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Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution ☆

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ABSTRACT

During blood banking, erythrocytes undergo storage lesions, altering or degrading their metabolism, rheological properties, and protein content. Carbonylation is a hallmark of protein oxidative lesions, thus of red blood cell oxidative stress. In order to improve global erythrocyte protein carbonylation assessment, subcellular fractionation has been established, allowing us to work on four different protein populations, namely soluble hemoglobin, hemoglobin-depleted soluble fraction, integral membrane and cytoskeleton membrane protein fractions. Carbonylation in erythrocyte-derived microparticles has also been investigated. Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (2,4-DNPH) and quantified by western blot analyses. In particular, carbonylation in the cytoskeletal membrane fraction increased remarkably between day 29 and day 43 ($P<0.01$). Moreover, protein carbonylation within microparticles released during storage showed a two-fold increase along the storage period ($P<0.01$). As a result, carbonylation of cytoplasmic and membrane protein fractions differs along storage, and the present study allows explaining two distinct steps in global erythrocyte protein carbonylation evolution during blood banking.
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1. Introduction

Transfusion medicine is a very important field in human health, concerning millions of lives worldwide. Red blood cells (RBCs) processed from whole blood donation or from apheresis are stored as erythrocyte concentrates (ECs) at 4 °C during 42 to 49 days, depending on the additive solution used (saline–adenine–glucose–mannitol, SAGM, or phosphate–adenine–glucose–guanosine–saline–mannitol, PAGGSM, respectively). This cold storage allows slowing down the RBC metabolism in order to improve the storage duration, and preserve the product from bacterial contamination. In spite of the undeniable benefits of transfusion in human health, some unknown counterparts have to be considered, due to lesions accumulating in labile blood products during their storage [1–6], even under standardized optimal storage conditions [7,8]. Such storage lesions occur in ECs, altering biochemical [9] and biomechanical [10] properties of erythrocytes, as well as their protein content.

Understanding the mechanisms altering RBC quality during storage is of great interest since increasing post-chirurgical complications and reducing patient survival have been associated with the age of received blood products [11]. More recently, a meta-analysis of studies comparing transfusion outcomes with blood storage-times revealed that the risk of death significantly increased when using older blood products [12]. Such studies make the transfusion medicine community investigating new storage conditions in order to improve blood quality. In particular, new additive solutions have been proposed during the last decade [13–15], as well as special banking conditions such as anaerobic storage [16–18].

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Among the storage lesions, it is known that RBCs are subjected to oxidative stress [19]. Protein oxidation [20–22], and particularly carbonylation, an irreversible post-translational amino acid residue modification, is a hallmark of oxidative stress [23,24]. It has been evidenced as, and is now commonly used as a marker of aging of stored RBCs [8,25,26] and other eukaryotic cell populations [27–29]. Several methods of detection and quantitation of protein carbonylation have been developed [30,31], the most common ones being based on the derivatization of carbonylated proteins with 2,4-dinitrophenylhydrazine (2,4-DNPH) [32–37]. In particular, Kriebardis et al. have shown via immunodetection of 2,4-DNPH derivatized proteins that the oxidative index of non-leukodepleted CPDA-stored erythrocyte membrane proteins increased along the storage period [25], later emphasized to the cytoskeletal proteins [26]. Moreover, Zolla et al. have shown that total erythrocyte protein carbonylation increased uniformly during the first four weeks of storage in leukodepleted, CPDA-collected, SAGM-stored RBCs, followed by a steady state evolution or slight decrease [38]. They have also measured an accumulation of ROS and lipid oxidation increasing parallel to protein carbonylation. Metabolomic investigations allowed them to correlate these findings with an over-activation of the oxidative phase of the pentose phosphate pathway, leading to the conclusion that protein carbonylation is due to an increasing oxidative stress along storage, reaching a maximum level after 3 weeks [38].

Another consequence of storage lesion consists in the release of microparticles (MPs) by erythrocytes [39]. MPs originating from different cell types have been observed, raised or lowered in several pathological conditions [40,41], and are known to play a role in some biological processes [41]. These RBC-derived MPs are small vesicles of less than 1 μm, containing part of its "mother cell" cytoplasmic content, jailed into membranes from the same cell [42]. During RBC storage, it has been shown that MP release presents a 20-fold increase at the end of the storage period [39]. The RBC microparticulation is thought to be a protective mechanism since erythrocyte-derived vesicles have been shown to be enriched in altered band 3 [42,43]. Clusters of band 3 were detected during RBC storage [44] and are responsible for the generation of senescent cell antigens which are recognized by naturally occurring auto-antibodies [45] leading to RBC clearance by hepatic macrophages [46].

In this study, we assessed the protein carbonylation status of RBCs during their storage as ECs in blood banking conditions. Different cellular (two soluble and two membrane fractions) compartments were analyzed, aiming at determining whether a particular protein population was more subjected to carbonylation or not. Protein carbonylation was also evaluated in the erythrocyte extracellular MP compartment, in order to correlate the observed cellular carbonylation variations with the microparticulation process.

2. Material and methods

2.1. Chemicals

Aminocaproic acid, β-mercaptoethanol (β-ME), BSA, *n*-dodecyl β-D-maltoside (DDM), Imidazole, NaCl and Ponceau S were from Sigma (Sigma-Aldrich, Steinheim, Germany) and Coomassie Brilliant Blue R250 from Fluka (Fluka Chemie, Buchs, Switzerland). Deoxycholate (DC) and EDTA were bought from Merck (MSD Merck Sharp & Dohme, Luzern, Switzerland). Tris–HCl was purchased from Biorad (Hercules, CA, USA), Tween 20 from Roche Diagnostics (Mannheim, Germany), SDS from MP Biomedicals (Illkirch, France), 0.9% NaCl from B. Braun Medical (Sempach, Switzerland), 10× PBS from Laboratorium Dr. Bichsel (Interlaken, Switzerland), BenchMark Protein Ladder (prestained or not) from Invitrogen (Carlsbad, CA, USA) and FITC mouse anti-Human CD47 antibody from BD Pharmingen (BD Biosciences, Franklin Lakes, NJ, USA).

2.2. Samples and extracts

2.2.1. EC preparation

RBC and MP samples were obtained from SAGM-stored ECs, prepared at the Service Régional Vaudois de Transfusion Sanguine by the blood component preparation laboratory, according to the standard procedure. Briefly, whole blood donation (450±50 mL in 63 mL of Citrate Phosphate Dextrose anticoagulant solution) is left at 22 °C overnight afterwards blood components (RBCs, plasma and white blood cell- and platelet-containing buffy coat) are separated upon centrifugation at 3500 *g* for 14 min. Components are then distributed among the sterile inter-connected blood bags by applying a semi-automated pressure on the centrifuged original blood donation bag. Erythrocytes are thus transferred into a SAGM-containing bag, to a total volume of 275±75 mL and a hematocrit of 0.6±0.1. As for RBCs, a leukodepletion step is performed by filtration. Only ECs that did not meet the quality criteria making them suitable for transfusion (low hemoglobin content, or small volume for example) were used here, under the signed assent of blood donors.

2.2.2. Subcellular fractionation of erythrocytes

Three ECs (namely EC #A, #B and #C) were followed weekly during their storage in blood banking conditions. Samples were taken at days 2, 5*, 7, 14, 21, 29, 36 and 43 days (*: note that for EC #A, a sample was taken at day 5 instead of days 2 and 7, due to a provision delay of this concentrate after its rejection from the transfusion bank). The global preparative process of samples is summarized in Fig. 1. Erythrocytes were first spun down at 2000 *g* during 10 min and at 4 °C. When needed, the supernatants were collected for the preparation of MP samples (see the last paragraph of this section). RBCs were then washed two times in a physiological 0.9% NaCl solution and spun down with the same conditions as before, to get them free of additive solution. RBCs were lysed by incubation of at least 1 h at 4 °C in a hypotonic 0.1× PBS solution under agitation. Then, cytoplasmic extracts and membranes were separated by centrifugation at 18,000 *g* during 30 min at 4 °C.

Hemoglobin (Hb) was depleted from the soluble cytoplasmic extracts by nickel-based IMAC chromatography with a HisTrap HP 5 mL column (GE Healthcare, Fairfield, CT, USA) on a BioLogic Workstation (BIO-RAD, Hercules, CA, USA), based on ref. [47]. The flow through (RBC soluble Hb-depleted fraction) was collected in a single fraction, and the corresponding Hb fraction was eluted with a gradient of imidazole-containing PBS buffer, from 10 to 100 mM imidazole in 20 min.