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 $\alpha$I domain of spectrin, which is involved in the SpD–SpD contact (HE SpD$\alpha$–SpD$\alpha$). 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 $N$-ethyl-maleimide (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.

As recently described,1 the major elements of the RBC membrane skeleton are the $\alpha$ and $\beta$ chains of spectrin (each &~100 nm long) which spiral around each other to form spectrin $\alpha\beta$ heterodimers (SpD). At their proximal ends, the SpD molecules self-associate into spectrin tetramers (SpT) and spectrin oligomers (SpO). This SpD self-association, both in solution and in the membrane, is governed by a simple thermodynamic equilibrium which depends on the spectrin concentration, ionic strength, and temperature.2,3 In the normal RBC membrane, this equilibrium strongly favors SpT and SpO, whereas only small amounts of spectrin are present in the form of SpD.4 At their distal ends, SpD are connected to oligomers of actin. The erythrocyte membrane exhibits a viscoelastic behavior.5 To provide a structural basis of the remarkable deformability of the RBC, the molecular model of the cytoskeleton must be correlated with the biophysical behavior of the RBC membrane.

Recent investigations of molecular defects of membrane skeleton in patients with hereditary elliptocytosis (HE) and a related disorder, hereditary pyropoikilocytosis (HPP), led to identification of distinct molecular defects of spectrin in all HPP patients yet studied and in a subset of patients with common HE.6-8 These abnormal spectrins are characterized by a defective assembly of SpD into SpT or SpO in solution, ghosts, or inside-out vesicles and by an increased percentage of SpD in the crude spectrin extract of the membrane. Functionally, this defective SpD self-association is accompanied by an increase in the mechanical fragility of membrane skeletons.9 Three different types of structural defects were identified in these abnormal spectrins by means of limited proteolytic digestion followed by two-dimensional analysis of the tryptic peptides.6,10 All three structural spectrin variants involve the 80,000-dalton peptide ($\alpha$I/80 fragment) which is derived from spectrin $\alpha$ chain and has been identified as the SpD self-association site.11 Patients who are either homozygous or double heterozygous for this defect exhibit severe hemolytic anemia with marked RBC fragmentation. In contrast, heterozygote carriers of these abnormal spectrins either are asymptomatic or manifest clinically as mild common HE; ie, they have minimal or no hemolysis and their blood smears contain 20% to 100% of elliptical cells.7

The purpose of the present investigation was to relate the viscoelastic properties of the RBC membrane to the state of spectrin in the membrane, using RBCs from the HE subjects, specifically the SpI/74 variant,9 and an asymptomatic carrier with the same molecular defect. SpI/74 is a subfragment of $\alpha$I/80 and does not self-associate. We also performed parallel studies on normal RBCs incubated with millimolar concentrations of $N$-ethyl-maleimide (NEM), which results in the same alteration of spectrin.12 With the micropipette test, we determined the viscoelastic parameters of the erythrocyte membrane during deformation at constant surface area for these HE RBCs and NEM-treated RBCs.

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.9 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 SpI/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 nondenaturating agarose/PAGE were performed as described previously. Erythrocyte ghosts prepared by the method of Dodge et al. 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 (BzSOF) 0.1, mM Na-tosyl-L-lysine chloromethyl ketone HCl (Tos-Lys-CH2Cl) 0.1, diisopropylfluorophosphate (iPr2 P-F) 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., 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 enter the gel. Quantitative analysis was performed by densitometry of the Coomassie blue-stained gels. The proportion of spectrin dimer in the extracts was expressed as the percentage of SpD over the total amount of proteins in the extract (SpD + SpT + HMW).

Deformability measurements. Micropipettes with a radius (R) 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. 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, D/R. 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, (τ)R, and the strain (D/R). The membrane viscosity of the initial rapid phase of deformation I (n) varies inversely with the level of deforming stress, (AR)R, as well as the degree of deformation, D/R. The n values of control and experimental (HE or NEM-treated) cells cannot be compared directly unless the stress or strain is specified. Hence, each experimental n value obtained at a given D/R is divided by the control value at the same D/R (read from a curve of control n = D/R)n, and this ratio is termed the viscosity index. This index equals 1 when the experimental value equals the control value obtained at the same D/R. For each blood shipment, the same micropipette was used throughout the experiment. A control sample was studied at the beginning and end of the experiment, and the variation between these two determinations was <20%. Six to 12 cells were examined for each sample. All measurements were made at room temperature (21 to 24°C). Statistical analysis of the data was performed by analysis of variance followed by the Bonferroni test for the pairwise comparisons among groups. The measurements of spectrin dimer content in the membrane extract were performed concurrently on the same blood sample as that used for the micropipette test.

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-SpT-SpO 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.

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 of 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

<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>Probands J.D. (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 J.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.
Each viscoelastic parameter is expressed as the ratio of the value of the parameter after the 20-second period of aspiration on the cell surface to that for the control cells. No residual bumps were observed on the cell surface on release of pressure after the 20-second period of aspiration into the micropipette.

**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 30% SpD). 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. 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 elastic modulus. No residual bumps were observed on the cell surface on release of pressure after the 20-second period of aspiration into the micropipette.

**Table 2. Membrane Viscoelastic Moduli of NEM-Treated RBCs**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Elastic Modulus (10^-3 dynes/cm)</th>
<th>Viscosity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10 ± 2</td>
<td>3.58 ± 0.22</td>
</tr>
<tr>
<td>NEM 0.5 mmol/L</td>
<td>38 ± 8</td>
<td>7.73 ± 0.36*</td>
</tr>
<tr>
<td>NEM 1 mmol/L</td>
<td>56 ± 6</td>
<td>6.52 ± 0.32*</td>
</tr>
<tr>
<td>NEM 2 mmol/L</td>
<td>68 ± 8</td>
<td>9.33 ± 0.41*</td>
</tr>
</tbody>
</table>

Values are ± SE. *P < .001, Anova and Bonferroni test for significance of difference from controls.
elastin 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 elastin modulus not significantly different from the control cells (ratio to control = 0.90, \( P = 0.17 \)) 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 elastin modulus of his RBCs. These findings, added to the fact that nucleated erythroid precursors of HE cells are normally round, 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

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