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A Novel Mouse Model of Red Blood Cell Storage and Post-Transfusion in Vivo Survival

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Abstract

Background—Storage of RBCs is necessary for an adequate blood supply. However, reports have identified potential negative sequelae of transfusing stored RBCs. An animal model would be useful to investigate the pathophysiology of transfusing stored RBCs. However, it has been reported that storage of rat RBCs in CPDA-1 resulted in an unexpected sudden decline in post-transfusion survival. We developed a mouse model of RBC storage and transfusion to assess survival kinetics of mouse RBCs.

Study Design and Methods—RBCs expressing green fluorescent protein were collected in CPDA-1, filter leukoreduced, adjusted to a 75% hematocrit, and stored at 4°C. At weekly intervals, stored RBCs were transfused into C57BL/6 recipients. RBC survival was measured by flow cytometry and ⁵¹Chromium labeling. Phosphatidylserine externalization and CD47 expression was also evaluated.

Results—Mean 24-hour survival of transfused RBCs was 99%, 91%, 64%, 54%, 30%, and 18% following 0, 7, 14, 21, 28, and 35 days of storage, respectively. Stored RBCs showed an initial rapid clearance with subsequent extended survival. Increased surface phosphatidylserine and decreased CD47 expression was also observed.

Conclusions—Mouse RBCs showed a progressive decline in survival, as a function of storage time, unlike the precipitous loss of viability previously reported for rat RBCs. Moreover, changes in the measured surface markers were analogous to trends reported for human RBCs. Together, these findings provide an initial characterization of a novel mouse model of RBC storage with the potential to serve as an experimental platform for studying the pathophysiological consequences of transfusing stored RBCs.

Introduction

In order to ensure an adequate supply of transfusable blood, red blood cells (RBCs) are maintained at 4°C for various times depending on the particular storage solution. CPDA-1,

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an FDA-approved solution, allows for the storage of human RBCs for up to 35 days. FDA standards currently require that following transfusion, an average of >75%, SD $\pm$ 9% of RBCs circulate for at least 24 hours ¹. Clinical use of RBCs stored under these or similar conditions has been the standard of care for decades. RBC transfusions can rapidly reverse the symptoms of anemic patients suffering from clinically evident hypoxia. However, there are studies to suggest that transfusion of stored RBCs may have deleterious effects in certain patient populations 2,3. Although these studies are of potential importance, cohort studies may contain confounders, such as severity of illness in the study population. Thus, whether transfusing stored RBCs directly leads to adverse outcomes remains controversial 4.

The potential negative sequelae of blood transfusion may be explained by several hypotheses, including accumulation of molecules that prime leukocyte inflammation ⁵, oxidized proteins and lipids ⁶, and perturbations of nitric oxide biology ⁷. To test these and other hypotheses under controlled and ethically permissible settings, an appropriate animal model of RBC storage and transfusion would be of great utility. Murine models would be particularly valuable, due to the availability of numerous immunological tools, strains, and genetically manipulated variants. However, murine models involve several challenges, the most important of which is whether the biology of stored murine RBCs accurately reflects that of human RBCs. This is particularly relevant in light of published data reporting that rat RBCs stored in CPDA-1 rapidly deteriorate with only a 26% 24-hour survival after 15 days ⁸. In contrast, a published abstract reported a 24-hour post-transfusion survival of 80% for rat RBCs stored for 28 days in CPDA-1 ⁹. The reason for this difference is unclear.

Given the considerable advantages that a murine model would provide, and in the context of the discrepancy regarding stored rat RBCs, we tested the properties of mouse RBCs processed and stored under similar conditions to human RBC storage. A transgenic mouse strain expressing enhanced green fluorescent protein (H2K^b-eGFP) ¹⁰ in RBCs was utilized to allow for detailed post-transfusion analysis of stored RBCs. We demonstrate that 24-hour post-transfusion survival of stored mouse RBCs showed relatively similar kinetics to that reported for human RBCs, when indexed for the normal lifespan of mouse RBCs. We also report that mouse RBCs developed changes in membrane molecules with a trend comparable to those described for human RBCs. Together, these results demonstrate the potential utility of a mouse system to study the effects of storage and transfusion of RBCs.

Methods

Mice

Adult 6- to 8-wk-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H2K^b-eGFP mice ¹⁰ were a generous gift from Dr. Derek A. Persons (St. Jude Children's Research Hospital, Memphis, TN), and were bred by the Emory Division of Animal Resources Animal Husbandry service. All studies and procedures were carried out in accordance with Emory University and/or Columbia University Institutional Animal Care and Use Committee guidelines.

Red blood cell harvest and storage

For each experiment, eight to ten donor H2K^b-eGFP mice were exsanguinated by orbital enucleation into a 50 mL conical tube containing 2.8 mL of CPDA-1, yielding roughly 8 to 10 mL of blood. The total volume was then brought up to 20 mL with additional freshly obtained blood from wild-type C57BL/6 donors resulting in a final CPDA-1 concentration of 14%. The total collection time was approximately 20 to 30 minutes. Blood was passed through a Neonatal High Efficiency Leukocyte Reduction Filter for Red Blood Cells (Pall Biomedical Products Company, East Hills, NY). The leukoreduced blood was centrifuged

for 10 minutes at 1350 rpm and supernatant was partially removed to obtain a hematocrit of approximately 75%. The blood was then transferred to multiple 500 μ L Eppendorf tubes, leaving a small residual air space, and stored in the dark at 4°C until use. The CPDA-1 in these studies was obtained directly from DEHP-PVC human blood storage bags.

Phenotypic analysis

At the indicated time points stored RBCs were assessed for CD47 expression and Annexin V binding. Freshly collected blood was used as a baseline control. Eryptotic RBCs were generated by incubating freshly isolated RBCs for 2 hours at 37°C in RPMI-1640 containing 0.4 mM CaCl_2 , then stimulated for an additional 30 minutes with 8 μ M ionomycin. All RBC suspensions were washed three times with PBS and diluted 1:1000 in FACS buffer (DPBS supplemented with 0.5% bovine serum albumin, 1 mM EDTA and 50 mM Hepes buffer, pH 7.2) for anti-CD47 staining or binding buffer (10 mM Hepes buffer, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4) for Annexin V staining. For CD47 staining, diluted RBCs were incubated with biotinylated anti-mouse CD47 or biotinylated goat IgG (isotype matched control) (R&D Systems, Minneapolis, MN) at 4°C for 30 minutes. RBCs were washed twice with FACS buffer and then incubated with streptavidin APC (BD Pharmingen, San Jose, CA) at 4°C for 30 minutes. RBCs were washed twice with and re-suspended in 500 μ L FACS buffer prior to analysis. For Annexin V staining, 100 μ L of diluted RBCs were incubated with 5 μ L Annexin V-APC (BD Pharmingen) at room temperature in binding buffer for 20 minutes. An additional 400 μ L of binding buffer was then added prior to analysis. Anti-CD47 and Annexin V binding were assessed by flow cytometry and analyzed using FlowJo Software (Treestar, Ashland, OR).

Assessment of RBC survival by flow cytometry

Following 0, 7, 14, 21, 28, and 35 days of storage, RBCs were diluted 1:5 in PBS and 500 μ L was transfused by tail vein injection into each recipient mouse (3–4 mice per experimental group). At 10 and 30 minutes, 1, 2, 18 and 24 hours, and weekly post transfusion time points, recipients were retro-orbitally bled into heparinized tubes and 3 μ L of blood was diluted into 1 mL of FACS buffer. The percentage of donor eGFP⁺ RBCs was determined flow cytometry. The percentage of donor RBCs at time 0 was calculated based on a best-fit regression curve over the initial 24 hours (GraphPad Software, La Jolla, CA). 24-hour survival was determined by the ratio of the percentage of eGFP⁺ RBCs at 24 hours divided by the percentage of eGFP⁺ RBCs estimated for time 0.

Assessment of RBC survival by ⁵¹Chromium labeling

Fresh RBCs and RBCs stored for 14 days in CPDA-1 were each resuspended to a 33% hematocrit with endotoxin-free normal saline and 1 mL aliquots were then incubated with 140 μ L of a 1 mCi/mL solution of ⁵¹Chromium (GE Healthcare, Piscataway, NJ) for 30 minutes at room temperature. Labeled RBCs were washed 3 times with normal saline and re-suspended to a 50% hematocrit. C57BL/6 mice were transfused with 100 μ L of ⁵¹Chromium-labeled RBCs by tail vein injection. Blood was collected by retro-orbital bleed immediately following transfusion and at 1, 2 and 24 hours post-transfusion. The collected blood was centrifuged and the height of the packed RBC column was measured. Percent survival was calculated as 100 multiplied by the ratio of counts per minute per millimeter of the RBC column height at each time point versus the immediate post-transfusion time point.

Statistical Analysis

To determine the significance of the flow cytometry survival data, we utilized a two way ANOVA analysis with a Bonferroni post-test for individual value comparison. For Annexin

V and CD47 data analysis, a non-parametric test (Kruskal-Wallis) was utilized with Dunn's post-test for individual value comparison. A two-tailed unpaired Student's t test was used for $^{51}\text{Chromium}$ survival data. A level of significance of $\alpha=0.05$ was set for all tests. All analysis was performed using the GraphPad Prism Software Suite (GraphPad Software).

Results

Use of eGFP transgenic RBC donors allows tracking of RBC survival in vivo

In the current study, it was imperative to minimize deviations from the collection and storage conditions used with human RBCs. Moreover, it was important to avoid possible biochemical alterations to the RBCs. Therefore, we developed a method to track transfused RBCs, using H2K^b-eGFP donor mice ¹⁰.

To establish detection parameters, eGFP⁺ RBCs from freshly obtained blood were characterized by flow cytometry. An RBC gate was established using forward and side scatter properties. Cells in the RBC gate were uniformly positive when stained for the mouse RBC surface marker Ter-119, as compared to an isotype matched control antibody (Figure 1A). Essentially all RBCs from H2K^b-eGFP mice express a high, uniform level of eGFP fluorescence; no eGFP is detected in C57BL/6 control mice (Figure 1B). Transfusion of H2K^b-eGFP RBCs into wild-type mice resulted in a distinct and traceable population of eGFP⁺ donor RBCs. These events are not due to non-specific background, as significant numbers of eGFP⁺ events are not detected in untransfused recipients (Figure 1C).

H2K^b-eGFP donors were exsanguinated and whole blood was collected to a final concentration of 14% CPDA-1 by volume, to mimic the conditions used for human RBC storage. Prior to storage, the blood was leukoreduced using a Pall neonatal leukoreduction filter. To decrease the number of H2K^b-eGFP mice required, the total blood volume was increased by mixing C57BL/6 and H2K^b-eGFP blood prior to leukoreduction. This did not interfere with the subsequent analysis, as eGFP-negative RBCs are excluded by our flow cytometry gating methods as described above (Figure 1C). After leukoreduction, the blood was centrifuged and supernatant was removed to adjust the final hematocrit to approximately 75%. The blood was then aliquoted into 500 μL Eppendorf tubes, allowing a small pocket of air at the top. These "units" were sealed and kept at 4°C for up to 35 days.

Analysis of phosphatidylserine externalization and CD47 expression

Increased surface expression of phosphatidylserine ^{11,12} and decreased CD47 expression occur with stored human RBCs ^{12,13}. To analyze these parameters following storage of mouse RBCs, levels of anti-CD47 and Annexin V staining were assessed on Ter-119⁺ eGFP⁺ events within the established RBC gate and were quantified as mean fluorescence intensity. Each stained sample was compared to freshly isolated C57BL/6 RBCs and a fold change was calculated. Analysis of the combined data of 4 independent experiments demonstrated that the stored eGFP⁺ RBCs did not exhibit a statistically significant increase in Annexin V binding until a 2-fold increase by day 28 and a 3-fold increase by day 35 (Figure 2A). A representative histogram is shown displaying the shift that occurred during storage. *In vitro* induced eryptotic RBCs are presented in the shaded histogram as a positive control for Annexin V staining.

There was a progressive decline in CD47 expression that reached statistical significance by 28 days and continued through day 35 (Figure 2B). The representative histogram shows the gradual loss of surface CD47 levels on the RBC population following storage. The decline in CD47 was not an artifact of a nonspecific decrease in cell size, as no decrease in the intensity of Ter-119 staining was observed on this same population (data not shown). The

induced eryptotic RBCs did not show a significant decrease in CD47 expression, suggesting that the loss of this molecule is due to other mechanisms than Ca^{2+} flux.

Survival in vivo of transfused stored mouse RBCs

H2K^b-eGFP RBCs stored for the indicated times were transfused into wild-type recipients and survival was monitored by enumerating eGFP⁺ RBCs at 10 and 30 minutes, and at 1, 2, 18, and 24 hours post-transfusion. Long term survival was assessed at weekly intervals post-transfusion. Representative survival curves are shown in Figure 3. Similar to humans, the clearance of transfused stored RBCs occurs in two distinct phases: an initial rapid clearance (Figure 3A) followed by an extended survival to at least 50 days (Figure 3B). Combined analysis of 4 independent experiments demonstrated that the mean 24-hour survival of transfused eGFP⁺ RBCs was approximately 99%, 91%, 64%, 54%, 30%, and 18% following 0, 7, 14, 21, 28, and 35 days of storage in CPDA-1 (Figure 3C).

To compare flow cytometry to the clinical method used in human patients, additional studies were performed using ⁵¹Chromium-labeled freshly isolated RBCs and 14-day stored RBCs. Like flow cytometry, this method detected a rapid initial clearance of stored RBCs immediately following transfusion (Figure 4A). The 24-hour survival of 14-day stored RBCs was found to be comparable as measured by either flow cytometry (i.e. 64% SD $\pm$ 5.0%) (Figure 3C) or ⁵¹Chromium labeling (i.e. 65% SD $\pm$ 7.7%) (Figures 4B).

Discussion

RBC transfusion is a common treatment in a variety of clinical settings with over 14 million units of RBCs collected and distributed per year in the United States alone. Recent evidence suggests that transfusion of RBCs stored for greater than 14 days may increase the risk of medical complications^{2,3}. For these reasons, a tractable animal model for studying the adverse effects of transfusing stored RBCs would be of great utility. Because the storage of rat RBCs in CPDA-1 was reported to result in highly accelerated aging *in vitro*⁷, which differs from stored human RBCs, rodent models have been considered to be of only limited utility. However, our current results demonstrate that, in contrast to what was reported for rats, stored mouse RBCs do not undergo a similar precipitous decline in post-transfusion survival.

In evaluating the significance of our findings, we interpreted mouse RBC storage in the context of their normal lifespan. Mouse and human RBCs have an average normal survival of 55 and 120 days *in vivo*, respectively. Therefore, 35-day storage of human RBCs in CPDA-1 represents approximately 29% of the normal RBC lifespan. The analogous 29% of the normal mouse RBC lifespan is 16 days. One might argue that this thinking is inappropriate, and that absolute time of preservation at 4°C ought to apply equally to either population. However, as RBCs continue to age *ex vivo*, it is our position that it is appropriate to assess the properties of stored mouse RBCs as a function of their normal rate of aging *in vivo*. By these criteria, our data indicate that mouse RBCs age somewhat more quickly in CPDA-1 than do human RBCs. However, the general pattern of progressive decline is similar between the two species. The decreased survival of mouse RBCs may represent a qualitative difference in biology. Alternatively differences in survival may be due to quantitative variables such as the fact that CPDA-1 has been optimized to storage of human RBCs, and known differences in mouse blood chemistry (e.g. osmolarity)¹⁴. Moreover, the absence of plasticizer, present in human blood bags and reported to extend human RBC survival, may also be responsible¹⁵.

It is important to note that the FDA requirement for 75% post-transfusion survival at 24 hours represents an average. Indeed, some units of RBCs stored under approved conditions

for the appropriate length of time have substantially worse survival¹⁶. Such differences can be donor specific. For example, in a study by Mishler et al., RBCs collected on two separate occasions from the same donor had a 24-hour survival of 33% and 41%, respectively¹⁷. This was in contrast to RBCs from other donors that had a mean survival of 74% and 75%. Thus, survival appears to be based upon donor specific factors. Similar to the above observations in humans, it would be reasonable to predict that genetic background and/or environmental factors may likewise affect survival of transfused mouse RBCs. This was not evaluated in the current study because the eGFP transgene is not yet backcrossed onto different strains.

Although phosphatidylserine externalization increased with storage, it does not statistically differ until after day 28. Likewise, the loss of CD47 expression does not reach significance until day 28. However, the 24-hour post-transfusion survival data clearly indicate that the *in vivo* survival is significantly decreased as early as day 14. These results demonstrate that neither phosphatidylserine nor CD47 expression is significantly altered at a storage time when the post-transfusion survival is decreased, suggesting that clearance of the transfused RBCs is due to other factors. However, one cannot rule out a sudden alteration in cell surface expression immediately after transfusion that may not be apparent in the stored RBC unit itself. A description of an apoptotic-like pathway in RBCs (i.e. eryptosis) that induces sudden changes of this nature has been previously described¹⁸.

Substantial efforts were made to model human RBC storage conditions accurately. For example, the ratio of CPDA-1 to blood, the use of filter leukoreduction, and the final hematocrit were all the same as those used with human blood. However, the storage conditions are not completely ideal. In the current study, RBC units are stored in 500 μ L Eppendorf tubes. Clearly, the plastic of the Eppendorf tubes differs from the storage bags used for human RBCs. As plasticizer continuously leaks from the bag material itself, our attempt to introduce the plasticizer into our stored mouse units by obtaining CPDA-1 from DEHV-PVC bags did not lead to the same concentrations present in stored human units. Because plasticizer has been shown to increase the post-transfusion survival of stored human RBCs, this may have a significant effect^{19,20}. In addition, collection time from mice necessitated a longer exposure of initial RBCs with undiluted CPDA-1, which is known to have a potential to shock the early collected RBCs. Also of note is the fact that mouse blood has a slightly higher osmolarity as compared to human blood. Thus, a preservative such as CPDA-1, designed for human use, may not be optimal for mouse RBC storage.

The current studies focus on 24-hour post-transfusion survival and alterations in surface phosphatidylserine and CD47. Although these parameters reflect a few of the known properties of stored human RBCs, several other well characterized biochemical changes exist, including alterations in ATP, 2,3-DPG, changes in shape, alterations in membrane deformability, increased osmotic fragility, and changes in hemoglobin (e.g. altered S-nitrosohemoglobin). Ongoing evaluation of this model would benefit from analysis of these parameters.

The current findings indicate that when stored under similar conditions to those used for humans, mouse RBCs do not display a precipitous decrease in post-transfusion survival as reported for rats⁷. Use of the eGFP⁺ transgenic mouse allows for characterization of RBCs during storage as well as tracking following transfusion. Together, these data suggest that this is a valid model that may be a useful platform to study pathophysiological effects of transfusing stored RBCs.

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Figure 1. Mouse RBCs expressing transgenic eGFP provide a traceable donor population following transfusion into wild-type recipients

Fresh whole blood from H2K^b-eGFP and C57BL/6 mice was analyzed for the expression of eGFP on Ter-119⁺ RBCs. A distinct forward and side scatter RBC gate was established (**A, left panel**). The validity of the RBC gate was assessed by staining with anti-Ter-119 or isotype matched control (**A, right panel**). Using the forward and side scatter RBC gate established in panel A, eGFP fluorescence was measured in H2K^b-eGFP RBCs as compared to the background fluorescence in wild-type C57BL/6 RBCs (**B**). When H2K^b-eGFP RBCs were transfused into C57BL/6 recipients, donor RBCs were clearly discernable from recipient RBCs; only minimal background fluorescence was seen in untransfused recipients (**C**).

Figure 2. Storage of mouse RBCs in CPDA-1 induces a progressive increase in phosphatidylserine externalization and decrease in CD47 expression

At weekly intervals stored mouse RBCs were stained with Annexin V (A) or anti-CD47 (B). The analysis was restricted to Ter-119⁺ eGFP⁺ events within the RBC gate (see Figure 1C). Representative histograms are shown to the right. The data is presented as a fold change between the indicated stored RBC specimen and fresh RBCs. Anti-CD47 staining was calculated by subtracting the mean fluorescence intensity of an isotype matched control stain from the mean fluorescence intensity of staining with anti-CD47 for each specimen. The presented data represents a combination of 4 separate experiments. Error bars indicate standard deviation. (*p value < 0.05)

Figure 3. Survival of stored mouse RBCs transfused into wild-type recipients

Donor RBCs were monitored by flow cytometry using eGFP fluorescence as a means to distinguish donor from recipient RBCs. Survival was monitored as a percent of donor cells within the RBC gate. Representative results from one experiment are shown from 14-days of storage. An initial rapid clearance occurred within the first several hours (**A**) followed by markedly slower clearance of the remaining donor RBCs over 50 days (**B**). At the indicated time of storage, eGFP⁺ RBCs were transfused into wild-type C57BL/6 recipients and 24-hour survival was monitored by flow cytometry (**C**). The presented data is the compilation of four independent experiments. (*p value < 0.05).

Figure 4. $^{51}\text{Chromium}$ 24-hour survival of transfused mouse RBCs

14-day stored RBCs were labeled with $^{51}\text{Chromium}$ and transfused into wild-type C57BL/6 recipients. Survival of transfused RBCs was determined using a gamma counter to measure radioactivity. An initial rapid clearance of stored RBCs was observed compared to freshly isolated RBCs (A). The 24-hour survival was 65% (*p value < 0.05), which was comparable to the flow based determination (64%) (see Figure 3C)