in such cells (not shown).

Furthermore, we have examined the propensity of HPP erythrocytes to form such a complex during in vitro incubation at 37°C. We employed anaerobic incubation with adenine, inosine, and glucose to maintain red cell ATP and GSH levels in a near normal range and hence prevent changes in membrane protein crosslinking which occur during ATP and GSH depletion in vitro. Incubation of HPP red cells for 16 hr at 37°C under the above conditions followed by crosslinking with diamide produced considerable amounts of the 1 x 10^6 dalton complex in such cells (not shown).

Changes in Calcium Influx
Since red cells from a patient with a presumptive HPP were previously shown to have increased intracellular calcium and increased calcium influx into the cells, we tested the role of calcium in the increased susceptibility of HPP erythrocytes to thermal damage. The temperature dependence of shape transformation and spectrin aggregation of HPP and normal red cells was not affected by addition of calcium (2 mM) to the medium (not shown). Furthermore, a prior enrichment of normal red cells with calcium by incubating...
red cells with calcium (0.1 mM) + ionophore A23187 for 30 min at 37°C failed to increase the susceptibility of normal cells to thermal damage. We have further examined calcium influx into fresh HPP erythrocytes and found it to be higher than that of normal cells (Table 2). However, it was of considerably smaller magnitude than calcium influx into deoxygenated sickle erythrocytes which failed to exhibit increased susceptibility to thermal denaturation (not shown).

**Ultrastructural Studies**

The scanning electron micrographs of fresh HPP red cells, shown in Fig. 4, reveal a considerable poikilocytosis and microspherocytosis as previously shown.\(^1,2\) Incubation of HPP red cells at 46°C for 15 min resulted in a marked membrane instability characterized by vesiculation (Fig. 4B), endocytosis (not shown) and further shape distortion. In contrast, normal red cells or cells from other hemolytic anemias (including hereditary spherocytosis, homozygous hemoglobin S and C disease, glucose-6-phosphate dehydrogenase deficiency associated with chronic hemolysis and autoimmune hemolytic anemia) did not exhibit any morphological injury up to 49°C. Red cells from two patients with hereditary elliptocytosis were spiculated but did not undergo fragmentation at 47°C (not shown).

Membrane skeletons, prepared by a Triton X–100 extraction of immobilized ghosts from thermally damaged HPP red cells (46°C, 15 min) exhibited poikilocytic forms similar to those of intact HPP erythrocytes (Fig. 10, top panels). Skeletons prepared directly from immobilized, previously heat treated HPP red cells exhibited additional abnormalities that included irregularities of electron density, formation
of skeleton free regions and a breakdown of entire parts of the cytoskeleton. Electrophoretic analysis of solubilized cytoskeletons reported previously revealed that they contained all major skeleton constituents (e.g., spectrin, bands 1 + 2, actin, bands 2.1 and 4.1) while they were virtually devoid of lipids, integral proteins, or cytoplasmic proteins. Under the above conditions, skeletons from normal erythrocytes maintained an intact ultrastructure. However, the ultrastuctural alterations of thermally damaged HPP cells could be duplicated in normal red cells in which spheroctysis and vesiculation were produced by exposure to temperatures exceeding 49°C (not shown).

DISCUSSION

In this report, we demonstrate major abnormalities of spectrin assembly in HPP red cell membranes and propose their possible role in HPP red cell membrane instability in vitro and, possibly, in vivo. These alterations include the following. (1) Heat treated HPP red cells when subsequently subjected to intermolecular crosslinking of membrane proteins differed from normal erythrocytes or cells from other hemolytic anemias in an increased susceptibility to form a high mol wt membrane protein complex that was markedly enriched in spectrin. Both the formation of this complex and the mechanical instability of such cells in vitro exhibited the same temperature dependence. (2) HPP red cells, but not red cells from normals or other hemolytic anemias formed a similar spectrin enriched complex when subjected to crosslinking after a prolonged incubation at body temperature, even under conditions preserving red cell ATP and GSH levels. (3) Cytoskeletons prepared from heat treated HPP red cells were misshapen, unstable and lacked a uniform appearance of the cytoskeletal meshwork. (4) Fresh HPP erythrocytes lacked any of the above abnormalities (with the exception of a poikilocytic deformation of their cytoskeletons); however, they differed from red cells of normals and other hemolytic anemias in a low content of spectrin relative to band 3.

Table 2. 45Ca Uptake by HPP Red Cells

<table>
<thead>
<tr>
<th></th>
<th>C.E.</th>
<th>D.B.</th>
<th>Controls†</th>
<th>Other Hemolytic Anemias†</th>
<th>Sickie Cell Anemia†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Red cell dpm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supematant dpm</td>
<td>3.7</td>
<td>7.0</td>
<td>1.3 ± 0.4</td>
<td>3.4 ± 2.0</td>
<td>3.4 ± 2.0</td>
</tr>
<tr>
<td><strong>N2 incubation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34.0 ± 10.0</td>
</tr>
</tbody>
</table>

*Red cells were incubated for 3 hr at 37°C as 20% suspensions in isotonic NaCl-glycylglycine buffer, containing traces of 45Ca. 45Ca uptake is expressed as a ratio of red cell to supernatant desintegrations per minute (dpm).
†Means ± SD of 10 determinations.
crosslinking may suggest that in heat treated HPP cells spectrin rearranged from a “lattice like” structure into small clusters. The close contacts among the individual spectrin molecules in such aggregates may then account for an increased spectrin susceptibility to crosslinking into a high mol wt complex.

The ultrastructural studies of normal cells suggest that the cytoskeletal network is loosely arranged presumably not covering more than 50% of the internal red cell surface.17 Thus, it is likely that the above spectrin aggregation leads to a formation of spectrin free domains in the membrane, that may be the local site of membrane instability. This possibility is supported by our demonstration of ultrastructural abnormalities of heat treated HPP red cell membrane cytoskeletons, which were unstable and exhibited irregularities in their electron density. Although we can not exclude that the latter structural perturbations were artifacts of preparation of these unstable skeletons, it is possible that, at least in part, this nonuniform cytoskeletal appearance reflected the above noted spectrin clustering and a subsequent formation of spectrin depleted domains in the membrane.

Similar ultrastructural and crosslinking abnormalities were observed in normal red cells subjected to 49°C. Both in normal and HPP red cells, such heat induced spectrin rearrangement may be responsible for their thermal instability as suggested by a positive correlation between spectrin aggregation in membrane and discocyte-poikilocyte transformation during exposure of both normal and HPP red cells to increasing temperatures. It is likely that these changes in assembly of spectrin in the membrane are a consequence of a thermal denaturation of this protein. Studies employing differential scanning calorimetry and circular dichroism have revealed that spectrin both in solution and in ghosts undergoes an irreversible phase transition at the same temperature that is required for cell fragmentation and spherocytosis.29

It is not surprising that both in normal and HPP red cells, the spectrin rich aggregate contained several other membrane and cytoplasmic proteins. This can be due both to the close physical contacts among spectrin and other membrane and cytoplasmic proteins and a possible entrapment of certain proteins within the spectrin aggregate.

The increased propensity of HPP spectrin to undergo aggregation in the membrane appears to reflect a primary defect of this protein rather than its modification by altered intracellular environment as suggested by increased susceptibility of purified HPP spectrin to thermal denaturation.7 Furthermore, our data exclude a possibility that spectrin aggregation in HPP cells is a consequence of intracellular calcium accumulation. The latter is seen in considerably greater amounts in red cells of other hemolytic anemias such as sickle cell anemia yet these cells neither exhibit thermal instability nor a thermal aggregation of spectrin. It is likely that the calcium accumulation of HPP red cells is a nonspecific conse-
quence of HPP membrane damage, since similar calcium accumulation can be produced by exposure of normal red cells to temperatures producing spectrin aggregation in such cells (Liu, S.C., Liu, P.A., Palek, J., in preparation). Likewise, ATP level of heat treated HPP red cells did not decrease below 80% of preincubation value excluding the possibility that spectrin aggregation in HPP membrane was a consequence of ATP depletion during cell exposure of heat.15 Finally, we can exclude the possibility that the increased propensity of spectrin to aggregate in the membrane was due to increased HPP susceptibility to oxidant injury. This was indicated by an absence of thermal instability of spectrin in G6PD deficient red cells (although spontaneous reducible aggregates have been recently described in a few G6PD patients35 and by the fact that spectrin of heat treated HPP red cells could be crosslinked into a high mol wt aggregate with agents other than those producing intermolecular disulfide couplings (e.g., glutaraldehyde).

The contribution of spectrin aggregation to membrane instability of HPP erythrocytes in vivo is presently uncertain. The absence of spectrin aggregation, even in the densest fresh HPP red cells suggest that other alterations may be significant in a removal of such cells from the circulation such as decreased binding of spectrin to ankyrin, (a recently identified polypeptide anchoring spectrin to band 3, the major transmembrane protein24 actin or an altered spectrin dimer tetramer equilibrium. However, we did observe the high mol wt spectrin enriched complex in HPP red cells incubated at physiologic temperatures even under conditions maintaining intracellular ATP and GSH levels that prevent the formation of reducible membrane protein complexes characteristic of ATP depleted erythrocytes.15,26 Furthermore, fresh HPP red cells contained diminished amounts of spectrin relative to band 3. Although our data can not exclude the possibility of proteolytic degradation or a decreased synthesis of this protein, it is possible that the decrease in spectrin relative to band 3 is related to the fact that small amounts of such aggregates form in vivo and that they are subsequently removed from the cells during their splenic passage as is the case with Heinz bodies33 or other high mol wt membrane protein complexes.34

ACKNOWLEDGMENT

We are indebted to Jim Munroe and Gail Domico for their skillful technical assistance and to Paula Foley and Rona Goodman for the typing of this manuscript.

REFERENCES

34. Lux SE, John KM: Isolation and partial characterization of a high molecular weight red cell membrane protein complex which is normally removed by the spleen. Blood 50:625, 1977
Altered assembly of spectrin in red cell membranes in hereditary pyropoikilocytosis

J Palek, SC Liu, PY Liu, J Prchal and RP Castleberry