Duration of red blood cell storage and inflammatory marker generation

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Abstract

Red blood cell (RBC) transfusion is a life-saving treatment for several pathologies. RBCs for transfusion are stored refrigerated in a preservative solution, which extends their shelf-life for up to 42 days. During storage, the RBCs endure abundant physicochemical changes, named RBC storage lesions, which affect the overall quality standard, the functional integrity and *in vivo* survival of the transfused RBCs. Some of the changes occurring in the early stages of the storage period (for approximately two weeks) are reversible but become irreversible later on as the storage is extended. In this review, we aim to decipher the duration of RBC storage and inflammatory marker generation. This phenomenon is included as one of the causes of transfusion-related immunomodulation (TRIM), an emerging concept developed to potentially elucidate numerous clinical observations that suggest that RBC transfusion is associated with increased inflammatory events or effects with clinical consequence.

Keywords: red blood cell, inflammation, storage.

Introduction

An emerging transfusion community interest concerns the ability for blood transfusion to modulate the immune system of recipients[1-5]. Transfusion-related immunomodulation (TRIM) has been implicated in adverse clinical outcomes[6,7]. The present "gold standard" for maximum shelf-life of red blood cells (RBCs) is six weeks (42 days)[8]. Even if storage of blood at 4 °C is proposed to slow down RBC metabolism and the accumulation of soluble factors, amongst other main pointers of quality and safety of stored blood, it does not stop the overall process often referred to as storage lesions. Storage lesions have been extensively researched[9-11]. Classically, storage lesions in RBCs are categorised as either biochemical or rheology changes. However, inflammatory markers are poorly evaluated in the literature. Even if the rise in inflammatory markers observed in transfusion-related immunomodulation[12] may be improved through leucocyte reduction, several immunomodulatory factors stored in RBCs participate in inflammation[13-16]. The potential of other modes of processing for creating storage lesions, e.g. through degradation of nucleic acids to enhance pathogen safety of red cell concentrates, deserves to be fully established. Therefore, a goal of the current review is to summarise the biochemical or rheology changes occurring in relation to the duration and processing of RBC storage, focusing on the generation of inflammatory markers.

Biomechanical changes in stored RBCs

During the typical storage conditions of blood, abundant biochemical alterations take place[17-21]. Such changes primarily refer to the generation of aggregates and biochemical debris that accumulate in the supernatant during prolonged storage of RBCs (Figure 1). Biomechanical storage lesions occur in the cytoskeleton and cellular membranes, defined as membrane and cytoskeleton protein oxidation, membrane phospholipid loss, abnormal rearrangement of membrane phospholipids, and morphological changes[22,23]. As an example, increased storage induces an increased level of extracellular potassium, lactate, and a decrease of sodium and glucose which leads to acidosis, particularly obvious by the end of the second week of storage (approximately after day [d]14). Extended RBC storage is also identified, resulting in reduced levels of ATP and 2,3-diphosphoglycerate (2,3-DPG) (Figure 1). Taken together, the above events are useful markers that could be indicative of a storage lesion in a given stored unit of blood[17,18,24-28]. Moreover, stored RBCs reveal functional changes to RBCs during storage, and particularly reduced deformability and increased rigidity, which may affect the flow of transfused RBCs through micro-capillaries, cell-to-cell aggregation, and adhesion to endothelial cells. The decreased levels of 2,3-DPG

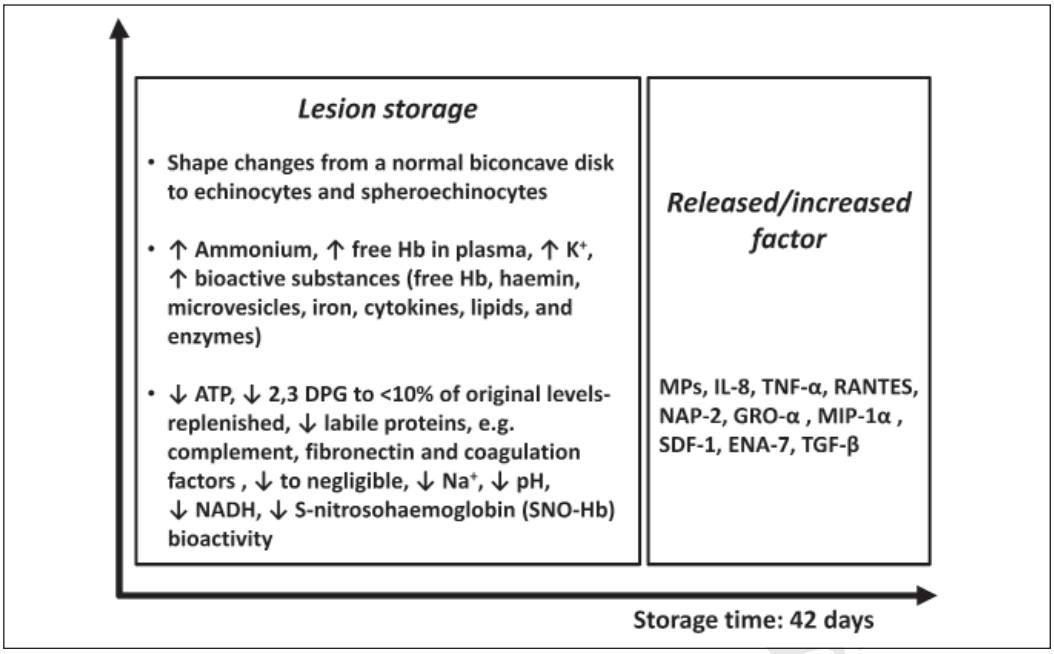

Figure 1 - Red blood cell product storage: lesion storage and biological response modifier release. Hb: haemoglobin; ATP: adenosine triphosphate; 2,3-DPG: 2,3-diphosphoglycerate; NADH: nicotinamide adenine dinucleotide; MPs: microparticles; IL-8: interleukine 8; TNF: tumour necrosis factor; RANTES: regulated on activation, normal T cell expressed and secreted; NAP: neutrophil-activating peptide; GRO: growth-related oncogene; MIP: macrophage inflammatory proteins; SDF: stromal cell-derived factor; ENA-7: epithelial neutrophil-activating protein 7; TGF: transforming growth factor.

increase the affinity of haemoglobin to oxygen, which results in reduced oxygen delivery[26,29-32]. D'Alessandro *et al*. performed comprehensive metabolomics and quantitative tracing metabolic experiments that revealed that mature RBCs can metabolise substrates other than glucose, such as citrate. This observation was highly relevant to Transfusion Medicine, influencing particularly the process of RBC preparation and the formulation of novel additives[33,34].

Red blood cell-derived lipids during storage

During RBC storage, the implications of RBC membrane breakdown and release of potentially harmful bioactive lipids could be quantified, and contributed to the quality assessment of RBC[18,22,26,28,35,36]. The damaging oxidative storage effects on the RBC lipid membrane have numerous functional implications. As an example, increasing oxidative stress on stored RBC is a determining primer to increase phosphatidylserine translocation to the RBC surface membrane[13,37,38]. This phenomenon could mediate adhesion of transfused RBC to endothelial cells and induce the shedding and accumulation of bioactive microvesicles (Mvs)[26,35,39,40]. These bioactive lipids have been implicated in transfusion-related acute lung injury (TRALI) pathogenesis due, mainly, to their[26] polymorphonuclear neutrophil (PMN) priming abilities. In 2011, Silliman *et al*. explored the effect of infusing d1 or d42 lipids that were isolated from healthy human donor into lipopolysaccharide (LPS) or saline-treated male rats[41]. The study evaluated the PMN-priming capacity of the lipids as well as the effect of their infusion on acute lung injury as part of the "two-hit" TRALI model[41-44].

Red blood cell-derived microvesicles during storage

One implication of RBC membrane failure could be the release of potentially injurious bioactive microvesicles[26,35,39,40]. Phospholipids of the membrane are released during the microvesiculation process, which was first defined by Rumsby *et al*. in 1977[45]. Microvesiculation is a cellular process that leads to intracellular communication and cell apoptosis. Membrane lipid oxidation and cytoskeletal protein oxidation can dislocate the plasma membrane and cytoskeleton[10,22,46-48]. This disturbance could be a key phenomenon contributing to the increased release, during the RBC storage, and accumulation of bioactive microvesicles. Recently, several studies investigating the composition of the RBC membrane, particularly during microvesiculation, revealed a significant increase of RBC-derived microparticles as storage exceeded day 42[22,40,49-53]. Moreover, these RBC membrane-derived microvesicles present a significant

physiological and inflammatory pathophysiological process, principally involving vascular dysfunction. However, there is little clinical and *in vivo* evidence linking the effects of microvesicles during transfusion. As defined and summarised elegantly by Michel Prudent *et al*.[54], analysis of *in vitro* data highlights the presence of reversible and irreversible storage lesions demonstrating that RBCs exhibit two limits during storage: one around two weeks and another one around four weeks of storage. Microvesiculations could be considered irreversible storage lesions as degradation/oxidation of proteins, protein aggregations, protein activation, such as the proteasome 20S, shape change and deformability[54].

Reisz *et al*.[55] hypothesised that routine storage of erythrocyte concentrates promotes metabolic modulation of stored RBCs by targeting functional thiol residues of GAPDH and identified ex vivo functionally relevant reversible and irreversible (sulfinic acid; Cys to dehydroalanine) oxidations of GAPDH without exogenous supplementation of excess pro-oxidant compounds in clinically relevant blood products. Palia *et al*. propose that 8 compounds (lactic acid, nicotinamide, 5-oxoproline, xanthine, hypoxanthine, glucose, malic acid, and adenine) strongly correlate with the metabolic age of packed RBCs, and can be prospectively validated as biomarkers of the RBC metabolic lesion[56]. In the same way, Wither *et al*. show that several of the oxidised residues identified play well-established roles in haeme iron co-ordination, 2,3-diphosphoglycerate binding, and nitric oxide homeostasis[57].

Recently, Straat *et al*. hypothesised that extracellular vesicles in RBC products during storage contribute to a pro-inflammatory host response in recipients, which is related both to their amount as well as to the storage duration[58]. The authors clearly demonstrate that incubation of whole blood with both fresh and stored supernatant containing extracellular vesicles induced a strong host response with production of tumour necrosis factor (TNF), interleukin(IL)-6 and IL-8. Moreover, once supernatant was depleted from extracellular vesicles, this host response was completely abolished.

Immunomodulatoty factors in stored red blood cell concentrates

The accumulation of immunomodulatoty factors in stored RBC concentrates has been implicated as a potential cause of transfusion reactions associated with the use of such products[8,9,25,26,29,32,59-61]. Data suggest high concentrations of TNF, IL-1 and IL-6 in random donor RBC concentrates[62-64], and an association between the period of storage of blood components and the risk of developing acute transfusion reactions to platelet concentrates (PCs) and RBCs[3,22,53,65,66].

Cytokine could orchestrate a systemic inflammatory response. Kristiansson *et al*. report that plasma-soluble immunomodulatory factor concentration increases on the first post-operative day after major surgical trauma, the Author observing a relationship between the amount of RBC concentrates transfused perioperatively and post-operative systemic plasma IL-6 concentration[67]. The interaction between these cytokines is complex, each being able to up-regulate and down-regulate their own expression as well as that of the other cytokines. Nevertheless, the cytokine content may reflect the presence of leucocytes, in other words, an association with the initial amounts of leucocytes in RBC concentrates. Leucoreduction may significantly decrease febrile non-haemolytic transfusion reactions and may decrease cardiopulmonary transfusion reactions (TRALI and transfusion-associated circulatory overload)[3,8,41,68,69]. Presumably, this ensues through reduced levels of bioactive lipids and soluble CD40L in leucoreduced RBCs, which would have been produced by leucocytes, had they not been removed from the blood product. Donor leucocytes release cytokines and lipid factors able to activate neutrophils in a time-dependent course during RBC storage[70]. Pre-storage leucoreduction decreases the release of metabolites and cellular components into the RBC product. TNF-α, a multipotent cytokine, perfoms several immunological functions and is involved in maintaining the homeostasis of the immune system. It is known, that TNF-α, like IL-1 and IL-6, suppresses erythropoiesis by direct inhibitory effects on bone marrow RBC production[71,72]. Moreover, TNF-α, being an endogenous pyrogen, is able to induce fever, apoptotic cell death, cachexia, inflammation and to inhibit tumourigenesis and viral replication, and respond to sepsis *via* IL-1 and IL-6 producing cells. IL-6 is an important mediator of fever and of the acute phase response. It is capable of crossing the blood-brain barrier and initiating synthesis of prostaglandin E2 (PGE2) in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilisation that leads to increased body temperature[73,74].

Therefore, Muylle *et al*. demonstrated a relationship between TNF-α and IL-6 levels and febrile transfusion reactions[75]. IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils, but also other granulocytes, causing them to migrate toward the site of infection. IL-8 is also known to be a potent promoter of angiogenesis[76,77]. Cases of TRALI have been consistently associated with high levels of cytokines/chemokines, specifically IL-8[78], which has been shown to promote assembly of cholesterol-enriched microdomains or so-called lipid rafts on human neutrophils[79]. Moreover,

McKenzie *et al.*, proposed that antibodies bind monocytes (instead of neutrophils), leading to increased IL-8, which results in neutrophilic pulmonary infiltrate with subsequent TRALI[80]. IL-1 is intensely produced by tissue macrophages, monocytes, fibroblasts, and dendritic cells, but is also expressed by B lymphocytes, natural killer (NK) cells and epithelial cells[81]. They form an important part of the inflammatory response of the body against infection. These cytokines increase the expression of adhesion factors on endothelial cells to enable transmigration (also called diapedesis) of immunocompetent cells, such as phagocytes, lymphocytes and others, to sites of infection[82,83]. They also affect the activity of the hypothalamus, the thermoregulatory centre, which leads to fever. IL-1, appears to be associated with the occurrence of febrile non-haemolytic transfusion reaction (FNHTR) and other transfusion reactions, such as urticaria, hypotension, anaphylaxis, or TRALI[62,84,85]. The main critical factors in determining the accumulation of cytokines are considered to be the WBC content and the age of the blood component; moreover, accumulation is heterogeneous and there is a large inter-individual variation related to donors' hereditary and social habits[86]. Interestingly, the cytokines/chemokines in RBCs might be caused by haemolysis of the cells. This could be a comparable phenomenon to that detected in haemolytic transfusion reactions *in vitro* where there are high concentrations of cytokines/chemokines[87,88].

In the majority of the cases, antibodies against HLAs and/or human neutrophil antigen (HNA) present in the transfused product are thought to be responsible for initiating TRALI. TRALI is assumed to result from two hits, the first hit being caused by the underlying clinical condition of the patient, whereas the second occurs when the antibodies or factors are transferred to the recipient during transfusion[89]. Peters *et al*. investigated 18 healthy male volunteers (aged 18-35 years) infused with LPS to induce systemic inflammatory response syndrome. Two hours later, each participant received either one unit of 2-day stored autologous RBCs, 35-day stored autologous RBCs, or an equal volume of saline. Every 2 hours up to 8 hours after LPS infusion, haemoglobin, haemolysis parameters, and iron parameters, including non-transferrin bound iron (NTBI), were measured. The author concluded that 35-day stored autologous RBCs do not cause haemolysis or increased levels of NTBI during human endotoxemia[90,91]. Production of cytokines/chemokines could originate from an activation of RBC contact with the storage bag system during the storage period, indicating that these storage lesions should also be considered for future evaluations. Foreign material may stimulate cytokine synthesis and release, though this may be less likely during storage at a temperature of 4 °C.

Recently[13], our group focused on the characterisation of stored RBC with regard to cytokine/chemokine content, and investigated the possible influence of storage time (Figure 1). Individual RBC concentrate (RBCC) supernatants were processed by double centrifugation at 2,600 *g* for ten minutes. Samples were kept frozen at −80 °C and shipped on dry ice to the sample-processing laboratory. Levels of soluble cytokines growth-related oncogene (GRO)-α, IL-16, epithelial-derived neutrophil-activating protein 78 (ENA-78), macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), stromal cell-derived factor 1 (SDF-1) and transforming growth factor (TGF) β1, 2, and 3 were measured in triplicate from aliquots using Luminex technology[92], and amounts were expressed in ng/RBC unit. Supernatants from RBCCs were collected over time and tested for the presence of a variety of soluble chemokines and cytokines. GRO-α, IL-16, ENA-78, MIP-1α, MCP-1, SDF-1 and TGF β1, 2, and 3 were selected on the basis of previous reports[25,59,93]. There were no differences in ENA, GROα, MIP1α, MCP1, SDF1, IL-16 or TGF β3, either between the groups or over time[13]. However, TGF β1 and TGF β2 decreased over time in both RBCC groups, with a significant difference at d0 *vs* d42[13]. The biological activities of TGF-β are not species-specific. TGF-β isotypes share many biological activities and their actions on cells tend to be qualitatively similar, though there are a few examples of distinct activities. In some systems, TGF-β3 appears to be more active than the other isotypes. TGF-β2 is the only variant that does not inhibit the growth of endothelial cells. TGF-β2 and TGF-β3, but not TGF-β1, inhibit the survival of cultured embryonic chick ciliary ganglionic neurons. TGF-β is the most potent known growth inhibitor of normal and transformed epithelial cells, endothelial cells, fibroblasts, neuronal cells, lymphoid cells and other haematopoietic cell types, hepatocytes, and keratinocytes. Although TGF-β inhibits endothelial cell growth, it promotes angiogenesis in several bioassays, though TGF-β may also inhibit angiogenesis under certain circumstances[94,95]. At higher concentrations, TGF-β stimulates the growth of these cells. TGF-β has mainly suppressive effects on the immune system by inhibiting the IL-2 dependent proliferation of T cells and B lymphocytes. TGF-β inhibits the proliferation of B lymphocytes and thymocytes induced by IL-2 and IL-1, respectively, and inhibits the maturation of B cells[96]. It also suppresses interferon-induced cytotoxic activity of NK cells, cytotoxic T-lymphocyte activity, and the proliferation of lymphokine-activated killer cell precursors. TGF-β also inhibits the synthesis of immunoglobulin (Ig)G and IgM by B lymphocytes and

stimulates the synthesis of IgA. TGF-β1 is the most potent known chemoattractant for neutrophils[97,98].

In this same report[13], we performed a functional assay of RBCC supernatant on EA.hy926 endothelial cells. The human endothelial hybrid cell line EA.hy926 was obtained by fusion of primary umbilical vein endothelial cells with the human lung carcinoma cell line A459/8 (ATCC #CRL-2922). EA.hy926 cells were cultured in Dulbecco's modified MEM medium supplemented with 10% foetal calf serum and 1% penicillin-streptomycin and then incubated at 37 °C in a humidified atmosphere in 5% CO_2 until the cell monolayer reached confluence. The cells were then exposed to stored RBCC supernatants. IL-6, sCD141 and sCD62E levels were measured by enzyme-linked immunosorbent assay. In this *in vitro* model, we investigated the bioactivity of soluble immunomodulatory factors in endothelial cells *in vitro*. We tested the potential bioactivities of the soluble immunomodulatory factors from RBCs over time using EA.hy926. There was a difference in the expression of marker molecules generally associated with EA.hy926 cell activation (CD141) during storage (d1-d42) and similar results were observed for the expression of soluble markers generally associated with EA.hy926 cell activation (sCD141, sCD62E and IL-6). This result, revealing the bioactivities of soluble immunomodulatory factors in the supernatant of RBCCs on endothelial cells *in vitro*, suggests a potential generation of inflammatory markers during RBC storage. Further investigation could be carried out to determine the nature of these inflammatory markers.

Conclusions and perspectives

The increase of inflammatory soluble markers observed in transfusion-related immunomodulation[12,99] is reduced by leucocyte reduction[60,63,100-103]. However, stored RBCs deliver large quantities of iron to the monocyte/macrophage system and could thus induce inflammation, and transfusion of older, stored RBCs, therefore, produces a proinflammatory response associated with increased iron levels in the liver, spleen, and kidney, and increased circulating levels of non-transferrin bound iron[3,104].

However, it is currently unclear whether the storage lesions simply reflect an accelerated ageing of the RBCs, or something else, and the consequences *in vivo* (after transfusion) remain largely unknown. In addition, RBC preparation and storage processes (cryopreserved for extended periods of time, cryoprotectant, plastic bag, etc.) could be investigated to quantify the RBC inflammatory soluble markers observed in transfusion-related immunomodulation. In this context, we note that several current concepts of intervention (reduction of biological response modifiers) focus on methods to attenuate the cytokine response[105-108]. Another characteristic could be considered concerning the transfusion-related immunomodulation, as for platelet component[2,109-112]. The variability in cytokine/chemokine concentration in RBCs could reveal a biological variation in donors with regard to their capacity to synthesise and release mediators. Moreover, differences in measured cytokine/chemokine concentrations associated with various commercial immunoassay kits should be considered and standardised in the future.

The clinical implications of transfusing RBCs containing cytokines/chemokines to critically-ill patients have not been clarified[113]. It might be that the cytokine content of transfused RBCs may fuel the systemic inflammatory reaction in conditions of trauma and infection, and simulate non-haemolytic transfusion reactions. Investigations have confirmed that stored RBC transfusions seem to up-regulate proinflammatory gene expression in the leucocytes of the transfusion recipient[114]. Moreover, McFaul *et al.*[115] observed an *in vivo* inflammatory effect of transfusion with an increasingly proinflammatory RBC function of storage.

Numerous studies have evaluated a wide variety of photosensitisers and alkylating agents as candidates for a pathogen inactivation process of RBC suspensions, but few with a focus on the inflammatory role of RBC. Consequently, future questions could probably investigate:

i) how this blood component differs from classical RBC components in use;
ii) what are the benefits to the patients of possible pathogen inactivation processes to be used for RBC suspensions;
iii) whether there a reduction in acute transfusion reactions in patients receiving future pathogen-reduced RBC (febrile non-haemolytic and/or allergic transfusion reactions, TRALI).

Future animal and clinical studies could probably increase knowledge of the effect of RBC storage on post-transfusion outcomes and TRALI, with a specific focus on the inflammatory soluble markers observed in TRIM. Moreover, knowledge of TRIM could help answer questions concerning a possible difference between fresh and old blood, and, more interestingly, the medical effects of transfusing stored RBCs. As elegantly defined in animal models by James C. Zimring[16], the question now is to understand the "induction of cytokine storm" on RBCs during storage, and the potential promotion of acute transfusion reactions.

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