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Membrane protein carbonylation in non-leukodepleted CPDA-preserved red blood cells

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Abstract

Transfusion of allogeneic blood products is associated with adverse reactions and complications. Some of the negative effects of RBC transfusion are associated with the storage lesion. The importance of RBC oxidative damage in the storage lesion is not well documented. We monitored the storage-induced membrane protein oxidation in CPDA-preserved non-leukodepleted RBCs units from five blood donors in the course of the storage period, as assessed by protein carbonylation levels estimation. Carbonylated protein content was determined following 2,4-dinitrophenylhydrazine derivatization and SDS-polyacrylamide gel electrophoresis coupled with Western blotting. Immunoblotting with dinitrophenol-specific antibody revealed increased RBC membrane protein carbonyls with prolonged storage in CPDA units. This finding supports the idea of oxidation as a part of the storage lesion.

Keywords: RBC membrane proteins; CPDA; Storage; Carbonylation; Oxidation

Introduction

Storage of red blood cells (RBCs) in preservative mediums is associated with accumulation of bioreactive substances in the medium and a series of metabolic, biochemical and molecular changes to erythrocytes. These changes, which collectively referred to as "storage lesion", influence the viability of stored RBCs, especially in the absence of prestorage leukoreduction [1,2].

Several factors appear to contribute to erythrocyte injury including metabolite depletion, WBC derived factors and, probably, lipid and protein oxidation. Despite the high antioxidant capacity of the RBC, many factors increasing the demands on the antioxidant capacity can be observed in the stored blood of donors, like the high glucose concentration, the exposure to light and agitation, the leakage of free radicals from leukocytes, etc. [3]. Consequently, oxidative damage of erythrocytes may occur and can be indirectly manifested by different ways, like potassium release, increased malondialdehyde (MDA) [4–6], phosphatidylserine externalization [2], reduction in reduced glutathione concentration [5,7], decreased activity of superoxide dismutase, glutathione peroxidase, glutathione *S*-transferase and glutathione reductase as well as decreased total antioxidant activity of plasma, that were evident in prolonged storage [4,5,7].

Evidence of oxidative damage to specific RBC membrane proteins in storage concerns mostly the spectrin [8] and the spectrin–actin–protein 4.1 complex formation [9]. RBCs banked in Adsol are characterized by a time-dependent increase of protein clustering and carbonyl modification of band 4.1 [5]. Maintaining cellular reduced glutathione in Adsol-banked RBCs protects membrane proteins and Hb from oxidative stress [10].

In spite of this indirect documentation regarding the oxidation in banked RBCs, the relative importance of both

Abbreviations: DNP, 2,4-dinitrophenol; DNPH, 2,4-dinitrophenylhydrazine; MW, molecular weight; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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lipid and protein oxidation in the storage lesion remains unclear [2,11]. Many studies on oxidant injuries and antioxidant capacity during storage have been performed with controversial results, regarding the accumulation of Heinz bodies, the levels of reduced glutathione [11,12], the activity of antioxidant enzymes [7], protein deficiencies and aggregation, lipid peroxidation and phosphatidylserine externalization [1,2,13].

Consequently, the present study was undertaken to determine the membrane protein storage-induced oxidation, as assessed by the carbonylation estimation.

Materials and methods

Collection and processing of blood

Five eligible male blood donors who had passed the donor selection criteria (average age of 25 years, body weight 79 Kg, Hb 16.4 g/dl, Hct 44%) were bled according to the standard operating procedures used in Greece. All of them were Rh positive (+) and Kell negative (−), with blood groups of: A (ccEe), A (Ccee), B (CCee), AB (CCee) and O (ccee). Whole blood (450 ± 50 ml) was collected in 63 ml of a CPDA double-pack container system. After centrifugation, most of the plasma was removed and packed RBCs were produced, with a final Hct of 70%. The units were stored at 4°C for 43 days. WBC reduction was not performed.

Each unit was sampled in sequential time intervals of 2–7 days, beginning from the day of donation (day 0), for the whole storage period (35 days) and 1 week after the expiration time (43 days). As controls (C), ghost samples of days 0–2 of the five packed RBC units, together with ghosts freshly prepared from blood samples of 10 healthy volunteer subjects of matching sex and age, were used. Each unit was fitted with a sterile sampling-site coupler (MacoPharma) and mixed gently. Aliquots of 6 ml of RBC concentrate was withdrawn through the sampling site by use of a 19-gauge needle and attached syringe in order to avoid any mechanical damage of the cells during aspiration. All procedures were carried out at 4°C.

Preparation of ghosts

White ghosts were prepared by hypotonic lysis of RBCs in phosphate buffer at 4°C as previously described [14], with the addition of 0.3 mmol/L phenyl-methyl-sulfonyl-fluoride to the lysis buffer to inhibit protease activity. Protein concentration was assayed using the Bradford protein assay reagent with bovine serum albumin as a standard (Bio-Rad).

Estimation of membrane protein carbonylation

The OxyBlot™ Protein Oxidation Detection Kit (S7150, Chemicon) is a reliable and reproducible procedure in revealing protein oxidation [15]. It was employed for the comparative quantitative analysis of the membrane protein carbonylation during the RBC storage period according to the manufacturer's instructions, with minor modifications.

2,4-Dinitrophenylhydrazine (DNPH) derivatization was carried out for 15 min following the manufacturer's instructions on 3 μg of white ghost protein. After the addition of the volume of the neutralization solution proposed by the manufacturer (1.5 vol), some of the derivatized ghosts were not stained as expected. The same samples presented with an unexpectedly high oxidation signal. We assume that this event was probably indicative of an incomplete neutralization (for unknown reasons) and, consequently, of a progressive derivatization reaction over the predetermined time interval of 15 min. To ensure complete neutralization along with identical experimental conditions, we doubled the dose of the neutralization solution in all samples.

One-dimensional SDS-polyacrylamide gel electrophoresis was performed under reducing conditions (0.72 M 2-ME) using the discontinuous buffer system of Laemmli and 5–15% linear gradient slab gels. Equal amounts (3–6 μg) of protein were loaded per track of each gel. The gels were stained with Coomassie blue R-250 or immunoblotted as previously described [16]. In briefly, the proteins were electrophoretically transferred to nitrocellulose membranes in fresh transfer buffer containing 40 mM glycine, 50 mM Tris, 0.04% SDS and 20% methanol. Following transfer, non-specific protein binding was blocked by incubation with 5% non-fat powdered milk dissolved in PBS (10 mM phosphate, 150 mM NaCl, pH 7.5) containing 0.1% Tween 20. Afterwards, the proteins were incubated with the anti-DNP antibody (specific to the DNP moiety of the proteins) and the suitable horseradish-peroxidase-conjugated secondary antibody, appropriately diluted (according to the manufacturer's instructions) in blocking buffer, for 1 h each. All steps were carried out at room temperature. Finally, the protein bands were detected by chemiluminescence (ECL Western blotting detection system, Amersham Biosciences) and exposed on X-ray films. Rabbit anti-human erythrocyte spectrin (diluted 1:5000, Sigma S-1515), monoclonal anti-human erythroid spectrin (diluted 1:300000, clone SB-SP1, Sigma S-3396) and rabbit antiserums against human erythrocyte protein 4.1 and/or band 4.2 (diluted 1:5000, kindly provided by Prof. J. Delaunay, Service d' Hématologie, Hôpital de Bicetre, Le Kremlin-Bicetre, France) were used as internal loading controls. For comparative purposes, the oxidative status of each sample was quantified by using the oxidative index as previously defined [15], namely, the ratio of the densitometric values of the total oxyblot bands either to those stained with Red Ponceau S (0.2% w/v Ponceau S in 3% w/v trichloroacetic acid for 3 min) or to those of the same sample immunostained with a red cell membrane reference protein-specific antibody by scanning densitometry (Gel Analyzer v.1.0 image-processing program, Biosure, Athens, Greece). Any measurement out of the value width of: [(average of the control samples) ± 2 SD], where SD is the standard deviation of the controls, was characterized as pathological. The average of the five units examined was used to compare carbonylation levels each day of storage.

Results and discussion

Oxidant stress, in vivo or in vitro, is known to induce oxidative changes in human RBCs [11]. To evaluate the possible consequences of storage in RBC membrane protein oxidation, red cell membrane preparations from five concentrated RBC units stored in CPDA at 4°C were analyzed at established intervals of the storage period.

The formation of carbonyl groups (aldehydes and ketones) on protein side chains (especially of Pro, Arg, Lys and Thr) is a common and widely studied oxidative-stress-induced modification of proteins. The OxyBlot kit performs the immunoblot detection of carbonyl groups introduced into proteins by oxidative reactions with ozone or oxides of nitrogen or by metal-catalyzed oxidation. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins or by secondary reaction of the side chains of Cys, His and Lys with aldehydes, like malondialdehyde (MDE), produced during lipid peroxidation. The use of protein carbonyl groups as biomarkers of oxidative stress has some advantages compared to the measurement of other oxidation products because of the relatively early formation and the relative stability of carbonylated proteins. Derivatization of protein carbonyls with DNPH is highly quantitative and labels each carbonyl with DNP, thus making it amenable to immunodetection [17]. Consequently, protein carbonylation was used for the comparative quantitative analysis of the membrane protein oxidation during storage.

The SDS-polyacrylamide gel electrophoresis densitometry and immunoblotting analysis of the stored RBC membranes for the skeletal proteins spectrin, band 4.1 and band 4.2 (pallidin) exhibited the anticipated normal pattern without any quantitative or qualitative aberrations (data not shown, apart from Fig. 1B). Therefore, these skeletal components were used as reference proteins for the estimation of the oxidative index [15].

A quantitative and statistical important difference in carbonylation was detected by immunoblotting analysis of membrane proteins isolated from RBCs stored for different time periods. In comparison with control membranes, there was an evident increase in the number and the intensity of the carbonylated protein bands appearing in the immunostained gel, ranging from MW 240 kDa to 15 kDa. Typical results concerning an examined unit are shown in Fig. 1A. The membrane protein carbonylation levels, reflected by the oxidative index, namely the ratio of the total oxyblot signal (Fig. 1A), either to the immunoblotting signal of a reference protein (Fig. 1B) or to the sum of the Red-Ponceau-stained immunoblotted proteins (data not shown), are significantly elevated from baseline throughout the storage period in CPDA, starting from day 10 (Fig. 1C). Analysis of the modified proteins revealed that the oxidation was not random but specific since it occurred systematically after 10 days of storage in the five examined RBC units. It was also evident that the degree of carbonylation was not correlated to the amounts of proteins: in fact, some proteins that appeared carbonylated were not among the largest bands (see arrowheads in Fig. 1A) and the opposite (see the trivial carbonylation levels of band 3, with MW 90–100 kDa). The higher levels of membrane protein carbonyl groups

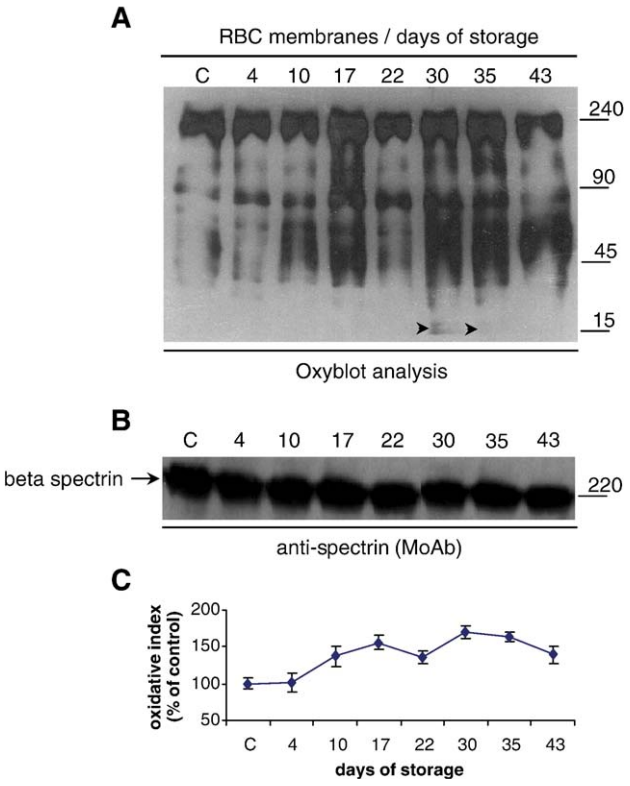

Fig. 1. Detection of RBC membrane protein carbonylation induced by storage in CPDA medium. (A) A representative oxyblot analysis, i.e. immunoblot analysis of ghosts from a donor stained with the anti-DNP antibody. Membranes (3 μg) were transferred to nitrocellulose and analyzed for carbonylation as described in Materials and methods. Arrowheads: low MW carbonylated bands in samples of prolonged storage. MW of the proteins is shown in kDa (right side). (B) A typical immunoblot analysis of the ghosts isolated from the RBCs of a donor stained with anti-human spectrin monoclonal antibody which was used as one of the internal loading controls. MW in kDa (right side). (C) The estimation of the oxidative index, i.e. quantitative analysis of the total protein carbonylation that is shown in immunoblots like the one in (A) against the Red Ponceau signal intensity or the immunoblotting signal of a reference protein like the spectrin shown in (B), after normalization to control values. Data are means ± SD (error bars), $n = 5$ blood donors.

were observed after 28–30 days in CPDA (Fig. 1C). The small "improvement" of protein carbonylation of the following days could be partially attributed to the hemolysis of the most severely damaged cells, as previously described [18]. The short storage in CPDA causes no changes in membrane protein carbonylation profile. The ROS-induced increase in protein carbonylation after 10 days of storage is consistent with previous reports showing that diminution in total antioxidant activity of RBCs was evident after a storage period of 10–12 days in CPDA-1 [7]. Since the stored RBCs convey less glucose to the pentose phosphate pathway, due to the subsequent decrease in NADPH and ATP levels, they are expected to be less protected against oxidative stress [19].

The increased protein carbonylation is a common oxidative effect in RBCs subjected to chronic oxidant stress, like in diabetes [20]. The specific carbonylation of a set of RBC membrane proteins with prolonged storage in CPDA is shown for the first time and supports the concept of protein oxidation as a part of the storage lesion. Almost all types of ROS can

induce protein carbonyl groups, and hence their formation does not shed light on the source of the oxidative stress. Furthermore, carbonyl groups are relatively difficult to induce compared to Met sulfoxide and cysteinyl derivatives, and thus these may reflect more severe cases of oxidative stress. Indeed, detection of elevated levels of protein carbonyls is generally a sign not only of oxidative stress but also of a protein dysfunction [17].

Although the sources of membrane protein oxidation in the storage remain unclear, it is well established that hemoglobin is a target for ROS. The Hb, and possibly the oxidized hemichromes as well, that accumulates in association with the red cell membrane during the storage period is capable of mediating oxidation reactions [11]. The induced by hemolytic agents ROS production in RBCs is accompanied by increased association of oxidized and denatured Hb to the membrane proteins, with no concomitant oxidative alterations of lipids [13]. Apart from intracellular Hb, the oxidative stress in stored RBCs could be further mediated by release of free radicals by the WBCs present in the unit [2,3].

Protein oxidation results in loss of protein function, fragmentation or aggregation [10,21]. Consequently, the carbonylation of membrane proteins may affect the architecture and the function of RBC membrane and lead to destabilization of the cytoskeleton, thereby compromising RBC survival.

The presented study revealed the increased carbonylation of membrane proteins after prolonged storage of RBCs in CPDA. This establishes that storage generates ROS in sufficient quantity to support specific carbonylation of certain target proteins in the membrane. The oxidative modification of membrane proteins evoked by RBC storage suggests a clear role for oxidation in the storage lesion. These data could give additional, useful information in evaluating improved conditions for storage of RBCs intended for transfusion.

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