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Storage Lesion. Role of Red Cell Breakdown

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Abstract

As stored blood ages intraerythrocytic energy sources are depleted resulting in reduced structural integrity of the membrane. Thus, stored red cells become less deformable and more fragile as they age. This fragility leads to release of cell-free hemoglobin and formation of microparticles, sub-micron hemoglobin-containing vesicles. Upon transfusion, it is likely that additional hemolysis and microparticle formation occurs due to breakdown of fragile red blood cells. Release of cell-free hemoglobin and microparticles leads to increased consumption of nitric oxide (NO), an important signaling molecule that modulates blood flow, and may promote inflammation. Stored blood may also be deficient in recently discovered blood nitric oxide synthase activity. We hypothesize that these factors play a potential role in the blood storage lesion.

The Storage lesion

The storage lesion refers to changes in red cells during storage. Over time, glucose in stored blood is consumed, levels of 2,3-diphosphoglycerate (DPG) and ATP decrease, while potassium levels increase.[1-9] As a result, there is red cell membrane loss during storage that leads to substantial changes in rheological properties.[10-15] This loss of red cell integrity results in hemolysis and formation of microparticles[4,16-19] that may contribute to complications associated with transfusion. Several studies have found that transfusions using older blood are associated with adverse clinical outcomes[20-27]. It should be noted, however, that others have not found these types of associations.[9,28-34] Although the impact of transfusion of old blood is a matter of debate, the fact that transfusion represents one of the most common medical therapies suggests that further large-scale study of its impact is warranted, and that the mechanisms involved should be elucidated. In this mini-review, we suggest how disturbance of nitric oxide homeostasis and its consequences may underlie, to some extent, the storage lesion. An overview of the mechanisms we propose to be involved are shown in Figure 1.[35]

Nitric Oxide

Nitric oxide (NO) is a neutral, radical molecule that has several important roles in physiological signaling. NO is the endothelial-derived relaxing factor (EDRF); it is made in

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endothelial cells and plays a major role in controlling blood flow by effecting smooth muscle relaxation adjacent to the blood vessels.[36-39] It is made by endothelial nitric oxide synthase (eNOS) from arginine and diffuses to the smooth muscle where it activates soluble guanylyl cyclase to produce cGMP, initiating a signaling cascade leading to vasodilation. In addition, via this ENOS, the two other isoforms (inducible NOS and neuronal NOS), or other mechanisms of formation, it plays a role in homeostasis through inhibition of platelet aggregation, acts a toxic agent in host defense, decreases expression of adhesion molecules, and has anti-oxidant properties.[40-42] More recently, a red blood cell NOS has been discovered.[43] Importantly then, NO is seen to contribute to many functions that could be linked to the storage lesion including blood flow, inflammation, and thrombosis.

Nitric Oxide Scavenging by Hemoglobin

Nitric oxide reacts with hemoglobin in a reaction that is rate-limited by diffusion to the heme group within hemoglobin,[44-46]

$$\mathrm{HbFe^{II}{-}O_2{+}NO \rightarrow HbFe^{III}{+}NO_3^-}. \quad (1)$$

This dioxygenation reaction occurs when oxygenated hemoglobin reacts with NO to form methemoglobin (Fe^{III}) and nitrate, and effectively destroys NO activity. Nitric oxide can also bind to a ferrous, vacant heme but once it comes off it is likely to undergo dioxygenation, so this pathway would be a poor mechanism in itself to preserve NO bioactivity. In 1994, Lancaster pointed out that given the rate of the dioxygenation reaction and the large amount of hemoglobin in blood, NO could not possibly act as the EDRF; it would undergo too much dioxygenation.[47] It has been subsequently determined, however, that the degree of NO dioxygenation that one would predict based on the amount of hemoglobin present is much less due to the fact that NO reacts with red cell encapsulated hemoglobin much more slowly than when the hemoglobin is free in solution or plasma.[48-58] In vitro measurements where NO is mixed with red cells or hemoglobin show that red cells react up to 1000 times slower than free hemoglobin, [48,49,58] primarily due to the reaction becoming rate-limited by the time it takes for NO to diffuse to the red cell through “an unstirred layer.”[58] In addition, a finite permeability of the red cell membrane to NO may play a role.[53] In vivo, a major contribution of reduced NO scavenging by red cells is thought to be due to the cell-free zone, where blood flow leads to a pressure gradient that pushes red cells to the center of vessels and away from the endothelium where NO is made.[50,52,59] Regardless of the relative contribution of these mechanisms, it is important to point out that they all breakdown upon hemolysis.[60]

Pathology associated with RBC breakdown

Given the many important functions of NO, it is not surprising that diminished NO bioavailability contributes to pathology in many diseases. Many of these, including atherosclerosis, obesity, diabetes, peripheral artery disease and coronary artery disease in general, result from endothelial dysfunction that is often due to reduced NO synthesis by NOS.[61-65] Besides reduced production, NO bioavailability can also be reduced by increased consumption; one way for this reduction to occur results from scavenging by cell-free hemoglobin that is released upon hemolysis.[66] As noted above, cell-free hemoglobin reacts with NO much faster than that encapsulated in a red cell. Nitric oxide scavenging has been a major contributor to pathological consequences of many blood substitutes that involve hemoglobin-based oxygen carriers.[67-70] In these cases millimolar amounts of hemoglobin were infused. One might think that in other conditions, such as hemolytic anemias like sickle cell disease, the amount of cell-free hemoglobin present is too low to substantially

affect NO bioavailabilty in the background of red cell encapsulated hemoglobin (normally about 10 mM in heme). However, in studies initially performed in patients with sickle cell disease, we found that the presence of even low micromolar amounts of cell-free hemoglobin in patients with sickle cell disease results in diminished blood flow response from NO.[71] That small concentrations of cell-free hemoglobin can affect blood-flow and have pathological consequences was subsequently confirmed in a canine hemolysis model,[72] and computational simulations suggest that as little as one micromolar cell-free hemoglobin (concentration in terms of heme, so in fact 0.25 micromolar hemoglobin tetramer) can substantially reduce NO bioavailabilty[73]. There is increasing evidence from transgenic animals, large animal and human epidemiological studies supporting a role for hemolysis in the pathobiology of sickle cell disease and other hemolytic anemias.[72,74-80] Despite this evidence, the fact that micromolar amounts of cell-free hemoglobin can affect NO bioavailability to an extent to which pathological consequences occur is not universally accepted.[81] Although we recognize that the extent of the impact of low amounts of hemolysis remains controversial, we feel the evidence strongly suggests the impact is substantial,[80] and that continuing research on this front is necessary. Moreover, the observation that larger amounts of hemolysis (resulting in tens of micromolar hemoglobin) leads to pathological consequences is not contested.[81]

Red cell breakdown and the storage lesion

Loss of red cell integrity during storage results in hemolysis and formation of microparticles.[4,16-19] There have been several studies that documented hemolysis as a function of time during storage. The levels of extracellular hemoglobin reported in the literature range from 28 μM (in heme) after 35 days of storage in citrate phosphate dextrose adenine (CPDA)[4] to 80 μM after 50 days of storage[17]. We recently reported similar findings.[82] Transfusion of just one unit of blood with this much hemolysis would result in plasma levels exceeding those of steady state sickle cell disease. As expected, we showed that NO consumption by the non-erythrocytic (plasma) fraction of older stored blood is dramatically greater than that from blood stored only one week and NO consumption is directly proportional to the extent of NO consumption.[82]

In addition to release of cell-free molecular hemoglobin, red cell breakdown leads to formation of hemoglobin-containing microparticles. In fact, when measuring cell-free hemoglobin in blood, no efforts are usually taken to separate microparticles from cell-free hemoglobin. In fact, in at least one case, the majority of hemoglobin in the supernatant after sedimentation of stored blood was found to be in the form of microparticles.[18] Due to their small size, we propose that microparticles will scavenge NO to a similar extent as cell-free hemoglobin. The extent to which external diffusion of NO to hemoglobin reduces the rate of NO scavenging depends on the average distance between vesicle (red blood cells or microparticles). Taking a red cell with an equivalent spherical radius of 3 μm and a microparticle with a radius of 0.075 μm, the time for NO to diffuse to the red cell would be roughly 2000 times longer on the average than to the microparticle.* In addition, due to their small size, it is unlikely that shear stress will result in removing microparticles from the cell-free zone. Any reduction in NO permeability that may exist in red blood cells could be absent or diminished in microparticles as the physical membrane barrier to NO entry is thought to be due to the underlying spectrin and other cytoskeleton proteins[83] and some of these are absent in microparticles [19]. Thus, all three mechanisms that contribute to reduced NO scavenging by red blood cells compared to cell-free hemoglobin are likely to be absent

*The time for diffusion is taken as x^2/D where x is the distance between the vesicles and D is the diffusion constant. The distance is given as, where r is the radius and Hct is the hematocrit. Here it is assumed that the concentration of hemoglobin inside red cells is the same as in microparticles. The calculation is performed for equivalent hematocrits.

or lessened for microparticles. Importantly, the effects of microparticles on NO bioavailability could be more extreme than those of cell-free hemoglobin as microparticles are not cleared by haptoglobin.

In addition to reductions in NO bioavailability that could result due to increased NO scavenging by cell-free hemoglobin and microparticles, NO bioavailability may be reduced due to decreased red cell or blood NOS. This reduction could be due to oxidative stress (oxidation of tetrahydrobiopterin and arginine deficiency) with red cell storage that is associated with functional uncoupling of the NOS protein. NO synthase uncoupling results in formation of superoxide from the NOS rather than NO. It is also possible that NO bioavailability is reduced due to infusion of stored, red cells from diminished NOS activity that is otherwise present in other blood cells. Future work is needed to explore this possibility.

Effects of Inflammation

Experimental evidence from murine studies suggests that transfusion of stored red cells can augment inflammation by various mechanisms.[84,85] We have previously shown that transfusion of red cells increased both systemic and lung inflammatory responses in endotoxemic mice to promote chemokine-mediated neutrophil accumulation and lung injury that were storage- and red cell-dependent.[85] Hod and colleagues have also shown that transfusion of stored red cells elicits systemic inflammatory cytokine responses related to the ingestion of membrane-encapsulated hemoglobin (Hb) by the mononuclear phagocyte system.[84] While human red cell units (PRBC) show increased hemolysis[86] and microparticle formation[87] with storage duration, little is known regarding the effects of free Hb and membrane-encapsulated Hb in the form of microparticles in altering inflammation in humans following transfusion. Our recent findings show that red cell microparticles in banked blood units demonstrate inflammatory chemokine binding and release ligand upon interaction with platelets in vitro.[87] Whether direct interactions between red cell microparticles and platelets actually occur to propagate or amplify inflammation in vivo is not currently known, although, insights from sickle cell disease (a disease characterized by hemolysis, reduced NO bioavailability, and inflammation) invoke a relationship between red cell microparticles and platelets.

Indeed, sickle cell disease shows increased circulating red cell microparticles expressing surface phosphatidylserine with thrombin generating potential[88] that trigger activation of the alternative complement pathway[89]. Platelet activation, an increasingly recognized component and propagator of persistent inflammation as observed in rheumatoid arthritis[90] or acute lung injury/acute respiratory distress syndrome[91], is negatively regulated by endothelial NO, ADPase, and PGI_2. Thus, in diseases of reduced NO bioavailability such as in hemolytic states, platelet activation is a characteristic feature as it has been observed for sickle cell disease[92] and paroxysmal nocturnal hemoglobinuria[93]. We suggest that transfusion of stored red blood may elicit a state of reduced NO bioavailability through the release of cell free Hb and microparticles and contribute to persistent inflammation and injury in susceptible hosts. However, other components of red cell breakdown may be involved to induce or amplify inflammation. Heme and free iron, the breakdown products of hemoglobin, may be involved in not only inflammation but also elicit pro-oxidant, cytotoxic effects.[84,86,94]

The epidemiological studies demonstrating an association between red cell transfusion and potentially worse outcomes were conducted in patients with underlying traumatic injury, the critically ill[95], or following cardiac surgery requiring support with cardiopulmonary bypass pump[27]. These findings invite the possibility that an underlying systemic inflammatory

response may predispose individuals to transfusion-associated complications. The only completed prospective, randomized controlled trial examining red cell transfusion and outcomes in the critically ill was an efficacy trial of transfusion using a restrictive versus liberal strategy[95]. In this study, a subset of patients, those younger and less ill in the ICU with stable anemia, showed a possibly superior outcome with less blood transfusions[95]. However, the etiology of this association is still unclear and whether hemolysis-related breakdown of the red cell during storage perpetuates ongoing inflammation following transfusion remains to be determined. In a recent study, Larsen and colleagues showed the deleterious effects of hemolysis during severe sepsis syndrome and implicated a central role for free heme in promoting death in patients[96]. Low serum concentrations of hemopexin, the counter-regulatory molecule that binds to free heme, predicted multiple organ failure and death in septic shock patients[96]. Thus, hemolysis is an increasingly recognized feature of severe sepsis, either directly caused by blood-borne pathogens or through microangiopathic hemolytic anemia from disseminated intravascular coagulation that often accompanies severe sepsis. Red cell transfusion may exacerbate inflammation in susceptible hosts through hemolysis and contribute to microvascular perturbations by reducing NO bioavailabiity, promoting platelet activation, inducing pro-oxidant effects and cytotoxicity through release of red cell breakdown products.

Planned Approach

Figure 2 summarizes our general hypotheses and approach. In Aim 1 of our proposed work we plan to examine the effects of blood storage on red cell integrity and how this affects NO bioavailabilty. We will look at NO deformability and fragility as a function of time during storage while monitoring hemolysis and microparticle formation. We will test our hypothesis that microparticles scavenge NO similarly to cell-free hemoglobin using time-resolved absorption spectroscopies and computational modeling in a similar manner to the approaches we have used in the past.[58,73,97] As red cell fragility increases during storage, we hypothesize that additional red cell breakdown occurs during and after transfusion. We will then test this hypothesis and the effects on NO bioavailabilty by examining blood flow and other physiological parameters upon infusion of older vs newer stored blood in humans and animals using methods similar to those previously reported.[71,72]

In Aim 2 of our proposed work, we plan to examine the role of the red cell NOS in the storage lesion. We will first examine the functionality using NOS knockout mice specifically in the endothelium or blood. The source of the blood NOS will be determined by immunodepleting platelets and removing leukocytes. After determining the contribution of blood NOS to NO homeostasis, we will examine the extent to which this activity is reduced in blood storage.

The last phase of our project focuses on therapeutics. We aim to explore mechanisms to both decrease NO scavenging and increase its production upon and post transfusion. To decrease NO scavenging we will explore preservation solutions with additives that decrease hemolysis while also examining additives that can neutralize the NO scavenging ability of cell-free Hb by preferential oxidation. Increased NO production may be accomplished by inclusion of additives that can be converted to nitric oxide in the blood such as nitrite[98], and compounds that may increase blood NOS activity will also be explored. By increasing NO bioavailaibility, inflammatory consequences of transfusion of old, stored blood will also be reduced.

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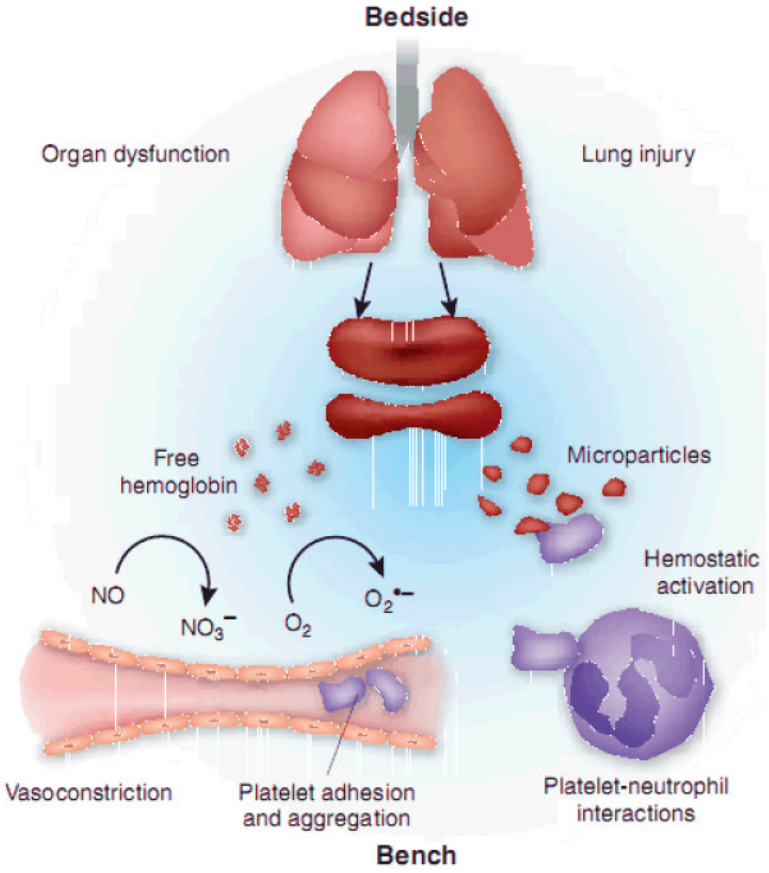

Figure 1.

Proposed mechanisms contributing to the storage lesion. Reprinted by permission from Macmillan Publishers Ltd:Nature Medicine (16(4):381-2), copyright (2010). Red cell breakdown leads to release of cell-free hemoglobin and red cell microparticles. These scavenge NO which leads to vasoconstriction, platelet activation and adhesion, and inflammatory pathways.

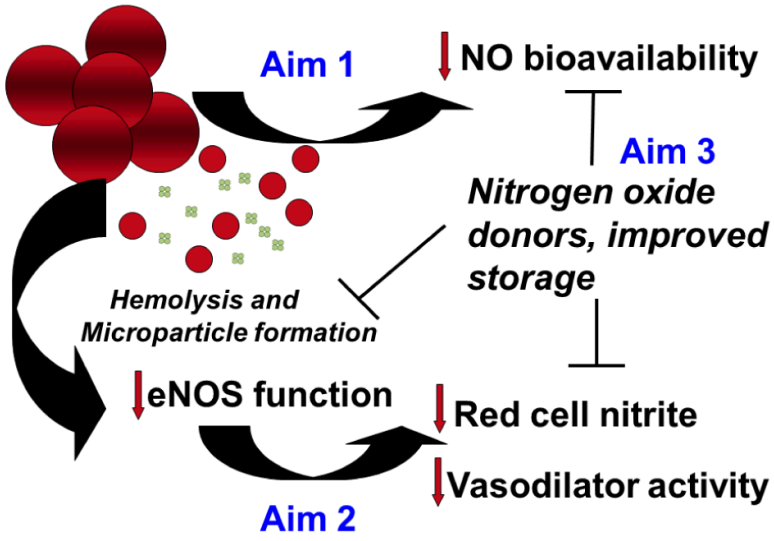

Figure 2.
General hypothesis and approach. Loss of red cell integrity during storage or upon transfusion (to be studied in Aim 1) results in release of free hemoglobin and red cell microparticles which scavenge nitric oxide leading to deleterious effects including susceptibility to platelet activation, inflammation, and poor control of blood flow. This loss of NO bioavailability may also be exacerbated by loss of red cell NOS activity, to be studied as part of Aim 2. In order to counteract these effects, in Aim 3, we will explore ways to reduce red cell breakdown during storage, reduce NO scavenging when there is red cell breakdown, and compensate for loss of NO activity using various donor substances.