Biophysical and Biochemical Markers of Red Blood Cell Fragility

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Keywords

Erythrocytes · Membrane defects · RBC aging · RBC lesion · RBC storage · Hemolysis

Summary

Background: Red blood cells (RBCs) undergo a natural aging process occurring in the blood circulation throughout the RBC lifespan or during routine cold storage in the blood bank. The aging of RBCs is associated with the elevation of mechanical fragility (MF) or osmotic fragility (OF) of RBCs, which can lead to cell lysis. The present study was undertaken to identify RBC properties that characterize their susceptibility to destruction under osmotic/mechanical stress. **Methods:** RBCs were isolated from freshly donated blood or units of packed RBCs (PRBCs) and suspended in albumin-supplemented phosphate-buffered saline (PBS). In addition, PRBCs were separated by filtration through a microsphere column into two fractions: enriched with rigid (R-fraction) and deformable (D-fraction) cells. The RBCs were subjected to determination of deformability, MF and OF, moreover, the level of cell surface phosphatidylserine (PS) and the stomatin level in isolated RBC membranes were measured. **Results:** In the RBC population, the cells that were susceptible to mechanical and osmotic stress were characterized by low deformability and increased level of surface PS. The OF/MF was higher in the R-fraction than in the D-fraction. Stomatin was depleted in destroyed cells and in the R-fraction. **Conclusion:** RBC deformability, the levels of surface PS, and membrane stomatin can be used as markers of RBC fragility.

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Introduction

Red blood cells (RBCs) have a lifespan of about 120 days, and undergo a natural aging process occurring in the blood circulation throughout their lifespan [1]. The reduction in their function occurs predominantly in the last days, and then they are removed from the bloodstream by the immune system (mainly dendritic cells). A similar aging process occurs during routine cold storage of RBCs in the blood bank as well, and some reports claim that it is even expedited under these conditions [2–6]. The aging-produced ‘senescent’ cells are characterized by the loss of cell surface area and cell morphology alterations [7], resulting from the shedding of hemoglobin (Hb)-containing vesicles [3, 6, 8, 9]. The cold storage of packed RBCs (PRBCs) is associated with a continuous increase in the percentage of cells with impaired functionality [10–12]. This included increased cell rigidity (reduced deformability) and aggregability [12–14], reduced level of cell membrane stomatin [15], and translocation of phosphatidylserine (PS) to the cell surface [9, 16], all leading to an increased adherence to endothelial cells [14].

It follows that, together with the above parameters, senescent RBCs will be more prone to osmotic (OF) and mechanical fragility (MF). Indeed, a few studies reported that RBC aging and storage duration are associated with increased OF and MF of the cells [13, 17–21].

In the present study we hypothesized that RBCs which are susceptible to osmotic/mechanical shock are characterized by changes in their cell properties that are considered markers of blood aging and altered during RBC storage, namely the cell deformability, the level of cell surface PS, and the membrane stomatin level.

The results clearly show that the RBCs with high MF and OF are characterized by high rigidity (low deformability) and surface PS level as well as by a low level of membrane stomatin.

Material and Methods

Materials

PMMA (polymethylmethacrylate) microspheres (27–32 µm diameter) were purchased from Cospheric (Santa Barbara, CA, USA) and antiaerosol pipette tip (1 ml) from Neptune Scientific (San Diego, CA, USA). Stomatin, GAPDH antibody, and the PS ligand annexin V were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stainless-steel beads were from Nation-Skander California Co (Anaheim, CA, USA). NP-40 and protease inhibitor cocktail were purchased from (Sigma, St. Louis, MO, USA); Bradford assay from Bio-Rad (Rishon Le Zion, Israel).

Experimental Design

The present study employed RBCs from two sources: Freshly collected human blood samples, and PRBCs following cold storage in the blood bank (upon donor's consent according to the Hadassah Hospital Ethics Committee approval). RBC samples were taken on donation day (day 1) and following 35 days of cold storage (day 35). The cells were isolated and subjected to determination of cell deformability and fragility, cell surface phosphatidylserine (PS), and membrane stomatin level.

To further explore the relation between RBC deformability and MF/OF, RBC samples were subjected to separation into rigid and deformable fractions (R-fraction and D-fraction), on a column of PMMA microspheres [22]. Then each fraction was subjected to determination of RBC deformability and fragility, and membrane stomatin level.

RBC Storage Conditions

Similar to our previous studies [14, 16], in the present study we have used non-leukoreduced PRBCs. Blood was drawn from 10 healthy donors in the Hadassah Hospital Blood Bank, following informed consent according to the Helsinki Committee Regulations Permit (98290, Hadassah Hospital, Jerusalem, Israel) and collected not-leukoreduced into standard sterile bags (Fresenius Kabi AG, Homburg, Germany), containing citrate phosphate dextrose (CPD). Immediately following collection, RBCs were isolated by centrifugation (Roto Silenta 630RS, Tuttlingen, Germany) for 6 min (2,367 rpm, 24 °C) followed by removal of the plasma. Units of concentrated non-leukodepleted RBCs were stored in citrate phosphate dextrose adenine (CPDA-1) under the standard conditions (2–6 °C for 5 weeks) at the Hadassah Hospital Blood Bank.

Isolation of RBCs

Preparation of RBC Samples from Freshly Collected Blood

RBCs from blood samples collected from healthy donors were isolated from plasma by centrifugation (500 × g for 10 min), washed (three times) from plasma by centrifugation in phosphate-buffered saline (PBS; pH 7.4), and suspended in PBS at 10% hematocrit.

Preparation of RBC Samples from Stored PRBCs

From 10 PRBC units, 5 ml were drawn, washed twice by centrifugation (500 × g for 10 min) in PBS, and suspended in PBS at 10% hematocrit.

RBC Fractionation into Rigid and Deformable Cells

In a previous study, we have shown that the percentage of rigid RBCs in PRBC units increases with storage duration [13, 14]. Accordingly, in this experiment, to obtain significant fractions of rigid cells, PRBCs stored for 35 days were separated into fractions of rigid (R-fraction) and deformable cells (D-fraction), by fractionation on column of PMMA microspheres (PMPMS), using a modification of the method of Deplaine et al. [22]: A total of 1 g of dry PMPMS were suspended in 6 ml PBS supplemented with 1% albumin (PBS-A). 600 µl of the

bead suspension was poured into an inverted 1 ml antiaerosol pipette tip and allowed to settle, leading to the formation of a bead layer above the filter. A total of 600 µl of RBC suspension (2% hematocrit) was placed on the PMPMS layer.

The PMPMS layer was then washed with 8 ml of PBS-A. The downstream sample, containing the D-fraction, was collected, and the RBC layer retained in the PMPMS, containing the R-fraction, was separated from the PMPMS by centrifugation (300 × g for 10 min) and washed twice in PBS.

Determination of RBC Hemolysis (% Hemolysis)

The Hb concentration in the supernatant was determined by its optical density at 540 nm. The hemolysis level at each mechanical or osmotic stress was expressed as % of the total lysis, according to the formula:

$$\% \text{ hemolysis} = 100 \times (\text{OD}_S - \text{OD}_0) / (\text{OD}_T - \text{OD}_0)$$

where OD_0 = OD of supernatant obtained from the control, untreated RBCs, OD_S = OD of supernatant obtained from RBC subjected to MS/OS, OD_T = OD of supernatant from RBC subjected to total hemolysis.

For total cell lysis, equal amounts of RBCs were incubated for 10 min in distilled water [13], and centrifuged (at 14,000 × g for 10 min) to remove the cell debris.

Determination of MF/OS

Mechanical Stress

Mechanical stress was induced using the conventional method previously described [13]. In brief: 3 ml of RBC suspension were rocked (at 40 cycles/min) for 1 h at room temperature in glass test tubes (13×100 mm), containing 5 steel beads (3.175 mm).

Osmotic Stress

Osmotic stress was applied using the common method, as previously described [13]: In brief: 10 µl of RBCs were suspended in 1 ml NaCl solution at increasing concentrations (0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 g/l). The cells were then centrifuged at 500 × g for 10 min at room temperature; the supernatant was collected and subjected to Hb determination as above.

The mechanical fragility index (MFI), was expressed as a percent of the total hemolysis. The osmotic fragility index (OSI), was defined by the concentration of NaCl that exerted 50% hemolysis.

Isolation of the Cells Surviving the MS/OS

Subsequent to application of MS or OS, the remaining intact cells were isolated by centrifugation, washed twice in PBS (500 × g for 10 min) and suspended at 10% hematocrit in isotonic PBS for determination of deformability, surface PS, and membrane stomatin. The level of hemolysis following treatments does not exceed 5.0%.

Determination of RBC Deformability

The present research employed the computerized Cell Flow-Properties Analyzer (CFA), designed and constructed in our laboratory [13, 14]. RBC deformability was determined by monitoring the elongation of RBCs, while adherent to a slide, under flow-induced shear stress [13, 14]. In brief, 50 µl of RBC suspension (1% hematocrit, in PBS supplemented by 0.5% of albumin) are inserted into the flow chamber (adjusted to 200 µm gap) which contains an uncoated polystyrene slide. The adherent RBCs are then subjected to controllable flow-induced shear stress, and their deformability is determined by the change in cell shape, as expressed by the axial ratio (elongation ratio): $\text{ER} = a/b$, with a/b = the major/the minor cell axes. $\text{ER} = 1$ reflects a round RBC, undeformed at the applied shear stress. The CFA image analysis program measures the ER for each individual cell, and provides the deformability distribution (ER-Dis) in a large RBC population (at least $3,500 \pm 300$ cells) as a function of shear stress [13, 14]. The accuracy of the axes measurement is about 10%. Therefore, RBCs with $\text{ER} \leq 1.1$ are defined as 'undeformable' cells (UDFC), namely the cells that do not deform under high shear stress (3.0 Pa in the present study). As shown

and discussed in our previous studies, when considering the potential of RBCs with low deformability to induce microvascular occlusion, the portion of undeformable cells is more clinically relevant than the shift in average values.

Determination of Stomatin Level in RBC Membranes

To examine the relation of the membrane stomatin level to the cell fragility, we determined the average RBC stomatin content and its change induced by MS and OS. To this end, RBC suspension was subjected to lysis (less than 5% of hemolysis, see above) by OS or MS, followed by mild centrifugation (5 min at $500 \times g$) to precipitate the intact cells. The membrane-containing supernatant was collected and subjected to membrane isolation by centrifugation (10 min at $14,000 \times g$), and determination of membrane stomatin level. Concomitantly, the same amount of control, untreated RBCs (no MS/OS) were subjected to complete hemolysis, membrane isolation, and stomatin determination.

For stomatin determination, membrane proteins were extracted by dissolving the membranes in 1% NP-40, containing protease inhibitor cocktail, while immersion in ice for 10 min (with frequent mixing), and the protein concentration was measured by Bradford assay. 20 μ g of the protein extract were subjected to determination of stomatin level using direct enzyme-linked immunosorbent assay (ELISA) with stomatin antibody. The lysates were incubated in parallel with GAPDH for normalization of protein content.

Determination of PS at RBC Surface

RBC surface PS was determined by the binding of the PS-specific ligand, fluorescein 5-isothiocyanate(FITC)-labeled human annexin V. The ligand (8 nmol/l) was incubated with RBCs (suspended in 200 μ l HEPES-buffered saline at 0.016% hematocrit) for 20 min at 37 °C in the dark. To remove unbound annexin V, the RBCs were washed and suspended in 500 μ l of the same buffer, and their fluorescence was measured in a fluorescence-activated cell sorter (FACS) to determine the number of PS-positive RBCs. Data acquisitions were performed on a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA, USA), and analysis was done with CellQuest software (Becton Dickinson). A total of 10,000 events were acquired for each sample. The percentage of annexin V-FITC-positive erythrocytes was determined from the fluorescence signal in excess of that obtained with a negative (unlabeled) control RBC sample.

Statistical Analysis

Data, analyzed and tested for statistical significance using the paired Student test, is presented as mean $\pm$ SD. Statistical difference, analyzed with the SPSS 21 software package (SPSS Inc. Chicago, IL, USA), was considered significant at $p < 0.05$.

Results

Osmotic and Mechanical Stress Destroyed Primarily RBCs with Low Deformability

To examine the susceptibility of rigid RBCs to stress, we analyzed the change in the distribution of RBC deformability (ER-Dis) exerted by MS or OS. Figure 1 shows that the ER-Dis for the RBC populations that survived the OS/MS-induced hemolysis shifted towards higher deformability (higher ER values), implying that the RBCs which were lysed by the MS/OS were the rigid cells. This is further supported by the results presented in table 1 showing that the percent of undeformable RBCs (% UDFC, having $ER \leq 1.1$) decreased markedly after application of MS/OS.

In addition, as demonstrated by figure 2, strong correlation ($r = 0.92$; $p < 0.0002$) between the % of UDFC and the MFI has been observed.

Fig. 1. The right shift of ER distribution of stored RBCs remaining after application of mechanical (2.8% hemolysis) or osmotic (4.7% hemolysis) stress.

Fig. 2. RBC MFI versus % of undeformable cells (UDFC) for freshly donated or stored RBCs.

Table 1. The population of RBCs that survived osmotic/mechanical stress are characterized by depletion of undeformable cells (UDFC, $ER \leq 1.1$)^a

Treatment	% UDFC		p value (pair test)
	before stress	after stress	
Osmotic stress	5.18 $\pm$ 4.44	2.35 $\pm$ 0.95	0.001
Mechanical stress	5.18 $\pm$ 4.44	3.12 $\pm$ 1.39	0.005

^aEach datum is the mean $\pm$ SD for 10 samples of PRBCs.

The susceptibility of rigid RBCs to MS and OS is further demonstrated by the fractionation of the RBCs into rigid and deformable cell fractions (R-fraction and D-fraction, see ‘Material and Methods’). Table 2 shows that the fragility indices (expressing susceptibility to stress) for the fractions of rigid cells (R-fraction) was markedly higher than for the deformable cells (D-fraction).

Table 2. Rigid RBCs exhibit high fragility and low stomatin level^a

Parameter	D-fraction	R-fraction	p value (pair test)
MFI	1.13 ± 0.39	3.15 ± 0.29	0.003
OFI	0.59 ± 0.19	0.68 ± 0.13	0.002
Relative stomatin concentration	1.0	0.805 ± 0.037	0.03

R-fraction = Rigid RBC-enriched fraction, obtained by separation on a micro-bead column; D-fraction = deformable RBC-enriched fraction, remaining after separation of R-fraction.

Table 3. RBCs that are destroyed under osmotic/mechanical stress exhibit low level of membrane stomatin.

Treatment	Relative stomatin concentration, %		p value
	RBCs before treatment	destroyed RBCs	
Osmotic stress	100	72.00 ± 15.36	0.0205
Mechanical stress	100	76.97 ± 18.89	0.0179

Rigid RBCs Are Characterized by Translocation of PS to the Cell Surface

PS is normally located in the inner leaflet of the RBC membrane, but under abnormal conditions, especially storage [16], that are associated with changes in cell mechanical properties [13] PS is translocated to the cell surface. The cells that exhibit surface PS were identified by the binding of the PS-specific ligand annexin V [16]. Figure 3, depicting the percent of undeformable RBCs (% UDFC) versus the percent of annexin V-labeled RBCs (expressing surface PS), shows a strong correlation ($r = 0.915$; $p = 0.0002$) between RBC rigidity and surface PS. Figure 4 shows that the % of PS-expressing (annexin V-labelled) RBCs decreased by the application of MS ($p = 0.0021$).

Osmotic or Mechanical Stress Destroyed Predominantly Cells with Low Levels of Membrane Stomatin

Table 3, depicts the stomatin level in the fraction of cells that were lysed by MS/OS relatively to the average in untreated RBCs (= 100%, see 'Material and Methods' for details). The table shows that the fragile RBCs are characterized by a low level (72–77%) of membrane stomatin.

In addition, the results presented in table 2 show that the stomatin level in the fraction of rigid RBCs (R-fraction) is lower than in the fraction of deformable cells (D-fraction).

Discussion

The aging of RBCs, occurring during their lifespan in the blood (in vivo) or during storage in the blood bank (in vitro), is characterized by the formation of microdefects in the RBC membrane

Fig. 3. The % of undeformable cells (UDFC) in the RBC population versus percent of cells labelled with the PS ligand annexin-V. **Fig. 4.** MS-induced decrease in the percentage of RBCs labelled with the PS ligand annexin-V.

[23–25]. The topological defects, studied by atomic force microscopy [23–25], appear normally as domains with grain-like structures ('grains') of up to 200 nm, which later merge to form large defects of 400–1,000 nm [24]. The formation of microdefects makes the cell susceptible to stress [24].

When RBCs are subjected to shear stress, the cell membrane deforms until the membrane reaches its 'yield point'. Beyond this threshold point, additional stress results in irreversible plastic deformation of the membrane, which accelerates with accumulation of microdefects in the membrane, leading to the cell destruction [26, 27]. Thus, this suggests that senescent (aged) RBCs will be destroyed under a relatively low level of mechanical stress.

A similar conclusion was presented by other groups. Sakota et al. [28] and Yokoyama et al. [29] studied RBC hemolysis during blood pumping (using rotary pump) and have speculated that the RBC hemolysis exerted by this procedure is due to selective destruction of aged, fragile cells under pumping-induced mechanical stress [28, 29].

In the present study, we demonstrated that the cells that were destroyed under low mechanical stress are characterized by low de-

formability, high level of surface PS, and reduced level of membrane stomatin.

Previous studies have linked reorganization of the RBC membrane [4, 7, 30], expressed particularly by changes in RBC deformability [31], PS translocation [16] and membrane stomatin level [3], to cell aging. In the present study, we show that these measures express the RBC susceptibility to mechanical stress, as expressed by the cell mechanical fragility.

In addition, the present study shows that RBCs with low deformability and stomatin level are markedly more susceptible to osmotic stress. This can be explained by the fact that the ratio of the cell surface area-to-volume, a key determinant of RBC rigidity [32] and osmotic fragility [33], decreases subsequent to the shedding of stomatin-containing membrane vesicles [9–11].

In conclusion, the present study shows that RBCs that are destroyed by mechanical/osmotic stress are characterized by low de-

formability and membrane stomatin level, and high level of surface PS, thereby suggesting that these cell properties, in addition to being markers of RBC aging, are measures of MF and OF.

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Disclosure Statement

The authors declare no conflict of interests.

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