Altered Erythrocyte Endothelial Adherence and Membrane Phospholipid Asymmetry in Hereditary Hydrocytosis

Running Title: Endothelial Adherence, Phospholipid Asymmetry, and Hydrocytosis

Patrick G. Gallagher, Seon Hee Chang, Michael P. Rettig, John E. Neely, Cheryl A. Hillery, Brian D. Smith, Philip S. Low

Word Counts: Abstract 137; Text 1200.

Scientific Section: Red Cells

Department of Pediatrics, Yale University School of Medicine, New Haven, CT, Department of Chemistry, Purdue University, West Lafayette, IN, Penn State University, Hershey, PA, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, and the Department of Medicine, University of Rochester School of Medicine, Rochester, NY.

Supported in part by grants from the National Institutes of Health (HL70981 CAH) and the March of Dimes Birth Defects Foundation.

Address correspondence to: Patrick G. Gallagher, Department of Pediatrics, Yale University School of Medicine, 333 Cedar Street, P. O. Box 208064, New Haven, CT 06520-8064. Tel: (203) 688-2896; Fax: (203) 785-6974; Email: patrick.gallagher@yale.edu.
Abstract

The risk of thrombosis is increased in patients with hereditary hydrocytosis, an uncommon variant of hereditary stomatocytosis. Erythrocytes from two hydrocytosis patients were studied to gain insight into the mechanism of thrombosis in this disorder. Erythrocytes demonstrated abnormal osmotic scan ektacytometry and decreased erythrocyte filtration rates. There was also a mild increase in adherence of erythrocytes to endothelial monolayers in a micropipette assay. Adhesion of erythrocytes to the subendothelial matrix proteins thrombospondin and laminin, however, was not significantly increased. The percentage of hydrocytosis erythrocytes and reticulocytes with phosphatidylserine exposed on the outer surfaces was increased in both patients relative to healthy controls and indicate altered membrane phospholipid asymmetry. Increased phosphatidylserine exposure accelerating thrombin-forming processes has been proposed as a mechanism for thrombosis in sickle cell disease and β-thalassemia, and may play a similar role in hereditary hydrocytosis.
Introduction

The risk of thrombosis is increased in many erythrocyte disorders including sickle cell disease and β-thalassemia.\textsuperscript{1,2} Thrombosis is also increased in the hereditary stomatocytosis syndromes, an uncommon, heterogeneous group of disorders characterized by mouth-shaped erythrocyte morphology on peripheral blood smear.\textsuperscript{3-5} The erythrocyte membranes of stomatocytosis patients exhibit abnormal permeability to sodium and potassium, with resultant modification of intracellular water content.\textsuperscript{4,5}

One rare stomatocytosis variant, hydrocytosis, is characterized by a net gain of sodium and potassium that leads to the retention of water, forming swollen, overhydrated erythrocytes. The molecular basis of hydrocytosis is unknown. We studied two of these very rare hydrocytosis patients, one whose affected father suffered from multiple thromboses, and another who has suffered from multiple thromboses, the first at 19-years-of-age.
Methods

Patients. Patient one is the 11-year-old son of the proband in the report by Zarkowsky et al. Typical red cell indices are hemoglobin 8-11g/dl, MCV 125-140fl, MCHC 26-27%, reticulocyte count 7-25%. Peripheral blood smear shows marked stomatocytosis. Incubated erythrocytes demonstrate increased osmotic fragility. He underwent splenectomy at 5-years-of-age and has not had any episodes of thrombosis. His father, who was splenectomized in the second year of life, suffered from multiple, chronic pulmonary thromboemboli which resulted in his death at 34-years-of-age.

Patient two has suffered from severe stomatocytosis with hemolytic anemia and jaundice since birth. He required multiple blood transfusions until age 4-years when he was splenectomized. He had fewer hospitalizations for hemolytic crises and was nearly asymptomatic from age 7-15 years. Hemolytic crises recurred during adolescence. At 19-years-of-age, he suffered from pulmonary embolus and was treated with heparin followed by coumadin. He has suffered from additional thrombotic events including deep vein thrombosis, myocardial infarction at age 29-years, and pulmonary hypertension attributed to chronic pulmonary emboli. Currently 36-years-of-age, he receives chronic erythrocyte transfusions. Prior to transfusion therapy, typical red cell indices were hemoglobin 11.9-12.8g/dl, MCV 124-136fl, MCHC 28-29%, reticulocyte count 26-28%. Incubated erythrocyte osmotic fragility was increased. Erythrocyte membranes demonstrate significantly decreased stomatin immunoreactivity.

Materials. Except where noted, whole blood was centrifuged, the plasma and buffy coat removed, and the remaining erythrocytes washed three times by repeated resuspension and centrifugation at 800xg, 600xg and 500xg, respectively. This washing procedure was shown to
efficiently remove platelets and leukocytes, which represented at most 0.05% of the residual cells. To collect a reticulocyte-rich fraction, buffy coat-free erythrocytes were centrifuged at 27000g for 60 minutes at 30°C. The top 5% of cells were harvested and reticulocytes were counted by the manual method. Samples from patient two were obtained 4 to 5 weeks after his last transfusion.

**Ektacytometry.** Packed erythrocytes were suspended in 4% polyvinylpyrrolidone solution and subjected to increasing osmolality (from 50-500mosmol/kg) at constant shear stress or to constant osmolality at increasing shear stress (0-250dynes/cm²). The axial ratio of the deformed cells was designated the deformability index (DI).

**Erythrocyte filtration rate.** Analysis of the rate of filtration of an erythrocyte suspension through a 4.6µm diameter nickel mesh filter was conducted by a gravity-based, vertical tube method that measures the rate of passage through the filter as a function of hydrostatic pressure (Tsukusa Sokken, Tokyo). Data shown are means ± SD of 4~6 measurements from 2 independent experiments.

**Phosphatidylserine exposure.** Unfractionated, reticulocyte-rich and reticulocyte-depleted erythrocytes were labeled with annexin V (Roche, Indianapolis, IN) and analyzed by flow cytometry using the method of Kuypers *et al.* with minor modifications. After incubation at 0.04% hematocrit for 15min with 4nM FITC-labeled annexin V, cells were diluted to 0.01% hematocrit and immediately analyzed by flow cytometry (Coulter XL-MCL, Hialeah, FL). Each sample was assayed in triplicate.

**Endothelial adhesion.** Adherence of erythrocytes to endothelium was quantified by measuring the shear force required to separate individual cells from cultured human umbilical
endothelial cell layers using a micropipette technique. Adhesion of erythrocytes to the subendothelial matrix proteins thrombospondin and laminin was quantified as described.
Results and Discussion

Ektacytometry. Purified erythrocytes were examined by ektacytometry to obtain information on cell water content, surface/volume ratio and membrane deformability.\textsuperscript{13,14} There was a reproducible, but mild increase in erythrocyte deformability as a function of increasing shear stress in both patients compared to controls (Figure 1A).

![Graph A: Whole cell deformability](image)

![Graph B: Osmotic deformability](image)

Figure 1. Evaluation of erythrocytes from patients with hereditary hydrocytosis and normal donors by ektacytometry. (A) Whole cell deformability was measured by subjecting isotonic erythrocytes to increasing shear stress. (B) Osmotic deformability was measured by subjecting erythrocytes to a moderate shear stress while increasing osmotic pressure. The data shown were highly similar in two experiments conducted on the patients’ blood collected on two separate occasions.
Osmotic scan ektacytometry demonstrated that the hypertonic, declining arms of the curves (Figure 1B) were displaced to higher osmolalities. The $O_{\text{min}}$, a measure of the average surface area/volume ratio of the erythrocyte, was increased in the hydrocytosis cells as was the $O_{\text{hyper}}$, which reflects the low mean corpuscular hemoglobin concentrations of these erythrocytes.\(^{13}\) The maximum value of the deformability index (DI$_{\text{max}}$), a measure of the maximal deformability at optimal cell hydration, was also slightly increased, suggesting possible expansion of the membrane surface area.

_Erythrocyte filterability._ Erythrocyte filterability, a direct measure of cellular rheology, was examined on purified erythrocytes. Filtration rates were reduced for both patients, indicating a marked decrease in the filterability of hydrocytosis erythrocytes (Table 1). This is probably due to the swollen nature of the hydrocytosis cells, which could cause them to traverse pores more slowly.\(^7\) Alternatively, considering the thrombotic tendencies in stomatocytosis patients, hydrocytosis erythrocytes may tend to aggregate, which would also lead to the increase in resistance during flow through a $4\mu$m pore.

_Erythrocyte phosphatidylserine exposure._ An increase in PS exposure has been observed in a subpopulation (2-3%) of erythrocytes from patients with sickle cell disease\(^{10,15}\) and $\beta$-thalassemia.\(^{16,17}\) This increase in PS-exposing erythrocytes has been proposed as a mechanism for the prothrombotic state seen in these disorders,\(^{10,15,17}\) as PS exposure may lead to the assembly of the contact coagulation factors by accelerating thrombin-forming processes.\(^{18}\) Unfractionated erythrocytes from both hydrocytosis patients demonstrated increased PS exposure (Table 1) in the range observed in erythrocytes of patients with sickle cell disease or $\beta$-thalassemia. These values represent the mean $\pm$ S.D of results from samples obtained on
separate dates. Reticulocyte-enriched and reticulocyte-depleted erythrocytes demonstrated significant increases in annexin V binding in both patients (Table 1).

**Endothelial adherence.** Adherence of hydrocytosis erythrocytes to endothelium was mildly increased in both patients as determined by a micropipette shear stress technique (Table 1). Under low flow conditions (1 dyne/cm²), there was slightly increased adhesion of erythrocytes from patient two to thrombospondin but much less than observed with sickle erythrocytes. Erythrocytes from the hydrocytosis patients did not demonstrate significantly increased adhesion to laminin.

Increased endothelial adherence of erythrocytes with altered membrane phospholipid asymmetry has been observed. Sickle erythrocyte phosphatidylserine exposure strongly correlates with adhesion to resting microvascular endothelial cells, in agreement with our observation of mildly increased adhesion of hydrocytosis erythrocytes to endothelium. Phosphatidylserine exposure may also contribute to erythrocyte adhesion to matrix bound thrombospondin. We did not observe a significant increase in thrombospondin binding, probably due to the limited number of patients and differences in experimental conditions. Cells in this earlier report were treated with calcium and A23187, elevating the number of PS-exposing erythrocytes from ~2% to ~5-10%. Hydrocytosis erythrocytes were not pretreated prior to the thrombospondin binding assay. It is still possible that significantly increased PS exposure leads to increased endothelial adhesion either via thrombospondin or directly to the endothelial cell *in vivo*.

This very small sample size precludes any definitive conclusions about the mechanisms of thrombosis in hydrocytosis. However, these initial data suggest that increased erythrocyte
endothelial adhesion, loss of membrane asymmetry, and exposure of phosphatidylserine may contribute to the thrombosis risk of hydrocytosis patients.
Table 1. Characteristics of Stomatocytic Erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Patient One</th>
<th>Control</th>
<th>Patient Two</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtration Rate (% buffer control)</td>
<td>21±12</td>
<td>92±9</td>
<td>67±5</td>
<td>90±2</td>
</tr>
<tr>
<td>Erythrocyte Adherence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shear Force (dynes/cm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (2000)</td>
<td>7 (2000)¹</td>
<td>9 (1000)</td>
<td>3 (1000)</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (2000)</td>
<td>1 (2000)¹</td>
<td>2 (1000)</td>
<td>0 (1000)</td>
</tr>
<tr>
<td>Thrombospondin Adhesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(erythrocytes/mm²)</td>
<td>&lt; 1</td>
<td>9²</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>Laminin Adhesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(erythrocytes/mm²)</td>
<td>&lt; 1</td>
<td>102²</td>
<td>180</td>
<td>150</td>
</tr>
<tr>
<td>Annexin V Binding (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unfractionated Erythrocytes</td>
<td>2.34±0.12⁴</td>
<td>0.80±0.16</td>
<td>2.77±0.25⁵</td>
<td>0.94±0.08</td>
</tr>
<tr>
<td>Reticulocyte Enriched⁶</td>
<td>2.25±0.36⁷</td>
<td>0.27±0.05</td>
<td>2.19±0.03⁸</td>
<td>0.58±0.02</td>
</tr>
<tr>
<td>Reticulocyte Depleted⁶</td>
<td>0.83±0.09⁹</td>
<td>0.08±0.01</td>
<td>1.71±0.16¹⁰</td>
<td>0.47±0.01</td>
</tr>
</tbody>
</table>

¹Sickle cell controls (total # adherent cells/total # RBCs observed): 20 dyne/cm² 10.9/1000, 30 dyne/cm² 6.5/1000, 30 dyne/cm² 9.1/1000. ²Sickle cell controls: thrombospondin 750 erythrocytes/mm²; laminin: 2500 erythrocytes/mm². ³Value for positive control erythrocytes treated with calcium and A23187 was 54±4%. ⁴p value (unpaired student t test) = 0.0002. ⁵p value = 0.0003. ⁶Manual reticulocyte counts (%): Enriched fraction: patient 1 and control 24±4% and 1±0.2%, patient 2 and control 32.3±8.1% and 1.96±0.8%; Depleted fraction: patient
1 and control 6.5±1.4% and 0%, patient 2 and control 1.71±0.01 and 0.47±0.01%. ⁷ p value = 0.0007. ⁸ p value = 0.0001. ⁹ p value = 0.0001. ¹⁰ p value = 0.0002.
Figure Legend

Figure 1. **Evaluation of erythrocytes from patients with hereditary hydrocytosis and normal donors by ektacytometry.** (A) Whole cell deformability was measured by subjecting isotonic erythrocytes to increasing shear stress. (B) Osmotic deformability was measured by subjecting erythrocytes to a moderate shear stress while increasing osmotic pressure. The data shown were highly similar in two experiments conducted on the patients’ blood collected on two separate occasions.
References


Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis

Patrick G Gallagher, Seon Hee Chang, Michael P Rettig, John E Neely, Cheryl A Hillery, Brian D Smith and Philip S Low