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Original Contribution

Red blood cell washing, nitrite therapy, and antiheme therapies prevent stored red blood cell toxicity after trauma–hemorrhage

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ABSTRACT

Transfusion of stored red blood cells (RBCs) is associated with increased morbidity and mortality in trauma patients. Pro-oxidant, pro-inflammatory, and nitric oxide (NO) scavenging properties of stored RBCs are thought to underlie this association. In this study we determined the effects of RBC washing and nitrite and antiheme therapy on stored RBC-dependent toxicity in the setting of trauma-induced hemorrhage. A murine (C57BL/6) model of trauma–hemorrhage and resuscitation with 1 or 3 units of RBCs stored for 0–10 days was used. Tested variables included washing RBCs to remove lower MW components that scavenge NO, NO-repletion therapy using nitrite, or mitigation of free heme toxicity by heme scavenging or preventing TLR4 activation. Stored RBC toxicity was determined by assessment of acute lung injury indices (airway edema and inflammation) and survival. Transfusion with 5 day RBCs increased acute lung injury indexed by BAL protein and neutrophil accumulation. Washing 5 day RBCs prior to transfusion did not decrease this injury, whereas nitrite therapy did. Transfusion with 10 day RBCs elicited a more severe injury resulting in ~90% lethality, compared to < 15% with 5 day RBCs. Both washing and nitrite therapy significantly protected against 10 day RBC-induced lethality, suggesting that washing may be protective when the injury stimulus is more severe. Finally, a spectral deconvolution assay was developed to simultaneously measure free heme and hemoglobin in stored RBC supernatants, which demonstrated significant increases of both in stored human and mouse RBCs. Transfusion with free heme partially recapitulated the toxicity mediated by stored RBCs. Furthermore, inhibition of TLR4 signaling, which is stimulated by heme, using TAK-242, or hemopexin-dependent sequestration of free heme significantly protected against both 5 day and 10 day mouse RBC-dependent toxicity. These data suggest that RBC washing, nitrite therapy, and/or antiheme and TLR4 strategies may prevent stored RBC toxicities.

Introduction

Transfusion with packed red blood cells (RBCs) is a front-line therapy for critically ill, anemic patients. However, recent studies have documented positive associations among the number of RBC units transfused, the age of the RBC unit being transfused, and increased transfusion-related morbidity and mortality [1]. For example, our studies have shown increased incidence of acute lung injury, acute kidney injury, pneumonia, and mortality in bleeding trauma patients, a population that receives a significant portion of the stored blood in tertiary care centers [2–4]. Importantly, storage lesion toxicity is observed in diverse patient populations, suggesting common mechanisms related to gain of toxic functions by stored RBCs. This understanding has fueled numerous research efforts aimed at elucidating the mechanisms by which stored RBCs may elicit injurious responses after transfusion [5]. Current thinking suggests that these are related to microcirculatory dysfunction, exacerbation of underlying inflammation, increased oxidative stress, and increased predisposition to

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nosocomial infections [2,6–11]. During storage, several structural, biochemical, and metabolic alterations occur to the RBCs. These changes include loss of metabolites (e.g., ATP), loss of RBC volume with accompanying formation of echinocytic forms and hemoglobin-containing microparticles, RBC degradation, and release of cell-free hemoglobin (hemolysis), iron, and cellular debris [12]. While potential toxic effects of each of these aspects of RBC storage have been studied (see below), little attention has been given to the potential of heme released during storage as a mediator of transfusion injury. Notably, recent studies suggest that heme is a potent inducer of inflammatory tissue injury in sickle cell disease and sepsis [13–18].

Loss of nitric oxide (NO) homeostasis has emerged as a mechanism underlying many features of transfusion toxicity associated with stored RBCs, including microcirculatory dysfunction and inflammation. Stored RBCs, including cells with altered morphology, hemoglobin-containing microparticles, and hemolysis-derived cell-free hemoglobin, all display ferrous heme-dependent NO scavenging with kinetics that are faster compared to freshly isolated RBCs [19–21]. This biochemical property also translates to a greater inhibition of NO-dependent signaling *ex vivo* and *in vivo* [19,20,22]. In addition to scavenging NO, older RBCs may also be less effective at stimulating endogenous NO formation. Stored RBCs display faster rates of nitrite oxidation *in vitro*, and transfusion of trauma patients with older cells, but not younger RBCs, results in lower circulating nitrite levels [20]. Since nitrite is a putative substrate for NO formation in hypoxic tissues, this reactivity would lower an endogenous substrate for NO formation, thereby further leading to inhibition of NO signaling. Moreover, RBC-derived ATP-dependent activation of endothelial nitric oxide synthase is lost in stored RBCs [23], and RBC–*S*-nitrosothiols have also been postulated as potential mechanisms for loss of NO homeostasis [24,25]. Collectively, these data provide mechanistic insights into how transfusions with stored RBCs inhibit endogenous NO signaling in the vasculature and suggest that NO-repletion strategies or approaches that remove components in stored RBCs that inhibit NO signaling could be beneficial. In the latter context, RBC washing immediately before transfusion to remove microparticles, cell-free hemoglobin, and iron, prevented many of the negative effects of storage in a canine model of transfusion and infectious lung injury [26] and attenuated cytokine levels in a mouse model of trauma–hemorrhage [27] . In the current study, we show similar protective effects of washing toward acute lung injury and lethality in a murine model of trauma–hemorrhage. In addition, we show that NO-repletion therapy using nitrite protects against storage-induced toxicity. Finally we provide evidence that free heme significantly increases during storage and is a key mediator of injury in this model.

Materials and methods

TAK-242, a small molecule inhibitor of TLR4, was purchased from InvivoGen and dissolved in Intralipid (Sigma). Hemopexin was purchased from Athens Research & Technology. All other materials were purchased from Sigma–Aldrich (St. Louis, MO) except MahmaNONOate which was obtained from Axxora Platform (San Diego, CA). Sodium nitrite (Sigma) used in resuscitation was dissolved in saline. Adsol was from Baxter Health Care Corporation. Human oxyhemoglobin was purified for healthy donors according to UAB Institutional Review Board-approved protocols and stored in the CO-ligated form as previously described [28]. Methemoglobin was synthesized and purified as previously described using potassium ferricyanide added at a 2-fold excess over oxyHb (in heme) and purifying metHb by gel filtration using Sephadex G-25 columns [29]. Similar protocols were used for preparing mouse hemoglobin. Male C57BL/6 mice weighing 22 to 30 g were purchased from Harlan Laboratories (Indianapolis, IN) at 8–10 weeks of age.

Preparation of hemin and hemoglobin standards for spectral deconvolution

To ensure that no COHb remained in oxyHb solutions and that all oxyHb was oxidized to metHb, reduction with sodium dithionite and $>99\%$ conversion to deoxyHb was verified. oxyHb and metHb solutions were diluted to 25 μM (in heme) into Adsol pH 7.4 or pH 6.8. Adsol was collected under sterile conditions on the day of experiments and pH adjusted from 5 (Adsol basal pH) to 6.8 or 7.4 using 0.1 N NaOH. Hemin was dissolved in 0.1 N NaOH at 10 mM (concentrations verified using $\varepsilon_{385\ \mathrm{nm}} = 58.4\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$). Then 5 ml of a 25 μM solution of hemin was prepared in Adsol pH 5.0 and pH adjusted to pH 6.8 using 0.1 N NaOH. For pH 7.4, hemin was diluted to 25 μM in Adsol (pH 5) and pH adjusted to 7.4 using 0.1 N NaOH. Standard spectra were acquired between 450 and 700 nm at room temperature. For preparation of hemin and oxyHb or metHb mixtures for validation of the deconvolution method, pHs of stock solutions were adjusted to 6.8 or 7.4 using Adsol (pH 5) or 0.1 N NaOH and then mixed to give final indicated concentrations.

Free heme measurement—Method development and validation

We developed a spectral deconvolution approach to simultaneously measure the concentrations of oxyhemoglobin, methemoglobin, and free heme in solution as described under Results.

Human and mouse RBC collection

Human RBCs stored for up to 42 day were collected from segments attached to blood bags from the UAB blood bank, and processed according to UAB Institutional Review Board-approved protocols. All human RBCs used in this study were leukoreduced and stored in Adsol-1. At the time of collection, RBCs (~0.5 ml) were placed into 1.7 ml Eppendorf tubes and pelleted by centrifugation (1500*g*, 10 min, 4 °C). The supernatant (~200 μl) was removed and indices of storage-related changes (described below) determined. For acquisition of spectra, the resultant supernatant fraction was centrifuged again (2000*g*, 30 s). Then 50 μl of the supernatant was taken and diluted 10-fold with distilled water and absorbance spectrum (450–700 nm) measured. For mouse RBCs, blood (~800 μl) was collected from healthy male C57BL/6 mice via cardiac puncture in 50 μl citrate buffer (trisodium citrate (22.0 g/L), citric acid (8.0 g/L), dextrose (24.5 g/L)). Blood was filtered through neonatal Sepacell filters or through Sephadex G25 microcellulose column to remove leukocytes by gel filtration. Columns were washed with 10 times the volume of PBS or Adsol to eluate RBCs (with no differences in hemolysis during processing observed (not shown). The eluent was centrifuged at 1500*g* for 5 min, 4 °C. The erythrocyte pellet was washed 3 times with cold Adsol and concentrated to a hematocrit of 60% with Adsol and stored in 0.7 ml Eppendorf tubes with a head space of approximately 300 μl. All solutions and procedures were performed under sterile conditions and used CPD anticoagulant and AS1 storage solutions obtained from the blood donation kit (Fenwal Express System, Lake Zurich, IL) used from human blood collection. RBCs were stored at 4 °C in the dark, for up to 10 days. LPS measurements in stored RBCs using the Limulus Amebocyte lysate assay (Cambrex, MD) indicated that levels were below the detection limit (not shown).