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Vascular effects of the red cell storage lesion

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Abstract

Transfusion of red blood cells (RBCs) is often clinically necessary, and life-saving, for anemic patients. RBCs can be stored for up to 42 days between the time of donation and transfusion. For many years, investigators have studied the biochemical changes that occur in RBCs stored prior to transfusion (the RBC "storage lesion"). More recently, clinical studies have suggested that RBC units stored for long periods (often described as > 14–21 days) may mediate adverse effects in the recipient, leading to morbidity and mortality. Unfortunately, these effects are difficult to identify and study because there are no agreed upon mechanisms for these adverse events, and few good assays to study them in individual transfusion recipients.

We have proposed the hypothesis of Insufficient NO Bio-Availability (INOBA) to explain the adverse events associated with transfusion of older RBC units. INOBA postulates that the combination of impaired NO production/increased NO scavenging by stored RBCs together with reduced NO synthesis by dysfunctional endothelial cells collectively reduce NO levels below a critical threshold in vascular beds. In this situation, inappropriate vasoconstriction occurs leading to reduced blood flow and insufficient O_2 delivery to end organs. If confirmed, the INOBA hypothesis can lead to improved methods for blood storage and collection, as well as new screening and matching tools for blood donors and transfusion recipients.

Keywords

Red blood cells; transfusion; nitric oxide

Background

Despite dramatic improvements in blood safety (particularly with respect to infectious disease transmission), post-transfusion complications still occur. Some of the more difficult of these complications to address, since their frequency, pathophysiology, and mechanism is unclear, are the purported adverse events associated with transfusion of aged ("non-fresh") RBC units compared to "fresh" units [1]. There are no effective clinical or laboratory assessments to determine whether these patients experience negative clinical consequences

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from stored blood. Nonetheless, there is epidemiologic data to suggest that such effects may be relatively common and are of major clinical significance [1].

RBC storage time and post-transfusion morbidity and mortality

A recent large retrospective study of 6002 cardiovascular patients, who received 19,584 transfusions, investigated adverse events related to the storage age of RBCs [1]. Patients that received older units (15–42 days of storage) had higher rates of in-hospital mortality (2.8% vs. 1.7%, P=0.004), as well as higher rates of extended intubation, renal failure, and sepsis as compared to those that received fresher units (< 14 days of storage). One-year mortality was also significantly greater in patients that received older units as compared to those receiving fresher units (11.0% vs. 7.4%; P<0.001). Similarly, in a retrospective review of 9 studies including over 2800 cardiac surgery, trauma, and ICU patients, there was an increased rate of mortality, multiorgan failure, infections or length of hospital stay in proportion to the age of the RBC units [2].

Not all studies are in agreement, however. Edgren, et al, performed a cohort study of transfusion recipients in Sweden and Denmark (1995–2002). A total of 404,959 transfusion episodes were evaluable. There was no significant increase in the 7-day risk of death for patients that received older versus fresher units (hazard ratio, 1.05; 95% confidence interval [CI], 0.97–1.12). A small, but statistically significant trend toward an increased 2-year risk of death in those that received older units (hazard ratio, 1.05; 95% CI, 1.02–1.08) was found, but it was ascribed to weak confounding [3]. Since the primary studies performed to date have been retrospective, there is the concern that their results have been affected by unintended biases.

A better estimate of the risks associated with transfusion of older versus fresher stored units likely awaits the results of prospective, randomized clinical trials (RCTs). The NHLBI-funded Red Cell Storage Duration Study (RECESS) is a multicenter RCT in which patients undergoing complex cardiac surgical procedures will be transfused with RBC units stored for 10 days or fewer versus RBCs stored for at least 21 days [4]. Approximately 1434 patients will be enrolled, and outcomes including Multiple Organ Dysfunction Score (MODS), all-cause mortality, and other measures of organ dysfunction will be tracked. The “Age of Blood Evaluation” (ABLE) study, a double-blind multicenter RCT, will compare results from transfusion of RBCs stored for 7 days or less (“fresh”) to those stored an average of 15 – 20 days (“standard issue”) for adult ICU patients [5]. The study will enroll 2510 patient with the objective of detecting a 5% absolute risk reduction by transfusion of fresh RBCs, Outcomes of interest include 90-day all-cause mortality, ICU mortality, organ failure, and nosocomial infections. The “Age of Red Blood Cells in Premature Infants” (ARIPI) RCT differs from RECESS and ABLE in that it will study transfusion outcomes in approximately 450 pediatric patients as a function of RBC storage interval (7 days or less versus standard practice) [6]. Outcomes of interest are mortality as well as disorders specific to neonatal patients, including necrotizing enterocolitis, retinopathy of prematurity, bronchopulmonary dysplasia, and intraventricular hemorrhage. The carefully vetted designs of these large RCTs should lead to much improved estimates of the efficacy and safety of fresh versus storage-aged RBC units for transfusion.

The endothelium controls vasodilation through regulation of NO signaling

The microcirculation functions as an actively-adjusting vascular circuit to match blood flow (and O_2 delivery) to local tissue oxygen needs [7, 8]. The endothelium, which releases mediators to regulate the contraction/relaxation of underlying smooth muscle and therefore vessel diameter, plays a very important role in microcirculatory function [9, 10]. These mediators include NO, thromboxane, endothelin, and endothelium-derived hyperpolarizing factor (EDHF) [7–10]. For our studies, We have focused on the role of NO (both synthesized/released by RBCs and endothelium) in the effects of stored RBCs.

NO production and signaling by the endothelium is fairly well-characterized. Endothelial NO synthesis is controlled by nitric oxide synthase (NOS) activity. Of the three isoforms of NOS (neuronal [nNOS], inducible [iNOS], and endothelial [eNOS]) eNOS is thought to be the major source of NO for regulating vasoregulation [9, 10]. Endothelial-produced NO diffuses to the underlying smooth muscle, where it activates guanylate cyclase and elicits muscle relaxation and vasodilation [9, 10].

Reduced NO production is seen in patients with endothelial dysfunction, a common finding in many ill patients including those with cardiovascular disease (CVD) [11]. Because a large number of transfusions are given to seriously ill patients, many transfusion recipients would be expected to have some degree of dysfunctional endothelial NO signaling. Reduced NO production due to endothelial dysfunction can be studies by non-invasive ultrasound techniques. In addition, these individuals can also be identified through their biomarker profiles, particularly those affected by oxidative stress (OS), which is involved in the pathophysiology of CVD. In patients with CVD, the total burden of risk factors including OS markers correlates with endothelial dysfunction [12–14]. These markers include hsCRP, $LpPLA_2$, and GSH/GSSG [15–18].

RBCs can also control arterial tone and regulate local blood flow

There are also substantial data to suggest that RBCs can control local blood flow (hypoxic vasodilation) through regulation of NO concentrations [19]. The ability of RBCs to monitor local O_2 concentrations and regulate blood flow to assure that O_2 is delivered to tissues in need is physiologically attractive. However, in contrast to the situation with endothelium, there is substantial disagreement regarding the mechanisms by which RBCs perform this function. Three different mechanisms have been forwarded to describe how red cells, upon sensing local hypoxia, could stimulate vasodilation and thereby increase blood flow: (1) release of ATP [20–23]; (2) release of NO from its storage form as S-nitrosylated hemoglobin (SNO-Hb) [7, 24]; and (3) reduction of circulating nitrite to NO during the R-to-T allosteric transition of Hb [25–27].

In the first mechanism, ATP released by RBCs binds to purinergic receptors on endothelial cells, stimulating the production of NO and other substances that produce vasodilation [21, 28]. This model is supported by data showing that physiologically meaningful levels of ATP are released from RBCs under hypoxic conditions, that direct ATP application to vessels can stimulate vasodilation, and that ATP release is susceptible to physiologically attractive feedback modulator including ADP and NO [21, 28].

The SNO-Hb model postulates that NO equivalents are carried in RBCs as S-nitrosylated moieties on cys β-93 of hemoglobin (SNO-Hb) [29, 30]. NO is then released from SNO-Hb during hemoglobin deoxygenation to promote vasodilation when O_2 tension is low (hypoxic conditions) [19, 31]. Problems with this model have been raised [32–34]. For example, if SNO-Hb were an important precursor of vasoactive NO then arterio-venous gradients in SNO-Hb should be identified, but have not. Furthermore, transgenic mice expressing human hemoglobin with an alanine substitution for cys β-93, preventing the synthesis of SNO-Hb, demonstrate normal RBC-dependent hypoxic vasodilation [35].

In the third model, Hb acts as an allosterically regulated nitrite reductase which catalyzes the formation of nitrite to NO [36, 37]. This has been recently reviewed in detail [38]. In addition, myoglobin can also reduce nitrite to NO, and the importance of this mechanism to cardiac function and response to ischemic events was shown with a myoglobin knock-out mouse [39–41]. Despite the elegance of the proposed allosteric mechanism [38] to regulate NO production based on local oxygen concentrations, this mechanism suffers from a problem also seen with the SNO-Hb model: how does the extremely short-lived NO molecule diffuse from the hemoglobin-rich environment of the red cell to the underlying smooth muscle without being consumed or scavenged in transit?

NO scavenging is another mechanism by which RBCs (and hemoglobin) may regulate NO signaling

Another mechanism, that may function in conjunction with (or instead of) those described above, is the scavenging of NO by Hb. With this mechanism, Hb either free in plasma (a consequence of RBC hemolysis), encapsulated in RBC-derived hemoglobin-containing microparticles (MP), or in intact RBCs (less likely, particularly since RBCs circulate in the middle of the blood stream away from the endothelium) consumes NO produced by endothelial cells, reducing NO levels and thus inhibiting the vasodilatory response [42, 43]. This mechanism is supported by studies in sickle cell disease and others that show that plasma free Hb can scavenge NO, reducing its bioavailability and causing clinical sequelae [44, 45]. Free hemoglobin increases as red cells breakdown during blood storage, suggesting that transfusion of long-storage age RBCs may lead to a bolus infusion of NO-consuming hemoglobin. Furthermore, red cell breakdown also leads to the formation of MP. Unlike intact red cells, MP can flow close to the endothelium, bringing hemoglobin near the sites of NO synthesis, which may further accentuate NO scavenging after transfusion [46].

Insufficient NO Bio-Availability (INOBA) may underlie adverse effects associated with transfusion of older RBC units

Disruption of NO production/signaling mediated by stored RBCs could account for adverse effects ascribed to transfusions of stored RBCs. Interestingly, the INOBA hypothesis not only addresses this requirement, but also provides a role for recipient factors including endothelial function. This unifying hypothesis can be stated: *When the cumulative effects of RBC transfusions and recipient factors reduce local NO bioavailability to levels below a*

critical threshold, tissue perfusion is insufficient to meet metabolic demands leading to morbidity and mortality in the transfusion recipient.

The INOBA hypothesis postulates that adverse events after transfusion are more likely to occur when RBC-specific dysfunction that occurs with blood storage is combined with endothelial dysfunction, surpassing a threshold where NO bioavailability is insufficient to produce appropriate vasodilation, causing reduced blood flow to vital organs, and morbidity/mortality. As described below, the INOBA hypothesis can be tested in both reductionist in vitro systems as well as in patients and healthy volunteers receiving transfusions.

Effects of stored RBCs on vasoreactivity *in vitro*

In the first series of studies we prepared leukoreduced AS-3 pRBC units from volunteer donors and stored the units for up to 42 days under standard conditions. At selected times we sampled the blood and performed aortic ring assays to determine the effects of blood storage on the ability of ACh-stimulated NO release from the endothelium to stimulate smooth muscle relaxation in rat aortic sections. These results showed that the presence of fresh pRBCs, even at low final hematocrits (~ 1%) could interfere with NO-mediated vasodilation in rat aortic rings. As compared to fresh RBCs, RBCs stored 3–14 days produced a significant shift in the dose-response curve, with a reproducible 50% greater inhibition of relaxation. The inhibitory activity further increased with extended storage: RBCs stored 28–42 days almost completely eliminated ACh-stimulated NO-mediate vasodilation. If the aortic rings were pre-relaxed first, subsequent addition of RBCs caused contraction indicating that the stored RBCs actively antagonize vasodilation.

One hypothesis to explain these effects is that stored blood hemolyzes, releasing free Hb that is known to scavenge NO. To address this possibility, we centrifuged the blood and removed the supernatant prior to adding the RBCs to the organ baths (mimicking a “volume reduction process” in the blood bank) or washed the cells 3 times with a excess of saline prior to testing the RBCs (mimicking blood component “washing”). The washing approach partially reversed the effects of stored RBCs. Washing 28–42 day RBCs prior to testing slightly abrogated their inhibitory effect on vasodilation, suggesting that some of the observed inhibition may be prevented by removing plasma free Hb, or other factors from the plasma that are released during RBC storage. It should be noted that MP are unlikely to be quantitatively removed by this washing approach; thus, residual MP remaining after washing may account for the residual inhibitory effects of washed aged RBC units. In addition, the membrane-permeable free radical scavenger Tempol (1mM) also partially blocked the inhibitory effects of stored RBCs, indicating that reactive oxygen species (ROS) may be involved in this effect.

Samples taken in parallel were tested for ATP and 2,3-DPG, and results were consistent with previously published reports.

Effects of stored RBCs on *in vivo* responsiveness to NO release

We sought to perform an in vivo equivalent of the aortic ring studies described above. These investigations are based on the hypothesis that while stored RBCs may have an effect on

vascular physiology, this effect may be exacerbated if transfusion recipients have underlying vascular dysfunction (resulting, for example, from atherosclerotic or diabetic vascular disease). These studies were approved by the IRB. Volunteer hospitalized patients for whom a transfusion had been ordered were randomized to receive either a fresh (<10 days old) or aged (>21 days old) blood transfusion. Endothelial function was assessed by flow mediated dilatation (FMD) assays of the brachial artery prior, during, 1 hour after, and the next day following transfusion. Twenty subjects have been studied to date. The mean age was 60±20, (SD), 50% were male, 7 had a cancer diagnosis, 6 required transfusion due to surgical blood loss, 3 due to chemotherapy, 2 due to GI bleeding and 3 due to other medical disease. Mean FMD prior to transfusion was 5.0±1.5% (SEM). During transfusion, FMD (corrected for shear rate) tended to increase ($p=0.056$) and returned toward baseline after an hour of transfusion and the following day. The pattern of change in those who received fresh and aged blood was not significantly different. Whereas FMD appeared to return to baseline or even lower after transfusion in those who received aged blood, it appeared to trend higher in those receiving fresh blood. Thus, there was a trend toward a difference in the endothelial functional response in those receiving fresh versus aged blood, although we plan to enroll a total of 30 subjects (based on power analysis) prior to final statistical analysis. The subjects enrolled to date have been heterogeneous in terms of baseline endothelial dysfunction, pre-transfusion hematocrits, medications received, time of day, and meal schedules (all factors that influence FMD).[47]

Global metabolic screening for biochemical changes in RBCs with storage

With few exceptions, the biochemical changes that occur in RBCs during extended storage, and which may underlie adverse effects of stored RBCs, are not known. We undertook a comprehensive metabolic screen to identify biochemical metabolites whose concentrations changed significantly during RBC storage as a first step to dissect the mechanistic aspects of the RBC storage lesion. 6 age-, race- and sex-matched volunteers donated whole blood units (500 mL+/−50 mL) that were processed and stored as AS-3 pRBCs. At defined times during storage (0, 3, 7, 14, 28, 42 days) aliquots were removed and snap frozen. All 36 samples were simultaneously extracted and analyzed by GC/MS and LC/MS/MS. Biochemicals were identified by comparisons to a library of mass spectra derived from purified standards. Proprietary visualization and interpretation software was used to confirm the consistency of peak identification between samples. A general linear statistical model analysis was applied to log-transformed data incorporating donor and time as main effects.

185 identified biochemicals were quantitated in RBCs. As internal confirmation of the methods 2,3-DPG declined to undetectable levels by storage day 14 while lactate increased progressively during storage, as previously documented. By 3 days of storage, 25 biochemicals had increased significantly ($p\leq0.05$) in concentration compared to day 0, while 6 had decreased significantly. By 42 days, 56 markers increased and 47 decreased significantly. Using a general linear model, 90 biochemicals changed in association with storage time. Intriguingly, changes in 132 markers were associated with donor-specific factors. Profound metabolic changes were identified in the Rapoport-Luebering shunt, glycolysis, glutathione synthesis, branched-chain amino acid metabolism, and adenine catabolism. Several biochemicals increased significantly only after 14 days of storage,

possibly in response to 2,3 DPG depletion, and may represent useful markers of RBC aging during storage. Interestingly, although there was a time-dependent decrease in glutathione levels, other biochemical data indicate that redox homeostasis in the RBCs was generally maintained over the storage period.

Summary and future directions

While RBC transfusion is a very commonly used clinical therapy, it can be associated with adverse events including the possibility of worse outcomes in patients transfused with older RBC units. The INOBA hypothesis postulates that some of the adverse events are due to time- and storage condition-dependant changes in NO production (and/or increased scavenging) by RBCs coupled with endothelial dysfunction in transfusion recipients, leading to reductions in blood flow, and thus oxygen delivery, to end organs. Our studies of this hypothesis have lead to the following observations: 1) older RBC units can block NO-mediated vasodilation in rat aortic ring models; 2) the mechanisms may involve mediators released into the supernatant of the blood component and/or ROS that are present at higher levels in older units; 3) there is a trend toward confirmatory findings in hospitalized patients treated by transfusion, although the studies are still ongoing; 4) global metabolic profiling has suggested some new targets to investigate regarding the RBC storage lesion and potential mediators that could impair vascular function. These results may be translated into improved preservative/storage solutions for RBC units and to better methods to match blood donors to recipients based on metabolic parameters.

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