Viscoelastic Properties of Red Cell Membrane in Hereditary Elliptocytosis

By A. Chabanel, K.-L.P. Sung, J. Rapiejko, J.T. Prchal, J. Palek, S.C. Liu, and S. Chien

The viscoelastic properties of the RBC membrane are in part determined by a submembrane network of proteins consisting of spectrin $\alpha\beta$ heterodimers (SpD) assembled head-to-head to form spectrin tetramers (SpT) and spectrin oligomers (SpO). SpT, in turn, are connected into a two-dimensional network by the linkage of distal ends of SpT to protein 4.1 and actin. With the micropipette technique, we determined the membrane viscoelastic properties of RBCs from a subset of patients with hereditary elliptocytosis (HE); these RBCs exhibit membrane skeletal instability, defective SpD self-association, and a molecular defect in the $\alpha_1$ domain of spectrin, which is involved in the SpD–SpD contact (HE SpDa–SpD). The elastic modulus and viscosity of the membrane were significantly higher for the HE RBCs than for the control cells. Incubation of normal cells with $N$-ethylmaleimide (NEM) produced a similar defective SpD self-association and a significant increase in the viscoelastic parameters of the membrane. The data provide evidence that the mode of assembly of membrane spectrin in the cytoskeletal protein network plays a major role in determining the rheologic behavior of erythrocyte membrane.

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MATERIALS AND METHODS

Clinical materials. Venous blood from eight normal subjects, five HE patients, and one carrier from two unrelated families was collected in sterile tubes containing citrate/phosphate/dextrose or citrate/dextrose. The blood specimens were transported in insulated containers with ice and analyzed within 24 hours; each shipment of HE or carrier sample was always accompanied by at least one normal sample for concurrent measurements as control. The clinical presentation and the functional and structural characterization of the abnormal spectrin have been described previously. Family S included two individuals with mild HE and one asymptomatic carrier. In family D, all three probands had mild HE at the time of the study; in the first year of life, all of them had poikilocytic hemolytic anemia which subsequently converted to mild HE. At the structural level, the abnormal spectrin was identified as the Spot1/74 variant, based on the abnormalities in tryptic peptide of spectrin,
analyzed by two-dimensional isoelectric focusing–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Treatment of erythrocytes with NEM. Human erythrocytes were obtained from freshly drawn blood of normal donors and washed three times with an isotonic "wash buffer" composed of 8 mmol/L sodium phosphate (pH 7.4), 145 mmol/L NaCl, and 5 mmol/L KCl. The washed cells were suspended in 5 mmol/L sodium phosphate isotonic buffer (pH 7.4), and aliquots were incubated with the buffer or buffer containing 0.5 to 2.0 mmol/L NEM for 15 minutes at 37°C. Then the cells were washed and incubated with 5 mmol/L dithiothreitol (DTT) for 15 minutes more at 37°C to remove the unreacted NEM. After DTT incubation, the cells were washed three more times with buffer.

Spectrin extraction and nondenaturing agarose/PAGE. Spectrin extraction and nondenaturing agarose/PAGE were performed as described previously. Erythrocyte ghosts prepared by the method of Dodge et al.12 were washed with 0.1 mmol/L sodium phosphate (pH 8.0) and centrifuged. The ghosts obtained from the pellet were then incubated at 0°C with an equal volume of low-ionic-strength buffer (pH 8.0) containing (in mmol/L) sodium phosphate 0.1, EDTA 0.1, phenylmethylsulfonyl fluoride (Bz1SO2F) 0.1, mM Na-tosyl-L-lysine chloride (Chen and Young10), 0.1, disopropylphosphorofluoridate (DFP) 0.1, and 2-mercaptoethanol 0.1. After incubation, the supernatant extracts and ghost residues were separated by centrifugation at 250,000 g for 35 minutes.

The low-ionic-strength extracts were subjected to electrophoresis in 0.3% agarose/2.5% acrylamide gels as described by Liu et al.16 except that the electrophoresis temperature was 2 to 6°C and SDS was omitted from the gel. The relatively high porosity of these composite gels allowed the high-molecular-weight complexes (HMWs), containing spectrin oligomers associated with actin, to pass through the gel. Quantitative analysis was performed by densitometry as described previously. Erythrocyte ghosts prepared by the method of Liu et al.15 were incubated with 10 mmol/L dithiothreitol (DTT) for 15 minutes more at 37°C to maintain near 0°C. At this temperature, the SpD + SpT + HMW was studied.

Deformability measurements. Micropipettes with a radius (Rn) of 0.4 to 0.6 μm were used to study the viscoelastic properties of the erythrocyte membranes. The methodology is described in detail by Chien et al.9 The membrane elastic, modulus, which measures the steady-state resistance to deformation, was calculated from the relationship between the stress applied, (P)R, and the strain induced, Dmn/Rs. The membrane viscosity, which reflects the dynamic rates of deformation or recovery in response to changes in stress, was calculated as the product of the time constant of the stress, (P)R, and the strain (Dmn/Rs).

Table 1. Membrane Viscoelastic Moduli of HE RBCs

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Clinical Presentation</th>
<th>Percentage of Spectrin Dimer</th>
<th>Elastic Modulus (10^1 dynes/cm)</th>
<th>Viscosity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (30)</td>
<td>No elliptocytes</td>
<td>5</td>
<td>3.63 ± 0.17</td>
<td>1</td>
</tr>
<tr>
<td>Patient M.S. (6)</td>
<td>HE, no hemolysis</td>
<td>25</td>
<td>8.99 ± 0.31*</td>
<td>3.30 ± 0.43*</td>
</tr>
<tr>
<td>Patient V.S. (12)</td>
<td>HE, no hemolysis</td>
<td>25</td>
<td>8.74 ± 0.16*</td>
<td>4.84 ± 0.56*</td>
</tr>
<tr>
<td>Asymptomatic carrier O.C. (6)</td>
<td>No elliptocytes</td>
<td>20</td>
<td>7.53 ± 0.04*</td>
<td>2.19 ± 0.06*</td>
</tr>
<tr>
<td>Proband Jd.S. (6)</td>
<td>HPP—HE, no hemolysis</td>
<td>15–20</td>
<td>5.37 ± 0.11*</td>
<td>1.77 ± 0.29</td>
</tr>
<tr>
<td>Brother Je.D. (7)</td>
<td>HPP—HE, no hemolysis</td>
<td>15–20</td>
<td>5.84 ± 0.21*</td>
<td>2.95 ± 0.34*</td>
</tr>
<tr>
<td>Sister D.D. (7)</td>
<td>HPP—HE, no hemolysis</td>
<td>15–20</td>
<td>5.70 ± 0.20*</td>
<td>2.83 ± 0.52</td>
</tr>
</tbody>
</table>

Values are ± SE. HPP—HE, hereditary pyropoikilocytosis-like presentation in the first year of life subsequently converted to HE. *P < .001, †P < .05, ‡P < .01, Anova and Bonferroni test for significance of difference from control.8

RESULTS

All of the HE patients and the asymptomatic carrier exhibited an increase of percentage of SpD in the low-ionic-strength extracts of RBC membranes (Table 1). During membrane extraction, the temperature was carefully maintained near 0°C. At this temperature, the Spd-Spd equilibrium is kinetically immobilized so that the relative proportion of the individual spectrin species in the crude spectrin extract reflects their relative distribution in the RBC membrane in situ. Neither qualitative nor quantitative abnormalities of membrane proteins in these HE cells was observed by SDS-PAGE.16

The micropipette technique was used to determine the intrinsic mechanical properties of the membrane. The results are not influenced by the ratio of cell surface area to volume and by the cell internal viscosity unless these are drastically altered. The results of micropipette tests for the HE samples are summarized in Table 1 and Fig 1; all these samples showed an increase in membrane elastic modulus (P < .001) above the normal controls. As shown in Table 1, phase I viscosity was also elevated in HE patients.

Incubation of RBCs with millimolar (0.5 to 2.0 mmol/L) concentrations of NEM for 15 minutes at 37°C resulted in an increase in the proportion of Spd in the spectrin extracted from the treated membranes. The protein composition of ghosts (SDS-PAGE) from treated cells was identical to that of control cells. The increase in the percentage of spectrin dimer extracted from the membrane was associated with significant increases in the membrane elastic modulus and viscosity (Table 2). In HE RBCs or in NEM-treated normal
Each viscoelastic parameter is expressed as the ratio of the value of RBCs, no residual cells was higher in the NEM experiments (determined at the low-ionic-strength extracts). An increase in the percentage of spectrin dimers appears to be the spectrin tetramers. An increase in the elastic modulus should decrease with the membrane elastic modulus.

DISCUSSION

In this study on erythrocyte membranes of subjects with HE and of NEM-treated normal RBCs, an increased percentage of spectrin dimers in the low-ionic-strength extracts of RBC membranes was associated with an elevation of membrane elastic modulus. The value of SpD for the control cells was higher in the NEM experiments (determined at Columbia University College of Physicians and Surgeons, New York) than that in the HE experiments (determined at St Elizabeth Hospital, Boston). Therefore, the experimental results in each series should be compared with their own control. A similar difference in control SpD values has been reported.16,23

Our results of micropipette tests and SpD determinations suggest that the mechanical properties of the erythrocyte membrane may be affected by a specific change in the state of the membrane spectrin. According to the elastomer theory, the membrane elastic modulus should decrease with the number of network subunits,24 which in the RBC membrane, appear to be the spectrin tetramers. An increase in the spectrin dimer proportion, and its concomitant decrease in spectrin tetrayers in the membrane, should lead to a decrease in elastic modulus. In this study, the opposite was found; the cause of this elevation in elastic modulus remains unknown. Liu et al.25 examining the isolated membrane skeleton of normal human erythrocytes by high-resolution negative-staining electronmicroscopy, described it as a network of hexagonal substructures. Each hexagonal subunit consisted of a short filament of F-actin at the center and spokes of six long filaments resembling SpT radiating from the center. In describing the various types of elastomeric networks, Mark26 described network irregularities such as dangling chains, which are attached to the network at only one end. In HE, the defect in spectrin self-association could result in such dangling spectrin dimers. This could generate increased local entanglement between the different layers of the network, bundling and aggregation of the spectrin filaments, or increased clustering around the junctional complexes (actin filaments). All these alterations of the skeletal structure may result in an increase in elastic modulus as observed in the present experiment. The molecular defect in the α domain may also affect the conformation of the spectrin molecule and, in consequence, modify its flexibility. This, in turn, could alter the deformability of the protein network.

Two previous studies demonstrated an increase in the mechanical fragility of RBC membranes in HE. Liu et al.16 observed that membrane skeletons produced from ghosts of type I (abnormal spectrin dimer–dimer association) or type II (normal dimer–dimer association) HE were abnormally fragile when mechanically shaken. Chasis and Mohandas,27 studying the fragmentation of whole cells and ghosts under shear stress, reported a significant reduction in the time required for fragmentation of HE RBCs to about one-half of the normal value. According to these observations and our present finding, the biochemical defects of the HE erythrocyte membrane skeleton appear to affect its mechanical properties, rendering it less deformable and more brittle, somewhat analogous to the mechanical behavior of aged rubber.

We have demonstrated that in one type of HE (type I HE, expressed as mild HE with no or minimal hemolysis) membrane fragility is associated with an increase in membrane rigidity. For two patients with the same type of HE, a similar finding was obtained in a recent study of membrane deformability assessed by ektacytometry of resealed RBC membranes.28 The one carrier studied (O.C., Table 1) has normal discocytes but also exhibits an abnormal spectrin dimer content in the membrane extract and an elevated membrane

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of Spectrin Dimer (%)</th>
<th>Elastic Modulus (10⁻² dynes/cm²)</th>
<th>Viscosity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10 ± 3</td>
<td>3.38 ± 0.22</td>
<td>1</td>
</tr>
<tr>
<td>NEM 0.5 mmol/L</td>
<td>28 ± 2</td>
<td>7.73 ± 0.36*</td>
<td>0.92 ± 0.18</td>
</tr>
<tr>
<td>NEM 1 mmol/L</td>
<td>56 ± 6</td>
<td>6.52 ± 0.32*</td>
<td>1.39 ± 0.16</td>
</tr>
<tr>
<td>NEM 2 mmol/L</td>
<td>83 ± 8</td>
<td>9.33 ± 0.41*</td>
<td>2.43 ± 0.28*</td>
</tr>
</tbody>
</table>

Values are ± SE. *P < .001, Anova and Bonferroni test for significance of difference from controls.
elast modulus. Thus, the increased rigidity of the RBC membrane appears to be associated with the spectrin defect rather than with the elliptical shape. Our observation that the RBCs from one patient affected with type II HE (normal spectrin dimer–dimer association) had an elast modulus not significantly different from the control cells (ratio to control = 0.90, \( P = .17 \)) supports this proposition. In addition, one patient with the type of HE characterized by a depletion of band 4.1 exhibited a decrease in the membrane elast modulus of his RBCs.29 These findings, added to the fact that nucleated erythroid precursors of HE cells are normally round,30 confirm the general belief that the abnormal elliptical shape is secondary to a combination of different nonspecific factors which appear to predispose the RBC to shear-induced permanent shape deformation in vivo.

Waugh and Agre\(^{31} \) showed that the membrane shear elasticity was directly proportional to the surface density of spectrin on the membrane. The present study provides evidence that the mode of assembly of the membrane spectrin proteins also plays an important role in determining the viscoelastic behavior of the membrane and that the micropipette test can be used to demonstrate altered rheologic parameters of RBC membrane in disease.

REFERENCES

Viscoelastic properties of red cell membrane in hereditary elliptocytosis

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