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Research Paper

Sickle Cell Trait Increases Red Blood Cell Storage Hemolysis and Post-Transfusion Clearance in Mice

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ABSTRACT

Background: Transfusion of blood at the limits of approved storage time is associated with lower red blood cell (RBC) post-transfusion recovery and hemolysis, which increases plasma cell-free hemoglobin and iron, proposed to induce endothelial dysfunction and impair host defense. There is noted variability among donors in the intrinsic rate of storage changes and RBC post-transfusion recovery, yet genetic determinants that modulate this process are unclear.

Methods: We explore RBC storage stability and post-transfusion recovery in murine models of allogeneic and xenogeneic transfusion using blood from humanized transgenic sickle cell hemizygous mice ($Hba^{tm1Paz}Hbb^{tm1Tow}$Tg(HBA-HBBs)41Paz/J) and human donors with a common genetic mutation sickle cell trait (HbAS).

Findings: Human and transgenic HbAS RBCs demonstrate accelerated storage time-dependent hemolysis and reduced post-transfusion recovery in mice. The rapid post-transfusion clearance of stored HbAS RBC is unrelated to macrophage-mediated uptake or intravascular hemolysis, but by enhanced sequestration in the spleen, kidney and liver. HbAS RBCs are intrinsically different from HbAA RBCs, with reduced membrane deformability as cells age in cold storage, leading to accelerated clearance of transfused HbAS RBCs by entrapment in organ microcirculation.

Interpretation: The common genetic variant HbAS enhances RBC storage dysfunction and raises provocative questions about the use of HbAS RBCs at the limits of approved storage.

1. Introduction

Sickle cell trait is the carrier status of sickle cell disease (SCD), a severe hemolytic disease that is caused by a point mutation in the gene encoding beta-hemoglobin (β6Glu → Val) that increases the hydrophobicity of this protein when deoxygenated. In SCD RBCs, this mutation causes Hb polymerization under small reductions in physiologic oxygen saturation leading to cell dehydration, increased membrane rigidity and hemolysis (Rees et al., 2010; Brittenham et al., 1985). These altered red cell properties promote vaso-occlusive events in microcirculation, causing severe pain and end-organ ischemia, infarction and progressive dysfunction. Red blood cells (RBCs) from individuals with sickle cell trait (SCT) contain 25–50% HbS that polymerizes only at low fractional oxygen saturations <50% (Brittenham et al., 1985). Thus, under normal physiologic conditions, individuals with sickle cell trait are asymptomatic. However, under extreme conditions of hypoxia and dehydration, vaso-occlusive events can occur (Statius van Eps and De Jong, 1997).

Historically, donor RBC genetic background is considered benign if the donor lacks clinically relevant symptoms but prolonged storage exposes RBCs to non-physiologic stress conditions and may amplify the effects of occult mutations (Dern et al., 1967; Latham et al., 1982).

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Sickle cell trait has a high prevalence in malaria endemic regions, which increases the probability that patients requiring RBC transfusions in these regions will receive stored HbAS RBCs. Current transfusion practices supporting the use of sickle cell trait RBCs are based on limited studies performed decades ago, which reported no differences in post-transfusion survival or recovery of sickle trait RBCs compared with normal RBCs. However, storage duration was relatively short (<21 days compared with present 42-day storage limits) and utilized less sensitive methods to evaluate RBC post-transfusion survival (Callender et al., 1949; Ray et al., 1959; Levin and Truax, 1960). Here, we show that sickle cell trait increases storage hemolysis and reduces red cell post-transfusion survival in mice, an effect that increases with increasing time in storage. Interestingly, transfused HbAS RBCs do not exhibit higher intravascular hemolysis compared to HbAA RBCs, but rather become entrapped in the systemic microcirculation. These findings raise concerns about the viability of stored sickle cell trait red blood cells after prolonged storage and suggest a need for further clinical evaluation of post-transfusion recovery of stored human HbAS containing RBCs.

2. Materials and Methods

2.1. Mice

8–12 week old wildtype (HbAA) mice (C57BL/6J), Berkeley hemizygous (HbAS) (*Hba*^{tm1Paz}*Hbb*^{tm1Tow}Tg(HBA-HBBs)41Paz/J) mice and transgenic mice expressing enhanced Green Fluorescent Protein in hematopoietic cells (C57BL/6-Tg(UBC-GFP)30Scha/J) were purchased from Jackson Laboratories. Berkeley hemizygous mice express human α-globin and sickle β-globin genes in addition to one copy of the murine β-globin, making them hemizygous for Sickle Cell Disease (HbAS). Our characterization studies and those of others suggest these mice express 20–30% HbS, which is similar to the 20–45% HbS distribution observed in sickle cell trait individuals (Noguchi et al., 2001; Steinberg and Embury, 1986). Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Both male and female mice were used as donors for RBC isolation. Recipient male mice were of C57Bl/6J strain and randomly assigned to experimental groups to receive either HbAA or HbAS blood. Experimenters performing the experiments was not blinded to group assignment but was blinded to outcome assessment. There was no exclusion of data from any animals.

2.2. Blood Collection and Storage

Leukoreduced human RBC units were stored under standard blood banking conditions from sickle cell trait donors and ABO blood type matched donors were obtained from Central Blood Bank (Pittsburgh, PA) and stored at 1–6 °C until specified times for testing. Whole blood (WB) was collected from mice via the inferior vena cava immediately following euthanasia using Citrate Phosphate Dextrose solution as an *anti*-coagulant (Sigma St. Louis, MO). Pooled WB from mice and human blood was leukoreduced using a Pall Purecell® NEO Neonatal High Efficiency Leukocyte Reduction Filter (Hod et al., 2010b). Leukoreduced blood was re-suspended in 14% CPDA-1 (Sigma St. Louis, MO), concentrated to a final hematocrit of 55%, and stored at 1–6 °C in glass vacutainers shielded from light for up to 11 days (Hod et al., 2010b).

2.3. In Vitro Hemolytic Assays

Storage hemolysis and stress-induced osmotic or mechanical hemolysis were measured by supernatant cell-free hemoglobin using Drabkin's assay (Moore et al., 1995). See Supplemental section.

2.4. Post-Transfusion Survival Studies

Fresh or 11-day stored murine RBCs (equivalent to 39–42 day stored human RBCs) (Mangalmurti et al., 2009; Gilson et al., 2009) for transfusion were labeled with lipophilic dyes 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate dyes DiI (D-383) or DiD (D-307) (Invitrogen, Carlsbad CA) prior to infusion in recipient mice by retro-orbital injection. RBC 24 h post-transfusion recovery was obtained by blood sampling via tail vein and enumerated by flow cytometry and analyzed using FlowJo (Ashland, OR). To confirm that fluorescent labeling dyes do not alter RBC post-transfusion survival, C57BL/6 mice expressing Green Fluorescent Protein (GFP) driven by the human ubiquitin C promoter (C57BL/6-Tg (UBC-GFP) 30Scha/J) were transfused with unlabeled fresh or stored HbAA and HbAS RBCs individually and analyzed using a negative FITC gate to quantify 24 h post-transfusion recovery.

2.5. Statistical Methods

To measure storage-related changes between HbAA and HbAS RBCs such as echinocyte formation, changes in hemolytic propensity and post-transfusion survival, two-way ANOVA with Bonferroni post-test for individual comparisons was used to perform statistical analysis. To determine differences in HbAA and HbAS RBC sequestration in tissues, Mann-Whitney *U* test was used for non-parametric analysis (GraphPad Prism 6, La Jolla, CA). Initial pilot studies examining RBC post-transfusion recovery provided the basis for sample size estimation of recipient mice. Details regarding statistics, technical and biological replicates performed are provided in the Figure Legends.

3. Results

3.1. HbAS Red Blood Cells Exhibit Higher Storage Hemolysis, Increased Resistance to Osmotic Stress

To assess the membrane properties of HbAA and HbAS RBCs, conventionally banked human and murine RBCs stored in standard preservative solution were assayed at the beginning and end of storage. Human RBCs under standard blood banking can be stored up to 42 days (Koch et al., 2008). Leukoreduced murine RBCs stored for up to 14 days show post-transfusion recovery that approximates what is observed with human RBCs at the limits of approved storage, suggesting that these shorter storage times represent a more appropriate and conservative model for day 39–42 human RBC storage (Hod et al., 2010b; Gilson et al., 2009). By 39–42 days of storage, conventionally banked human HbAS (h-HbAS) RBCs exhibited higher storage hemolysis compared to human HbAA (h-HbAA) RBC samples (1.0 ± 0.6% versus 0.15 ± 0.02%, $p = 0.0035$, Fig. 1a). RBCs from Berkeley hemizygous mice (m-HbAS) also exhibited higher storage hemolysis at the end of 11-day storage compared to murine HbAA (m-HbAA) RBCs (2.4 ± 0.16% versus 1.7 ± 0.1%, $p = 0.0022$, Fig. 1b). In both human and murine HbAS RBCs, free hemoglobin concentrations were higher at the beginning of storage compared to HbAA RBCs, a transient increase likely due to mechanical challenges suffered during leukoreduction (Fig. 1a–b) (Stroncek et al., 2002). The size distribution and hemoglobin content of human HbAA and HbAS RBCs showed no differences (Supplemental Fig. S1).

To determine membrane resilience to osmotic shock, packed RBCs were re-suspended in a hypotonic buffer for 3 h. Similar to prior findings in HbSS RBCs,(Franco et al., 2000) human and murine HbAS RBCs showed reduced % osmotic hemolysis, or increased resilience to osmotic shock, compared with HbAA RBCs (h-HbAS and h-HbAA RBCs: 9.0 ± 3.1% vs. 35.1 ± 10.3% hemolysis; $p < 0.0001$, Fig. 1c; m-HbAS and m-HbAA RBCs: 18.1 ± 5.4% vs. 55 ± 5.4% hemolysis; $p = 0.0001$, Fig. 1d). The osmolarity at which RBCs exhibited maximum deformability (Osm Max) was lower for HbAS RBCs compared to

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