Quantitative assessment of sensing and sequestration of spherocytic erythrocytes by human spleen: implications for understanding clinical variability of membrane disorders

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Abstract

Splenic sequestration of red blood cells (RBCs) with reduced surface area and cellular deformability has been long recognized to contribute to pathogenesis of several RBC disorders, including hereditary spherocytosis. However, the quantitative relationship between the extent of surface area loss and splenic entrapment remains to be defined. To address this issue, we perfused ex vivo normal human spleens with RBCs displaying varying degrees of surface area loss and monitored the kinetics of their splenic retention. Treatment with increasing concentrations of lysophosphatidylcholine resulted in a dose-dependent reduction of RBC surface area at constant volume, increased osmotic fragility and decreased deformability. The degree of splenic retention of treated-RBCs increased with increasing surface area loss. RBCs with a >18% average surface area loss (> 27% reduced S/V ratio) were rapidly and completely entrapped in the spleen. Surface-deficient RBCs appear to undergo volume loss after repeated passages through the spleen and escape from splenic retention. This study defines for the first time the critical extent of surface area loss leading to splenic entrapment and identifies an adaptive volume regulation mechanism that allows spherocytic RBCs to prolong their life span in circulation. This has significant implications for understanding the clinical heterogeneity of RBC membrane disorders.
Introduction

During their 120 day life span, human red blood cells (RBCs) repeatedly traverse capillaries of the vascular bed and inter-endothelial slits (IES) of the venous sinus of spleen red pulp, both of which are narrower than their smallest dimension \(^1\). This necessitates maintenance of the RBC ability to undergo repeated, extensive and reversible deformations. Repeated major membrane deformations induce ion and water permeability changes in the RBC \(^2\)–\(^5\). The biconcave discoid shape endows the human RBC with an advantageous surface area – to – volume (S/V) ratio, allowing the cell to undergo marked deformations while maintaining constant surface area \(^6\)–\(^8\).

Reduced RBC S/V ratio has long been recognized to contribute to pathogenesis of several RBC disorders \(^9\)–\(^11\) including hereditary spherocytosis (HS), the most common cause of inherited chronic hemolytic anemia in Northern Europe and North America with an estimated incidence of 1 in 2000 \(^11\). The clinical presentation can range from mild to severe hemolytic anemia \(^12\),\(^13\). The molecular basis of HS is heterogeneous, the common denominator being the loss of HS RBC membrane surface area due to specific molecular defects in several membrane proteins (\(\alpha\) or \(\beta\) spectrin, ankyrin, protein 4.2, and band 3), which result in the loss of cohesion between the lipid bilayer and the membrane skeleton \(^10\),\(^11\),\(^14\),\(^15\). The loss of membrane surface area results in the transformation of the biconcave discoid shape, first to a stomatocyte and finally to a spherocyte, with a progressive reduction in cellular deformability.

Although a major role for the spleen in pathogenesis of HS is well established \(^16\), there is currently no data on the quantitative relationship between the extent of surface area loss and the extent of splenic entrapment. The only indirect evidence comes from early studies, which documented reduced survival of Cr\(^{51}\)-labelled spherocytes infused into normal recipients,
whereas the survival of normal RBC in HS subjects was normal 17-19. This implied that the reduced life span and splenic entrapment was an intrinsic feature of HS RBC. Unfortunately, as neither surface area nor S/V ratio of infused spherocytes was measured in these studies, the extent of membrane surface area loss (or reduced S/V ratio) that leads to splenic retention of altered RBC remains undefined. As a result, there is no predictive biological parameter to estimate the risk of splenic entrapment of spherocytic cells and ensuing anemia. We addressed this important issue using the isolated human spleen system 20 perfused with human RBCs with defined extents of surface area loss.

RBCs with defined loss in membrane surface area can be experimentally generated by treatment with lysophosphatidylcholine (LPC) 6,7. By initially accumulating exclusively in the external leaflet of the RBC lipid bilayer, LPC induces in a dose-dependent manner, echinocytosis (spiculation) and eventually spherocytes through release of microvesicles 21,22. The effects of LPC on RBC morphology, surface area loss and S/V ratio 7,8,21,22 and cellular deformability 6,7,23,24 have been extensively documented. In the present study, we used LPC treatment to induce various degrees of surface area loss and assessed a number of cellular changes: i) cell shape ii) S/V ratio and iii) cell deformability. The ability of the treated RBCs to traverse human splenic sinuses was monitored using the isolated perfused spleen system 20 and the rate of sequestration was quantified. We established for the first time that RBCs with an average surface area loss of >18% (corresponding to > 27% decrease of S/V ratio) are rapidly sequestered in the spleen. Unexpectedly, kinetic analysis showed that a subset of RBCs with less than 18% surface area loss adapt to repeated passages through the spleen by decreasing their volume and hence their sphericity and thereby escape splenic entrapment.
Materials and Methods

This study was approved by the Ile-de-France II Investigational Review Board (Paris, France).

Human spleen retrieval

Spleens were retrieved and processed as we have previously described 20,25,26. Medical and surgical care was not modified, and patient written consent was obtained. All patients (5 females, 4 males), 47 to 72 years of age, underwent left splenopancreatectomy for pancreatic disease (neuroendocrine tumor, proven or suspected adenocarcinoma, cyst, unspecified tumor, or chronic pancreatitis). Spleens were macroscopically and microscopically normal in all cases. Following examination of the macroscopic aspect of the spleen by the pathologist during a 30 minute period of warm ischemia linked to the surgical procedure, the main splenic artery was cannulated. The spleens were flushed with cold RPMI 1640-albumin solution for transport to the laboratory.

RBC labeling with PKH67 and/or PKH26

Blood from a blood center (Etablissement Français du Sang, Rungis) was washed three times in RPMI 1640 to remove white blood cells. RBCs were labeled with lipophilic fluorescent probes PKH67 or PKH26 (hematocrit: 5%) as described by the manufacturer (Sigma-Aldrich) with some modifications in order to obtain 4 different populations of labeled RBC. Two populations of RBCs were labeled with one PKH (RBC/PKH67: PKH67 dilution 1/1000; RBC/PKH26: PKH26 dilution 1/250). Two other populations of RBCs were labeled with both PKH’s at different concentrations (RBC/KH26-67: PKH67 dilution 1/2000 plus PKH26 dilution 1/500; RBC/PKH67-26: PKH67 dilution 1/1000 plus PKH26 dilution 1/3000). These four populations of labeled RBCs can be distinguished by FACS and as such allowed
us to perfuse four distinct preparations in a single spleen and trace each population individually.

**Treatment of red blood cells with lysophosphatidylcholine**

PKH labeled RBCs were resuspended in LPC (0 - 15 µmol/L) in phosphate buffer saline or PBS at 1% hematocrit level to induce a controlled loss of membrane area. The LPC samples were incubated for 5 minutes at room temperature. Following incubation, samples were washed three times with PBS and resuspended in Krebs-albumin for further analysis.

**Measurement of RBC deformability**

RBC deformability was measured by ektacytometry using a laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Hoorn, The Netherlands) as previously described. The elongation index (EI, unit of RBC deformability) was defined as the ratio of the difference between the 2 axes of the ellipsoidal diffraction pattern and the sum of these 2 axes.

**Ex vivo spleen perfusion**

Isolated-perfused spleen experiments were performed as described previously, in a Plexiglas chamber maintained at 37°C by a regulated warm air flow. The perfusion of mixture of untreated (RBC/PKH) or LPC-treated (RBC/PKH/LPC) PKH-labeled RBCs (15 - 25% final hematocrit in Krebs-albumin medium) was done over a 1 to 2 hours period. Percentage of circulating LPC-treated was quantified by flow cytometric analysis (Fascalibur; BD Biosciences, San Jose, CA). Data were analyzed using CELLQuest software (BD Biosciences).

**Analysis of red blood cell morphology and dimensions**
Untreated or LPC-treated RBCs were fixed with PBS-paraformaldehyde (1%) for analysis. Image acquisition and data analysis were done as described previously. Images were acquired on the Imagestream® imaging cytometer (Amnis Corp., Seattle, WA). At least 10,000 images were collected for each sample. Post-acquisition data analysis was performed using IDEAS® image analysis software package (Amnis Corp.). Morphological (compactness, circularity, aspect ratio and shape ratio) and dimension (surface area, diameter and perimeter) parameters of RBCs were calculated using images of RBC by IDEAS software.

Osmotic fragility test

Osmotic fragility of RBC was determined according to the method originally described by Parpart et al. Untreated RBCs or LPC-treated RBCs were incubated for 30 minutes in hypotonic solutions with NaCl content ranging from 0.1 % to 0.9 % (hematocrit: 1%). After centrifugation, absorbance of the supernatants was measured at 540 nm using spectrophotometer and the percent hemolysis was calculated for each supernatant and plotted against NaCl concentrations.

Scanning electron microscopy

RBC specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1M) overnight at +4°C. The red cells were gently dropped onto a glass covership that had been treated with poly-lysine. After three cacodylate buffer rinses (10 min each), the specimens were fixed with 1% osmium tetraoxide for 1 hour. After three rapid washes in water, samples were processed through an ethanol dehydration series of 25% to 100% ethanol followed by critical point drying with CO₂. Dried specimens were sputtered with 22-nm gold palladium, examined, and photographed with a JEOL JSM 6700F field emission scanning electron microscope.
operating at 5 kV or 7 kV. Images were acquired with the upper SE detector and the lower secondary detector.

Results

RBC treatment with LPC results in a dose-dependent loss of surface area and deformability

RBCs were exposed to increasing LPC concentrations in order to generate RBC populations with progressively decreasing membrane surface area (Fig 1A). Surface area and diameter of the LPC-treated RBCs were significantly reduced (see also supplementary Fig 1A), and their volume was only slightly elevated (Table 1), resulting in a substantial increase in sphericity, with most cells becoming nearly perfect spheres at high LPC concentrations (Fig 1B and supplementary Fig 1B-D). The RBC populations were morphologically homogenous, even at low LPC concentrations, as indicated by the uniform shifts of RBC distribution patterns (Fig 1C). Due to the decrease in RBC surface area and a corresponding slight volume increase, there was a decrease of S/V ratio value (Table 1). RBC with a decreased S/V ratio exhibit increased osmotic fragility in hypertonic solution 7, a feature displayed by the LPC-treated RBC (Fig 2A-B).

Ektacytometric analysis showed that LPC-treated RBCs exhibited lower cellular deformability than control RBCs (Fig 2C), a feature which was not related to increased internal RBC viscosity 7, since there was little or no change in the mean corpuscular hemoglobin concentration (MCHC) and hence cytoplasmic viscosity upon exposure to LPC (Table 1). The deformability profiles were characteristic of RBC exhibiting a loss of surface area 7, implying that reduced RBC S/V ratio accounts for decreasing values of elongation index (EI, deformability parameter) of LPC treated RBCs. The maximum value of elongation index (EI
max) and percentage of surface area loss were inversely correlated ($r = -0.97; p < 0.0001$) (Fig 2D). It should be noted that LPC treated RBCs do not perfectly mimic HS RBCs. Besides surface area loss, there is the additional contribution of increased internal viscosity to reduced deformability of HS red cells due to the presence of a subpopulation of dehydrated cells with high MCHC 6.

Retention of LPC-treated RBCs by the isolated-perfused human spleen is determined by the extent of surface area loss

In order to understand how the human spleen handles RBCs displaying decreased surface area at constant volume (MCV) (i.e. with reduced S/V ratio), isolated-human spleens were perfused with a preparation containing RBCs exposed to different LPC concentrations and control RBCs, each differentially labeled with fluorescent PKH26 and/or PKH67 probes. Circulating cells were sampled from the perfusion medium over a two-hour period of perfusion and the various labeled RBC populations were monitored by flow cytometry. The LPC-treated RBCs were rapidly cleared by the human spleen, with a mean clearance half-time of 3.5 [range: 2.0 – 5.0] minutes (Fig 3A and supplementary Fig 2). The maximum level of clearance occurred between 10 to 20 minutes. The level of splenic retention increased (range: 18 to 96%) with increasing concentrations of LPC (0 to 10 μM) (Fig 3A) and with increasing extents of surface area loss (range: 1 to 28%) (Fig 3B). There was a positive correlation ($r = 0.95; p < 0.0001$) between the percentage of RBCs retained by the spleen and the extent of RBC surface area loss. A surface area loss of 3% (approximately 13% decrease of the S/V ratio) resulted in the retention of 27% of RBCs within the spleen (Table 1). Clearance of the RBC population becomes massive (> 90%) following surface area loss of > 18% (> 27% decrease of the S/V ratio) (Fig 3B). This retention cannot be explained by saturation of the splenic filtration function, given the small percentage of modified RBCs.
perfused (~4 to 5% of the total RBCs).

**Adaptation of a subset of surface-deficient RBCs during splenic transit**

Even at the highest extent of membrane surface area loss, a subset of LPC-treated RBCs exited from the spleen, as reflected by the existence of a plateau in cell clearance studies (Fig 3A). To obtain insights into the cellular features of this subpopulation, we compared the dimension and morphology of the RBCs at the onset of perfusion (time 0, T0) and those recovered from the perfusate after 40 minutes of perfusion (T40) (i.e., when the plateau was attained). Data from a representative study is illustrated in Fig 4, and supplementary Fig 3A-D. The dimensions and morphological parameters of untreated RBCs were unchanged between T0 and T40 (supplementary Fig 3C-D). For all concentrations of LPC, surface area, diameter and perimeter of the LPC-treated RBCs were similar at T0 and T40 and significantly lower than that of untreated RBCs (Fig 4A-C). In marked contrast, the circularity and aspect ratio values of the LPC-treated RBCs collected at T40 were different from values at T0, and closer to those of control RBCs (Fig 4D-E). Taken together, these findings suggest a likely reduction in the volume of subpopulation of LPC-treated RBCs circulating at T40 so as to compensate for the surface area loss. The acquisition of a more favourable S/V ratio due to decreased volume enabled the sustained circulation of this population of cells with reduced surface area.

**Discussion**

This study clearly demonstrated that cell surface area loss with reduced S/V ratio is a significant predictor of splenic sequestration of human RBC. Loss of 18% of cell surface area (corresponding to > 27% decrease of the S/V ratio) leads to rapid RBC entrapment in normal human spleen. The vast majority of these surface deprived RBCs were trapped during their
first passage through the isolated spleen. Thus, it appears that their spheroidal shape renders them incapable of undergoing the cellular deformation necessary to squeeze across the IES of the venous sinus, which have apertures of (0.2 – 0.5) x (2 – 3) µm. This determination of human spleen retention threshold of surface deficient RBCs has significant implications for diagnosis (spleen functionality) or prognosis studies in the field of RBC hemolytic disorders, and for drug screening (identification of compounds susceptible to induce severe anemia).

We found that even at the highest extent of membrane surface area loss, a subset of LPC-treated RBCs exited from the spleen. Several hypotheses might explain these results. Firstly, the heterogeneity of the initial population of LPC-treated RBCs which might include more spherical RBCs (preferentially entrapped in the spleen) and LPC-treated RBCs with dimensions closer to normal values. The fact that LPC-treated RBC dimensions (for all concentrations of LPC) were similar between T0 and T40, and both significantly reduced from that of untreated RBCs (Fig 4A-C) exclude the possibility that heterogeneity of the initial LPC-treated RBC population is responsible for continued circulation of this subpopulation of cells. Secondly, LPC-treated RBCs might acquire membrane lipid which increases their surface area and restores the cell S/V ratio; permitting them to escape spleen retention. In our study, all experiments were done without serum or plasma. Moreover, the fact that LPC-treated RBCs dimensions were similar between T0 and T40, and both different from that of control RBCs (Fig 4A-C), suggests that the reacquisition of membrane surface area by LPC-treated RBC membrane cannot explain the presence of un-cleared LPC-treated RBC subpopulation. Thirdly, the survival of LPC-treated RBC subpopulation might be due to their S/V ratio returning towards normal values through a decrease in cell volume as a result of regulation of ions and water permeability of the cell.
It’s well established that, when submitted to increased mechanical stress, normal RBC exhibit a reversible increase in permeability to monovalent\textsuperscript{2,5,32,33} and divalent\textsuperscript{5,34} cations, and to anions\textsuperscript{5}. Such phenomenon has been shown exacerbated when RBC membrane rigidity is increased\textsuperscript{3}, or when surface area was reduced such as in HS RBCs\textsuperscript{14,35}. This provokes a transient dehydration and increased loss of intracellular potassium, only partially offset by an increase in intracellular sodium\textsuperscript{14,34,36,37}. If such a process takes place in the circulating LPC-treated RBCs, it would lead to a decrease in cell volume creating a more favorable the S/V ratio, thus enabling the surface deficient RBCs to remain in the circulation. This hypothesis is supported by the fact that LPC-treated RBC dimensions were similar at T0 and T40, and both significantly lower than that of untreated RBCs (Fig 4A-C). However, the sphericity value of untrapped LPC-treated RBCs was different from values at T0 (Fig 4D-E), and closer to those of control RBCs (supplementary Fig 3C-D). This phenomenon of adaptive volume regulation mechanism might take place between 10 and 20 minutes of splenic perfusion, indicating that acquiring of a favourable S/V ratio by the surface-deficient RBC subpopulation takes a relatively short time, possibly through deformation-induced membrane permeability modifications. Similar findings have been noted in a mouse-based study\textsuperscript{8}, which has a non-sinusoidal spleen\textsuperscript{38}.

In summary, the present study enabled the definition of the relationship between S/V ratio of human RBC and splenic entrapment and documented the critical extent of surface area loss that leads to rapid splenic removal. Interestingly, it provides a novel paradigm, namely that surface-deprived RBCs might adapt and escape the splenic retention through regulation of cell volume. The human RBC plasma membrane and the membrane skeleton provide the RBCs with unique structural and functional properties, permeability characteristics that operate to regulate a favorable S/V ratio necessary for undergoing extensive deformations.
essential for optimal function.

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**Authorship Contributions**

Innocent Safeukui and Pierre Buffet: designed research, performed research, contributed vital analytical tools, analyzed data and wrote the paper; Guillaume Deplaine and Sylvie Perrot: designed research, performed research, contributed vital analytical tools and
analyzed data; Valentine Brousse: designed research, performed research and analyzed data; Alioune Ndour and Marie Nguyen: performed research and analyzed data; Odile Mercereau-Puijalon, Peter H David, Geneviève Milon and Narla Mohandas: designed research, analyzed data and wrote the paper.

Conflicts of Interest Disclosures

The authors declare no competing financial interests.

References


Table 1

Characteristics of untreated RBCs and RBCs treated with increasing concentrations of LPC (1-15 µmol/L).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hb</th>
<th>MCHC</th>
<th>MCH</th>
<th>MCV</th>
<th>Projected area</th>
<th>A/V</th>
<th>Sphericity</th>
<th>Elmax</th>
<th>Spleen retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated RBC</td>
<td>11.3 (1.8)</td>
<td>34 (2)</td>
<td>31 (3)</td>
<td>92 (2)</td>
<td>71 (1)</td>
<td>0.77 (0.01)</td>
<td>0.81 (0.01)</td>
<td>0.638 (0.01)</td>
<td>9 (4)</td>
</tr>
<tr>
<td>LPC-treated RBC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[1 - 2.5] µM</td>
<td>10.5 (0.8)</td>
<td>35 (2)</td>
<td>32 (3)</td>
<td>92 (1)</td>
<td>70 (0.3)</td>
<td>0.76 (0.01)</td>
<td>0.81 (0.02)</td>
<td>0.621 (0.01)</td>
<td>19 (7)</td>
</tr>
<tr>
<td>[3.5 - 5.0] µM</td>
<td>10.1 (0.3)</td>
<td>35 (1)</td>
<td>33 (1)</td>
<td>94 (0)</td>
<td>63 (3)</td>
<td>0.67 (0.03)</td>
<td>0.91 (0.03)</td>
<td>0.520 (0.04)</td>
<td>64 (16)</td>
</tr>
<tr>
<td>[6.0 - 7.5] µM</td>
<td>9.7 (0.2)</td>
<td>33 (2)</td>
<td>32 (1)</td>
<td>97 (1)</td>
<td>59 (3)</td>
<td>0.61 (0.04)</td>
<td>0.92 (0.02)</td>
<td>0.470 (0.05)</td>
<td>79 (13)</td>
</tr>
<tr>
<td>[8.5 - 15.0] µM</td>
<td>10.1 (0.1)</td>
<td>32 (1)</td>
<td>30 (2)</td>
<td>95 (0)</td>
<td>56 (3)</td>
<td>0.59 (0.03)</td>
<td>0.93 (0.01)</td>
<td>0.423 (0.04)</td>
<td>92 (4)</td>
</tr>
</tbody>
</table>

Hb, level of hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; MCV, mean cell volume; S/V, projected surface area – to – volume ratio; Sphericity; aspect ratio; Elmax, elongation index at a shear stress of 30 Pascal. Hb, MCHC, MCH and MCV were measured with ADVIA. All data presented are means with standard deviation (in bracket).
Figure legends

Figure 1

**Effects of LPC treatment on red blood cell membrane surface area and cell morphology.** Analysis of RBC dimension, morphology and morphological homogeneity using ImageStream technology. There is a dose dependent shift toward lower values of the distribution of RBC membrane surface area (A), while aspect ratio (sphericity) (B), increased with increasing concentrations of LPC. (C) The RBC populations are morphologially homogenous, even at low LPC concentrations, as characterized by the red blood cell distribution shifting as a whole. RBCs with a circular shape in the control group did not correspond to spherical cells but were indeed discocytes, since the images were captured face on.

Figure 2

**Monitoring of LPC-treated RBC features.** (A) Osmotic fragility test of LPC treated red cells (one out of three representative experiments). (B) Linear regression fit of the correlation between NaCl concentration which induce 50% of hemolysis and the extent of surface area loss (a representative experiment). There was a good correlation (r = 0.998; p < 0.0001) between the extent of surface area loss and the osmolarity value at which 50% of red cells hemolyze. (C) Deformability profiles of LPC-treated red blood cells used for splenic perfusion studies (a representative experiment). The RBC deformability changes that occurred upon LPC-treatment were examined by LORCA, a system which measures extent of cell deformation as a function of applied shear stress. LPC-treated RBC were less deformable than control/untreated RBC, with a dose dependent reduction in the maximum value of elongation index (EI, deformability parameter) (EI max). (D) Linear regression fit of the correlation between EI max and the extent of surface area loss.
Figure 3

Clearance of LPC-treated red blood cells by isolated-perfused human spleens. (A) Data from a representative experiment showing kinetics of circulating LPC-treated red blood cells recovered from the perfusate over 2 hours of splenic perfusion. RBC were differentially labeled with lipophilic fluorescent probes PHK26 and/or PKH67 enabling the distinction of four separate LPC-treated RBC populations by flow cytometry. (B) Linear regression fit of the correlation between EI max and the level of LPC-treated red blood cell retention within the spleen (n = 9 independent experiments).

Figure 4

Adaptation and survival of red blood cells with reduced surface area and S/V ratio during the isolated-human spleen perfusion. Dimension (A-C) and morphology (D-E) analysis of LPC-treated RBC before (T0) and 40 minutes (T40) after the start of the perfusion. Projected surface area (A), diameters (B) and perimeters (C) of LPC treated RBCs were similar between T0 and T40, and all significantly lower than that of untreated RBCs. Shifts toward normal values of the circularity (D) and the aspect ratio or sphericity (E) of LPC-treated RBCs at T40. See also supplementary Fig 3 for the deformability profile of LPC-treated RBC populations before the perfusion, and the kinetics of their clearance during 60 minutes of spleen perfusion. Projected surface area in µm²; diameter and perimeter in µm. One out 3 representative experiments.
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