Storage of Erythrocytes Induces Suicidal Erythrocyte Death

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Key Words
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Abstract

Background/Aims: Similar to apoptosis of nucleated cells, red blood cells (RBC) can undergo suicidal cell death - called eryptosis. It is characterized by cell shrinkage and phosphatidylserine translocation. Eryptosis is triggered by an increase of intracellular calcium concentration due to activation of nonselective cation channels. The cation channels and consequently eryptosis are inhibited by erythropoietin. Eryptotic RBC are engulfed by macrophages and thus rapidly cleared from circulating blood. In this study, we explored whether storage of RBC influences the rate of eryptosis. Methods: Flow cytometry was employed to quantify phosphatidylserine exposing erythrocytes from annexin V binding and cytosolic Ca\textsuperscript{2+} activity from Fluo-3 fluorescence. Clearance of stored murine RBC was tested by injection of carboxyfluorescein succinimidyl ester (CFSE)-labelled erythrocytes. Results: Storage for 42 days significantly increased the percentage of phosphatidylserine exposing and haemolytic erythrocytes, an effect blunted by removal of extracellular calcium. Phosphatidylserine exposure could be inhibited by addition of erythropoietin. Upon transfusion, the clearance of murine CFSE-labelled RBC from circulating blood was significantly higher following storage for 10 days when compared to 2 days of storage. Conclusion: Storage of RBC triggers eryptosis by Ca\textsuperscript{2+} and erythropoietin sensitive mechanisms.

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Introduction

Similar to apoptosis of nucleated cells, red blood cells (RBC) may enter eryptosis, a suicidal cell death characterized by cell shrinkage and cell membrane scrambling [1]. Eryptosis is triggered by opening of Ca\(^{2+}\)-permeable unselective cation channels and subsequent increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\text{\(i\)})\], which is followed by cell shrinkage due to activation of Ca\(^{2+}\)-sensitive K\(^+\) channels [2] and by breakdown of the phospholipid asymmetry of the cell membrane with phosphatidylserine translocation to the erythrocyte surface [1]. Stimulators of eryptosis further include ceramide formation [3], and caspase activation [4-8]. The Ca\(^{2+}\)-permeable unselective cation channels, and thus eryptosis are inhibited by erythropoietin [9].

Eryptosis is stimulated by a wide variety of xenobiotics [3, 10-42] and parallels several clinical conditions with anemia [1, 43].

The present study explored the possibility that storage of RBC for transfusion purposes modifies the susceptibility to eryptosis. In Germany, RBCs may be stored up to 49 days [44]. Thus, the impact of storage period on susceptibility to eryptosis was tested \textit{in vitro} in human RBC concentrates at different times of storage. To this end erythrocyte [Ca\(^{2+}\)]\text{\(i\)}, and phosphatidylserine surface abundance were quantified by flow cytometry. Moreover, the \textit{in vivo} life span of stored murine erythrocytes traced by CFSE-labelling was determined upon transfusion in animals.

Materials and Methods

Animals

C57BL/6 animals were used in this study. All mice were maintained under specific pathogen-free conditions and animal experiments were performed under approval of the LANUV NRW in accordance with German laws for animal protection.

RBC concentrates, solutions and chemicals

Leukodepleted human RBC concentrates provided by the Blood Center of the Heinrich Heine University Medical Center Düsseldorf were prepared by standard techniques from 450 ml whole blood donations collected into bags preloaded with 70 ml citrate-phosphate-dextrose (CPD). Leukodepletion (< 1 x 10\(^6\) leukocytes per unit) was achieved by inline filtration using LCR-5 leukocyte filters (Maco Pharma International, Langen, Germany); RBC concentrates were stored in additive solution composed of phosphate-adenine-glucose-guanosin-saline-mannitol (PAGGS-M) at 4 + 2 °C under quality controlled blood banking conditions for up to 49 days. Following storage for the indicated time periods, RBC were incubated \textit{in vitro} at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 MgSO\(_4\), 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl\(_2\); pH 7.4 at 37°C. Where indicated, RBC were exposed to Ca\(^{2+}\)-free Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1.2 MgSO\(_4\), 32.2 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 EDTA; pH 7.4 or to solutions containing 0.1 mU/ml erythropoietin (Sigma Aldrich, Schnelldorf, Germany). For murine erythrocyte transfer, RBC from C57BL/6 animals were labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen).

Analysis of annexin-V-binding and forward scatter

After incubation under the respective experimental condition, RBC were washed in Ringer solution containing 5 mM CaCl\(_2\), and then stained with Annexin-V-FITC (1:50 dilution; ImmunoTools, Friesoythe, Germany) at RT for 15 min under protection from light. Using flow cytometry, the forward scatter of the cells was determined, and annexin V fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Canto II (BD, Heidelberg, Germany).

Measurement of intracellular Ca\(^{2+}\)

RBC were loaded with Fluo3 by incubation with Ringer solution containing 1µM Fluo3/AM (Biotium, Hayward, USA) for 1 h at 37°C. After incubation, cells were washed with Ringer solution twice and exposed
to the respective experimental conditions. Changes in Fluo3 fluorescence compared to time point 0 was measured at indicated intervals at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Canto II (BD, Heidelberg, Germany).

### Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was performed using ANOVA with Tukey’s test as post-test or t test as appropriate; n denotes the number of different specimens studied. Since different RBC specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same RBC specimens have been used for control and experimental conditions.

### Results

The present study tested the hypothesis that storage of RBC enhances their susceptibility to eryptosis, the suicidal erythrocyte death. Eryptosis is characterized by cell shrinkage and phosphatidylserine translocation to the RBC surface.

In order to identify phosphatidylserine exposing erythrocytes, annexin V binding was determined using flow cytometry. As illustrated in Fig. 1A-D, incubation of erythrocytes in Ringer was followed by a gradual increase in the percentage of annexin V binding erythrocytes. The annexin V binding following incubation in Ringer gradually increased with storage time prior to the experiment, an effect reaching statistical significance at 42 days of storage.

For comparison, hemolysis was quantified by determination of free hemoglobin concentration in the supernatant. As illustrated in Fig. 1E, significant hemolysis of RBC stored for 49 days but not of RBC stored for 8 days was observed following extended (≥72 h) exposure times to Ringer solution.

**Fig. 1.** Phosphatidylserine exposure and hemolysis in human erythrocyte concentrates as a function of storage period. A-D. Arithmetic means ± SEM (n = 6-15) of the percentage annexin-binding erythrocytes (anx+) as a function of storage period in days (d) after subsequent 24 h (A), 48 h (B), 72 h (C) and 96 h (D) incubation in Ringer. E. Arithmetic means ± SEM (n = 13-14) of percentage hemolysis following erythrocyte storage for 8 days (closed circles) and 48 days (open squares) and subsequent incubation in Ringer for 24, 48, 72, and 96 h. **(p<0.01), *** (p<0.001) indicates significant difference from respective value of 8 days storage (ANOVA).
As cell membrane scrambling could have resulted from an increase of cytosolic Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]), additional studies were performed with Ringer solutions lacking extracellular Ca\textsuperscript{2+}. As illustrated in Fig. 2A-D, the effect of storage time on the percentage of annexin V binding RBC was virtually abolished in the absence of extracellular Ca\textsuperscript{2+}.

The Ca\textsuperscript{2+} permeable unselective cation channels mediating the influx of Ca\textsuperscript{2+} can be inhibited by erythropoietin. Thus, in an additional series of experiments erythropoietin (0.1 U/ml) was added. As illustrated in Fig. 3, the effect of storage time on the percentage of annexin V binding erythrocytes was virtually abolished in the presence of erythropoietin.

Additional experiments were performed in mice in order to quantify the impact of storage time on the \textit{in vivo} life span of transfused RBC. Similar to what had been observed in human erythrocytes, incubation of murine erythrocytes in Ringer was followed by a gradual increase of the percentage of annexin V binding erythrocytes, which was significantly enhanced following storage for 6 or 10 days (Fig. 4). Labelling of murine RBC with CFSE allowed tracing the number of transfused erythrocytes remaining in circulating blood. Following injection, CFSE-labelled RBC gradually disappeared from the circulation (Fig. 4). The clearance of CFSE-labelled erythrocytes from circulating blood was significantly accelerated by prior storage (Fig. 4).

**Discussion**

The present study reveals that the susceptibility of RBC to eryptosis, the suicidal death of erythrocytes, is significantly enhanced by prior storage under quality controlled standard blood...
banking conditions for RBC concentrates. Eryptosis of stored erythrocytes is evidenced from phosphatidylserine translocation to the erythrocyte surface. The susceptibility to eryptosis is not immediately apparent but becomes unmasked by subsequent incubation of RBC in Ringer solution. The present study further discloses that the effect of storage on eryptosis is virtually abrogated following removal of extracellular Ca\(^{2+}\) and in the presence of erythropoietin, which has previously been shown to inhibit the Ca\(^{2+}\) permeable unselective cation channels in the RBC membrane [9].
The effect of storage on a change of cell membrane asymmetry is paralleled by augmented increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)). As removal of extracellular Ca\(^{2+}\) disrupts the effect of storage on annexin V binding, the impact of storage on erythrocyte death is in large part secondary to an increase in [Ca\(^{2+}\)]\(_i\).

The experiments in mice illustrate the in vivo significance of a storage-associated lesion with induction of suicide susceptibility. Eryptotic RBC are phagocytosed and thus rapidly cleared from the circulation [1]. As the life span of stored RBC is shorter than that of fresh erythrocytes, the transfusion of stored erythrocytes is less efficient in the treatment of anaemia than the transfusion of fresh erythrocytes.

More importantly, phosphatidylserine exposing erythrocytes interact with endothelial CXCL16/SR-PSO thus adhering to the vascular wall [45]. Moreover, phosphatidylserine exposing erythrocytes can activate coagulation and thus induce thrombosis [46-48]. Accordingly, accelerated eryptosis may compromise the microcirculation [45, 46, 49-52]. To the extent that transfused erythrocytes are particularly sensitive to eryptosis, they may thus interfere with microcirculation of the recipient. It must be kept in mind that the susceptibility may vary depending on the donor. In view of the present observations, extended storage periods should be avoided. Alternatively, inhibitors of eryptosis could be added to extend the life span of stored RBC. For this purpose, the addition of erythropoietin could be considered as the hormone inhibits the cation channels and thus eryptosis [9]. According to the present observations, blockade of the Ca\(^{2+}\) permeable unselective cation channels by erythropoietin is indeed able to prevent the effect of storage on annexin V binding. Following in vivo administration, erythropoietin plays a dual role in the regulation of eryptosis. At the one hand, it directly inhibits eryptosis by downregulating cation channel activity, on the other hand it fosters the generation of RBC, which are particularly susceptible to eryptosis and prone to die as soon as the erythropoietin levels in blood decline [1].

Beyond susceptibility to eryptosis, storage of RBC may lead to depletion of 2,3-bisphosphoglyceric acid and ATP with subsequent impairment of oxygen transport by increased oxygen affinity [53, 54]. Storage lesions further include oxidative injury by reactive oxygen species (superoxide, hydroxyl radical, or hydrogen peroxide) and metabolically programmed cell death [54]. Erythrocytes with storage lesions can release toxic products including lysophospholipids and free iron and may lead to decreased availability of nitric oxide [53-56]. A decrease of S-nitrosohemoglobin (SNO-Hb) in stored RBC impairs their ability to exert hypoxic vasodilation and may thus compromise tissue perfusion and oxygenation [55, 56]. It is noteworthy that nitric oxide is a powerful inhibitor of eryptosis [57]. Consequently, nitric oxide depletion does not only lead to impaired tissue perfusion but presumably contributes to the enhanced susceptibility of stored RBC to eryptosis.

In conclusion, erythrocyte storage fosters the stimulation of Ca\(^{2+}\) entry into erythrocytes with subsequent change of erythrocyte cell membrane asymmetry. Thus, extended storage periods enhance the susceptibility of RBC for transfusion purposes towards eryptosis, the suicidal erythrocyte death.

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Disclosure Statement

The authors have declared that no competing interests exist.
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