Red blood cell aging markers during storage in citrate-phosphate-dextrose–saline-adenine-glucose-mannitol

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BACKGROUND: It has been suggested that red blood cell (RBC) senescence is accelerated under blood bank conditions, although neither protein profile of RBC aging nor the impact of additive solutions on it have been studied in detail.

STUDY DESIGN AND METHODS: RBCs and vesicles derived from RBCs in both citrate-phosphate-dextrose (CPD)–saline-adenine-glucose-mannitol (SAGM) and citrate-phosphate-dextrose-adenine (CPDA) were evaluated for the expression of cell senescence markers (vesiculation, protein aggregation, degradation, activation, oxidation, and topology) through immunoblotting technique and immunofluorescence or immunoelectron microscopy study.

RESULTS: A group of cellular stress proteins exhibited storage time– and storage medium–related changes in their membrane association and exocytosis. The extent, the rate, and the expression of protein oxidation, Fas oligomerization, caspase activation, and protein modifications in Band 3, hemoglobin, and immunoglobulin G were less conspicuous and/or exhibited significant time retardation under storage in CPD-SAGM, compared to the CPDA storage. There was evidence for the localization of activated caspases near to the membrane of both cells and vesicles.

CONCLUSIONS: We provide circumstantial evidence for a lower protein oxidative damage in CPD-SAGM–stored RBCs compared to the CPDA-stored cells. The different expression patterns of the senescence markers in the RBCs seem to be accordingly related to the oxidative stress management of the cells. We suggest that the storage of RBCs in CPD-SAGM might be more alike the in vivo RBC aging process, compared to storage in CPDA, since it is characterized by a slower stimulation of the recognition signaling pathways that are already known to trigger the erythrophagocytosis of senescent RBCs.

Aging of red blood cells (RBCs) is characterized by accumulation of structural, metabolic, and functional modifications.[1] RBC shrinkage, membrane remodeling, microvesiculation, and exposure of surface removal markers that triggers erythrophagocytosis are some of the typical changes observed in senescent RBCs. Powerful removal signals are the externalization of phosphatidylserine (PS)[2] and the binding of autologous immunoglobulin G (IgG) to senescence-specific neoantigens that originate from structural changes in Band 3 protein.[3] The RBC aging process is also associated to the operation of an apoptosis-like cell death program, that could be induced by Ca^{2+} influx and prevented by calpain and caspase inhibitors.[3-6] The activation of caspases 3 and 8 is, nowadays, considered as a sign of RBC senescence.[7,8] The induction of Fas/caspase–driven death program in RBCs has been associated with the fragmentation[7,9] or clustering[10] of Band 3. More recently, Bratosin and coworkers[11] reported that caspases are activated in vivo, in circulating senescent RBCs. Vesiculation probably represents a mechanism for the exocytosis of removal

ABBREVIATIONS: HMW = high molecular weight; Hsp = heat-shock protein; MW = molecular weight; PoAbs = polyclonal antibodies; Prx2 = peroxiredoxin-2; PS = phosphatidylserine; SAGM = saline-adenine-glucose-mannitol.

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signals, thereby postponing the untimely elimination of otherwise healthy RBCs.[12]

RBCs stored under blood bank conditions undergo structural and functional deterioration, collectively referred to as RBC storage lesions.[13] Most of the lesions are the result of the progressively effete RBC metabolism and are reversible in vivo. On the other hand, the structural changes and the loss of membrane are permanent and probably related to in vivo RBC survival and the adverse effects of transfusion.[14] Although some differences in the aging profile between in vivo and ex vivo conditions have been reported,[1] a part of stored RBCs progressively express some of the typical marks of senescence and erythrophagocytosis, like the formation of Band 3-IgG complexes,[15] the exposure of PS,[16] and the spheroechinocytosis.[17] The increasing accumulation of aging effects in a part of the transfused RBCs contributes not only to their rapid elimination[16] but also to the various adverse side effects of the transfusions.[1,17]

Our study focuses on answering the question of whether storage of RBCs in additive solution (AS) alters the aging process compared with storing RBCs in autologous plasma. We have previously published detailed biochemical analysis of senescence-related molecules during storage of RBCs in citrate-phosphate-dextrose-adenine (CPDA) plasma.[18-21] Here we report a similar analysis of RBCs stored in saline-adenine-glucose-mannitol (SAGM). In addition, we present the results of some new methods that we have not previously applied to RBCs, for RBCs stored in both SAGM and CPDA plasma.

MATERIALS AND METHODS

Collection and processing of blood

Whole blood (450 ± 45 mL) from 20 eligible young blood donors was collected in a quadruple CPD-SAGM top-and-bottom bag system (Baxter, Rome, Italy) and anticoagulated with 70 mL of CPD (26.30 g/L sodium citrate dihydrate, 3.27 g/L citric acid monohydrate, 2.51 g/L sodium phosphate dihydrate, 25.00 g/L glucose monohydrate, pH 5.6). After cooling and centrifugation most of the plasma and the buffy coat were removed and RBCs were produced. SAGM (100 mL) was then added to the RBCs. Leukoreduction was not performed. The units were stored for 7 weeks (and for an additional 10 days) according to the standard banking procedures at 2 to 6°C. From the very first 2 to 3 days of storage and weekly thereafter a sample (of 6-55 mL) was withdrawn by a 19-gauge needle and a syringe attached to a sterile sampling-site coupler (Maco-Pharma, Mouvaux, France) fitted in the bag, after gentle mixing of the unit content by inversion for approximately 5 minutes. Owing to the fact that the isolation of vesicles needs larger volume of RBC aliquots compared to the other preparations and to diminish any sampling effect that might compromise the results, the examined units were separated as following: 5 units of RBCs were used for the collection of vesicles and sampled in two groups for Days 17, 26, 40, and 23 through 33, 12 units were utilized for the biochemical analysis of RBC fractions, and 3 non-sampled units stored for a short (2-4 days) or long (33-42 days) period in CPD-SAGM were used for all the microscopy (fluorescence and electron) analyses.

The collection and processing of blood for the preparation of CPDA units of RBCs were performed as previously described.[21] Whole blood samples from 10 blood donors of matching age with those of the CPD-SAGM study were collected in CPDA double-pack container systems. After centrifugation, most of the plasma was removed and RBCs were produced. Leukoreduction was not performed. The units were stored for 5 weeks (and 1 week thereafter) at 2 to 6°C and sampled as mentioned above. As controls of nonstorage, RBCs freshly prepared from blood samples of 10 age-matched subjects were collected in ethylenediaminetetraacetate and heparin. Hematological analysis was performed with an automated analyzer (Sysmex XT-1800i, Roche Diagnostics, Mannheim, Germany). Informed consent was obtained from all donors participating in this study. Investigations were performed after approval by the appropriate research ethical committee.

Preparation of vesicles and RBC fractions

The vesicles were isolated from the plasma of the RBCs and quantified as previously described.[18] Briefly, the plasma collected from 55 mL of RBCs was ultracentrifuged twice (37,000 × *g*, for 1 hr at 4°C) after filtration through sterile 0.8-μm pore size syringe-driven nitrocellulose filter units (Millipore, Carrigtwohill, County Cork, Ireland). Light- and dark-field microscopy disclosed no residual RBCs or RBC membranes in the pellet of vesicles (data not shown). After the addition of protease inhibitors mix (protease inhibitor cocktail, Sigma, St Louis, MO), protein concentration was determined using a protein assay with bovine serum albumin (BSA) as a standard (Bradford protein assay, Bio-Rad, Hercules, CA).

For the preparation of RBC fractions, aliquots of RBCs were collected and washed with phosphate-buffered saline (PBS, pH 7.4) at 4°C. RBCs were lysed with hypotonic (5 mmol/L) sodium phosphate buffer (pH 8.0), containing a cocktail of protease inhibitors.[20] The supernatants (hemolysates without RBC membranes) were collected, recentrifuged twice under the same conditions to eliminate any residual membrane component, and stored in aliquots representing the membrane-free cytoplasm fraction. The pellets (RBC membrane ghosts) were repeatedly washed with the same buffer until they turned white (white ghosts, total RBC membrane largely free of hemoglobin [Hb]). RBC cytoskeletons were prepared from the

washed ghosts of stored RBCs by Triton X-100 extraction as previously described.[19] Protein concentration was assayed as for the vesicle samples. All solutions and buffers utilized in the biochemical and microscopy experiments were prepared using 18.2 MΩ water generated by a laboratory water purification system (Direct-Q3, Millipore Corp., Molsheim, France) and subsequently filtered through 0.22-μm filters (Millex-GV, Millipore Corp.).

Electrophoresis and immunoblotting analysis

Equal amounts (7 μg) of total protein preparations were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing (0.72 mol/L 2-mercaptoethanol) or nonreducing conditions. Immunoblotting analysis was performed as previously described.[22] Primary monoclonal antibodies (MoAbs) and polyclonal antibodies (PoAbs) for the following proteins were utilized: Band 3, spectrin, actin, and anti-human IgG horseradish peroxidase (HRP)-conjugated (Sigma, Munich, Germany); peroxiredoxin-2 (Prx2; Acris Antibodies GmbH, Hiddenhausen, Germany), sorcin (Zymed Laboratories, San Francisco, CA); flotillin-1, flotillin-2, and synexin (BD Biosciences, San Diego, CA); FADD, ubiquitin, caspase 8, and caspase 3 (detecting both the full-length caspases and the cleaved fragments), as well as cleaved caspase 3 and 8 (Cell Signaling Technology, Beverly, MA); Fas/CD95, heat-shock protein (Hsp) 70, Hsp27, and cathepsin E (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-HbA (Europa Bioproducts, Cambridge, UK). Protein 4.1R antiserum was provided by Prof. J. Delaunay (Service d'Hématologie, Hôpital de Bicetre, Le Kremlin-Bicetre, France) and MoAb against stomatin was provided by Prof. R. Prohaska (Department of Medical Biochemistry, Medical University of Vienna, Vienna, Austria). Species-specific HRP-conjugated secondary antibodies that were used were anti-rabbit (GE Healthcare, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), anti-mouse (DakoCytomation, Glostrup, Denmark), and anti-goat (Sigma). The immunoblots were developed using enhanced chemiluminescence reagent kits (ECL or ECL Plus Western blotting detection reagents, GE Healthcare, Amersham Biosciences). The molecular weight (MW) of immunoblotted bands was compared to a lane of MW protein standards (Fermentas, Hanover, MD). The immunoblots shown are derived from different representative donors. Quantitation of Coomassie blue–stained gels and ECL-developed immunoblots was performed by lengthwise scanning densitometry using a gel analyzer image-processing program (v.1.0, Biosure, Athens, Greece). Individual protein levels were quantified as percentage of total per day of storage and final normalization to the controls (% of controls), namely nonstored samples or samples stored for a short period (of 2-4 days).

Estimation of protein oxidative modification in RBC fractions

Equal amounts of total ghost, cytoskeletal, or cytoplasmic protein collected from the units of RBCs after various storage periods were processed for the detection of carbonyl groups using a detection kit according to the manufacturer's specifications (Oxyblot, Millipore, Chemicon, Temecula, CA), with minor modifications as previously described.[21] For quantification purposes, the oxidative index was utilized,[23] that is, the relative percentage of the densitometric values of the oxyblot bands to the total protein bands per day or to a reference protein immunoblotted band, further normalized to the controls (= 100%).[21]

Immunofluorescence and immunogold localization

Fresh aliquots of RBCs and vesicles were prepared from three nonsampled units of RBCs at two time points: very early (second to fourth days) and late (33rd-42nd days) in the storage period. Immunofluorescence assays were performed as previously described.[8,20] Briefly, the RBCs were fixed with 90% methanol in PBS and permeabilized in the same solution containing 0.05% Triton X-100. After being blocked with 3% BSA and 0.1% Tween 20 in PBS, the cells were probed with MoAbs to Band 3 and stomatin and PoAbs to spectrin, Hb, Fas, and caspase 3 (total or cleaved) for 30 minutes and incubated with secondary antibodies conjugated to fluorescein isothiocyanate or rhodamine (dilution 1:250, Santa Cruz Biotechnology). The slides were observed under a confocal laser scanning microscope (Digital Eclipse C1, Nikon, Melville, NY). Routine procedures were applied to demonstrate the specificity of immunostaining protocols: 1) the use of the preimmune serum instead of the antibody serum and 2) the omission of the respective primary antibody. No immunoreactivity was observed in the samples used in these procedures. All the micrographs shown are representative of results from six independent experiments, representing RBCs derived from three different donors and an equal number of controls, photographed at the same exposure time.

The immunoelectron microscopy analysis was performed as described elsewhere.[24] Shortly, the RBCs and vesicles were fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in PBS (pH 7.2) and embedded in unicryl acrylic resin (British Biocell International, Cardiff, Wales, UK). For immunogold labeling, the grids were blocked with BSA and incubated with MoAbs against Band 3, synexin, cleaved caspase 8, and PoAbs against cleaved caspase 3 followed by 10- or 15-nm gold-conjugated probes (British Biocell International). The specificity of the reactions was tested by a number of control experiments, including 1) mouse or rabbit normal serum instead of the reactive primary antibodies, 2) absence of primary antibodies, 3) a nonspecific secondary antibody, and 4) an additional

incubation of the sections with an unlabeled secondary antibody (anti-mouse or anti-rabbit IgG) between the primary and the specific gold-labeled secondary antibody. Sections were viewed in an electron microscope (TEM 900, Zeiss, Oberkochen, Germany; or TEM 300, Philips, Eindhoven, the Netherlands) at 60- to 80-kV accelerating voltage. The sources of all antibodies were those mentioned above.

Statistical analysis

Quantitative data from SDS-PAGE and immunoblotting densitometry analysis were presented in the graphs as mean ± standard deviation (SD) for the units examined each day of storage (stated under "Collection and processing of blood" and also in the legends to the figures), after normalization to the controls (percentage of control values, namely, fresh or stored for a short period samples). Mean values of each day of storage were compared to those presented by the reference samples. On occasion (as stated in the text), paired or unpaired t test was used to compare mean values between groups representing different mediums or different days of storage. A p level of less than 0.05 was considered significant.

RESULTS

Protein carbonylation index and membrane vesiculation

The oxidation index of both cytoskeleton and total ghost membrane, extracted from CPD-SAGM–stored RBCs, was pathologically increased after the 11th and 24th days of storage, respectively (Fig. 1A). On the last day of storage, both indexes presented a decrease that was significant only for the ghost proteins (Day 35 vs. Day 42, p = 0.009, n = 12). The relative percentage of carbonylated cytoskeleton proteins to total membrane proteins was dramatically increased after Day 35 of storage (Fig. 1A). The oxidation indexes of the CPD-SAGM and CPDA cytoplasmic samples increased in parallel until the 24th day of storage, while after that certain time point, the oxidation of the CPDA samples exceeded the one of the CPD-SAGM samples (375% vs. 256% on Day 26, Fig. 1B). Finally, there was a notable increase in the vesicular protein content of RBC units stored longer than 27 days in CPD-SAGM (Fig. 1C).

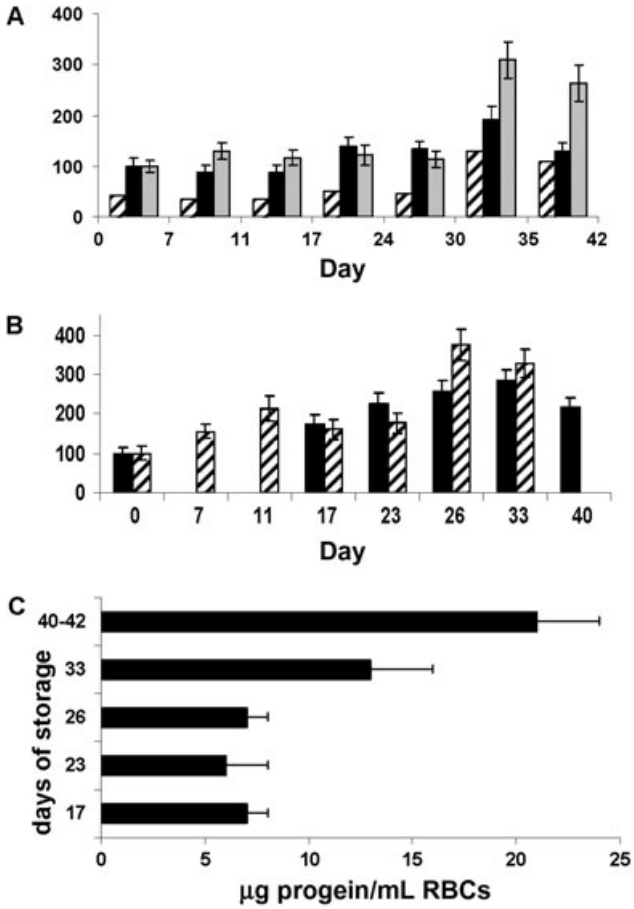

Fig. 1. RBC protein carbonylation and total vesicular protein content under storage in CPD-SAGM (A-C) or CPDA (B). (A) The variation of the oxidative index in membrane (■, n = 12) and cytoskeleton (▒, n = 12) samples of RBCs stored in CPD-SAGM, after normalization to the controls (100%, samples stored for short). (▨) Relative percentage of carbonylated cytoskeletal proteins to the total membrane carbonylation signal each day of storage. (B) Comparative indication of the oxidative indexes for cytoplasm samples of RBCs stored in CPD-SAGM (■, n = 12) or CPDA (▨, n = 10). (C) Total vesicular protein content in µg of protein per mL of RBCs during storage in CPD-SAGM (n = 2 for Days 23 and 33 and n = 3 for Days 17, 26, and 40). Each data point is the mean ± SD for the indicated number of samples, estimated as presented under Materials and Methods.

The magnitude of Band 3–associated modifications

Starting on Day 17, a decrease in the membrane expression of Band 3 was evident in the RBC membrane as a function of storage time (Fig. 2D), which coincides with an increase in mild proteolysis products (arrows in Fig. 2A) as well as in its relative content into the released vesicles. The Band 3 percentage of total vesicular protein is shown as an inset on Fig. 2D columns. High-molecular-weight (HMW) bands of nonreducible Band 3 oligomers were detected after the 24th day of storage and in an apparently lower proportion than the one previously observed on CPDA-stored RBC membranes[20] (currently run in a parallel comparative gel shown by asterisk in Fig. 2A). Subsequent analysis of Band 3 by microscopy techniques (Figs. 2B and 2C) verified the presence of Band 3 aggregates in a small proportion of cells

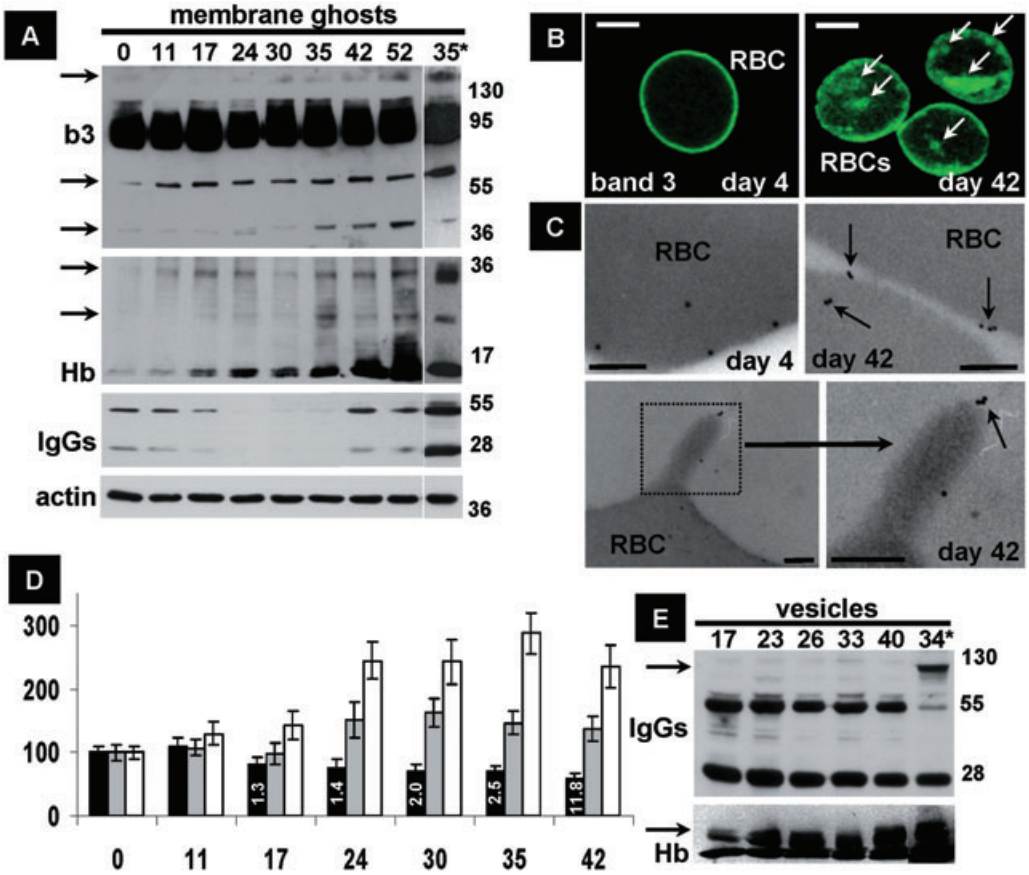

Fig. 2. Western blot and microscopy analysis of the Band 3–associated protein modifications observed under storage in CPD-SAGM. (A and E) Immunoblot analysis of representative RBC ghosts (7 μg; A) or vesicle (E) preparation for the detection of Band 3, Hb, IgG, and actin under reducing conditions. The duration of storage is indicated to days at the top of the blot figure. The corresponding samples of CPDA preparations, included for comparison purposes, are denoted by asterisk in a parallel (A) or in the same (E) gel. The aberrant bands are shown by arrowheads. The running of the MW markers is shown in kDa (right side). (B) Fluorescent micrographs of RBCs stored in CPD-SAGM after immunostaining with MoAb to Band 3. The arrows demonstrate the protein clusters after prolonged (42 days) storage. Bars = 3 μm. (C) Immunogold electron microscopy of RBCs after storage in CPD-SAGM, with MoAb specific for Band 3 and 10-nm gold–conjugated species-specific probes. Aggregates of gold particles representing Band 3 molecules were observed in the RBCs and their protruding spikes (arrow in the higher magnification micrograph) after a long storage period. The micrographs shown are representative of results from three independent experiments each using RBCs derived from different donors. Bars = 0.1 μm. (D) Densitometry analysis of Band 3 (■), Band 6 (▨), and Band 8 (□) proteins performed on ECL-developed films (for Band 3) or on Coomassie blue–stained gels (for the other proteins). Inset in the Band 3–indicating columns: relative percentage of the Band 3 into the released vesicles. Protein levels were quantified as percentage of total per day of storage and final normalization to the controls (% of controls). Error bars = the SDs of the tested units (n = 12).

and in their protruding spikes, formed after a long storage period in CPD-SAGM (arrow in Fig. 2C). As expected,[18,19,25] crosslinked Hb forms of probably oxidized/denatured Hb were increasingly associated with the membrane (Fig. 2A) and the vesicles (Fig. 2E) of stored RBCs, but, once again, in relatively lower amount compared to those of previously reported CPDA-stored RBCs (currently run in a parallel lane shown by asterisk in Figs. 2A and 2E). The membranes were further featured by progressively increased amounts of Band 6 and Band 8 proteins (Fig. 2D). IgG binding to the CPD-SAMG membrane was minimal, as previously suggested,[25] and slightly increased only on the last day of storage (Fig. 2A). The vesicles were enriched in IgG compared to ghosts (Fig. 2E). Nonreducible IgG was detected in the membranes of CPDA-stored RBCs (shown by asterisk in Fig. 2E).

Modifications of cytoskeletal proteins under storage in CPD-SAGM

HMW species of Hb, including the crosslinking product of Hb with the main cytoskeletal protein spectrin, was

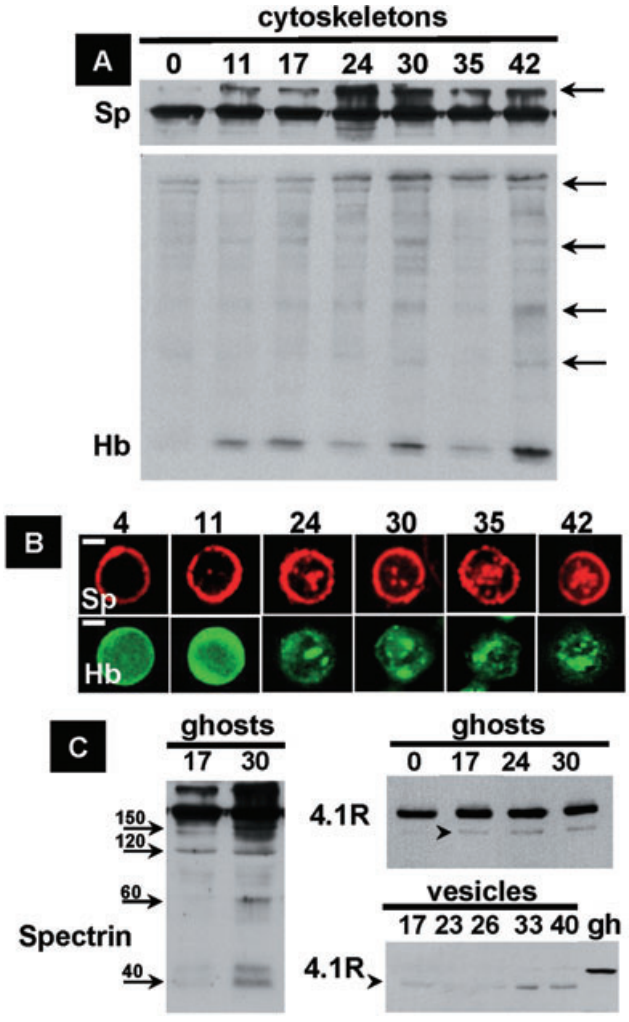

Fig. 3. Cytoskeletal protein modifications under storage in CPD-SAGM. (A) Immunoblot analysis of a representative RBC cytoskeleton preparation (7 μg) using anti-human–specific antibodies for spectrin and Hb, under reducing conditions. The duration of storage is indicated to days at the top of the blot figure. The aberrant bands representing crosslinkages are shown by arrowheads. (B) Fluorescent micrographs of RBCs stored for various periods in CPD-SAGM after immunostaining for spectrin or Hb. The signal was gradually transformed from smooth peripheral (spectrin) or cytoplasmic (Hb) to punctuated and aggregated over the storage. Bars = 3 μm. (C) Immunoblots of RBC ghosts and vesicles showing (by arrows or arrowheads) the proteolytic products of spectrin and 4.1R proteins.

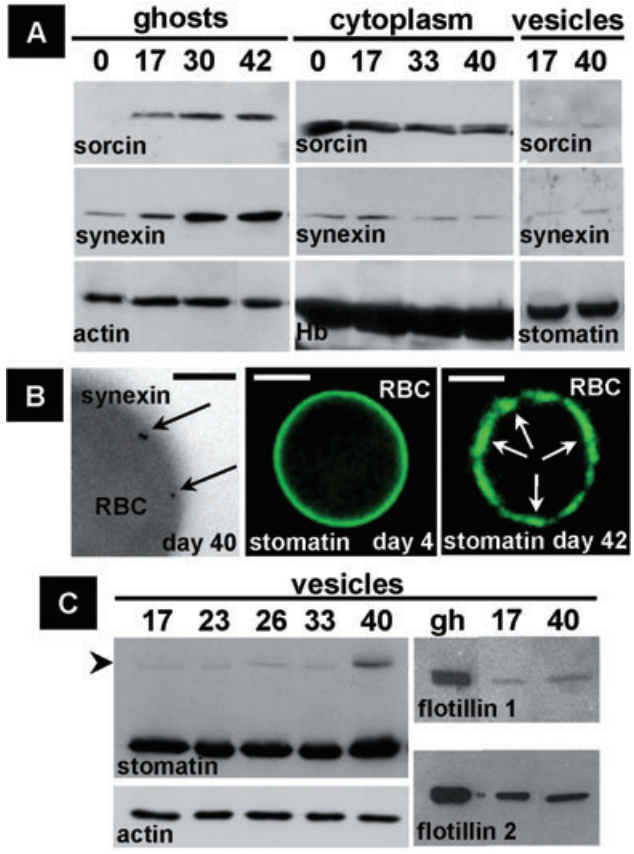

Fig. 4. Lipid raft–associated protein modifications under storage in CPD-SAGM. (A and C) Immunoblot analysis of representative RBC ghosts, cytoplasm, and vesicle preparations using antibodies for sorcin, synexin, stomatin, flotillin-1, and flotillin-2 (actin and Hb were applied as controls). The duration of storage is indicated to days at the top of blot figures. The HMW aggregates of stomatin in the vesicles are shown by an arrowhead. (B) Immunogold and fluorescent microscopy images denoting the membrane delivery of synexin (left) and the punctuate pattern of stomatin staining (right) in the membrane of RBCs stored for a long period in CPD-SAGM. Bars = 0.1 and 3 μm, respectively.

detected in CPD-SAGM–stored cells, soon after the beginning of the storage period (arrow in Fig. 3A). Immunofluorescence patches of spectrin and Hb were found in situ, especially after prolonged storage (Fig. 3B). The cytoskeletons were further characterized by a decrease in the spectrin as previously reported,[25] along with a decrease in 4.1R and actin (87%-40% of the normal values) after the 30th, 17th, and 24th day of storage, respectively (data not shown). Mild proteolysis products were detected in both membranes and vesicles (Fig. 3C). The actin was effectively exocytosed, as a component of the released vesicles (Figs. 4C and 5B).

Variation in lipid raft–associated proteins of CPD-SAGM RBCs

Vesicles collected from CPD-SAGM units seem to contain a repertoire of lipid raft–associated proteins, including flotillin-1, flotillin-2, synexin, sorcin, and especially, stomatin in progressively increased amounts (Fig. 4). Compared to the RBC membrane, both flotillins are depleted

from the released vesicles (Fig. 4C). As shown by immunoblotting (Fig. 4A) and electron microscopy approaches (Fig. 4B), the membrane binding of synexin and sorcin increases in parallel to a decrease in their cytoplasmic content (Fig. 4A). The exact opposite effect characterizes the membrane and the vesicular content of stomatin and flotillins (Fig. 4C). Upon prolonged storage, the membranes and the vesicles contain stomatin oligomers, detected by both immunoblotting (arrow in Fig. 4C) and immunofluorescence microscopy (Fig. 4B).

Cellular stress and proteasome proteins in CPD-SAGM and CPDA-stored RBCs

Although Hsp27 was found only in traces in the membranes of stored cells (data not shown), Hsp70 was detected in progressively increasing amounts (Fig. 5A). After normalization to the ghosts of the first days, the membrane association of Hsp70 was more pronounced in the CPDA samples (Day 30 CPDA vs. Day 30 CPD-SAGM, $p = 0.012$, $n = 10$ and 12, respectively). The relative proportion of CPD-SAGM membrane-associated Prx2 was also gradually increasing with storage duration but it remained invariable in the membrane of CPDA-stored RBCs until the end of the storage period where it was completely declined (Fig. 5A). Prx2, Hsp27, and Hsp70 were all detected in the vesicles from CPD-SAGM and CPDA units (Fig. 5B). The relative content of membrane-associated cathepsin E in stored RBCs was constantly decreasing until Day 24 of storage while from Day 30 it presented the opposite effect, by increasing in both mediums (Fig. 5A). The final increase in the membrane-bound enzyme was equivalent to the normal values only in the case of CPD-SAGM–stored RBCs. It should be noted that the vesicles did not contain cathepsin E (data not shown). The proportion of the proteasome protein ubiquitin followed a decreasing pattern in the membrane of stored RBCs

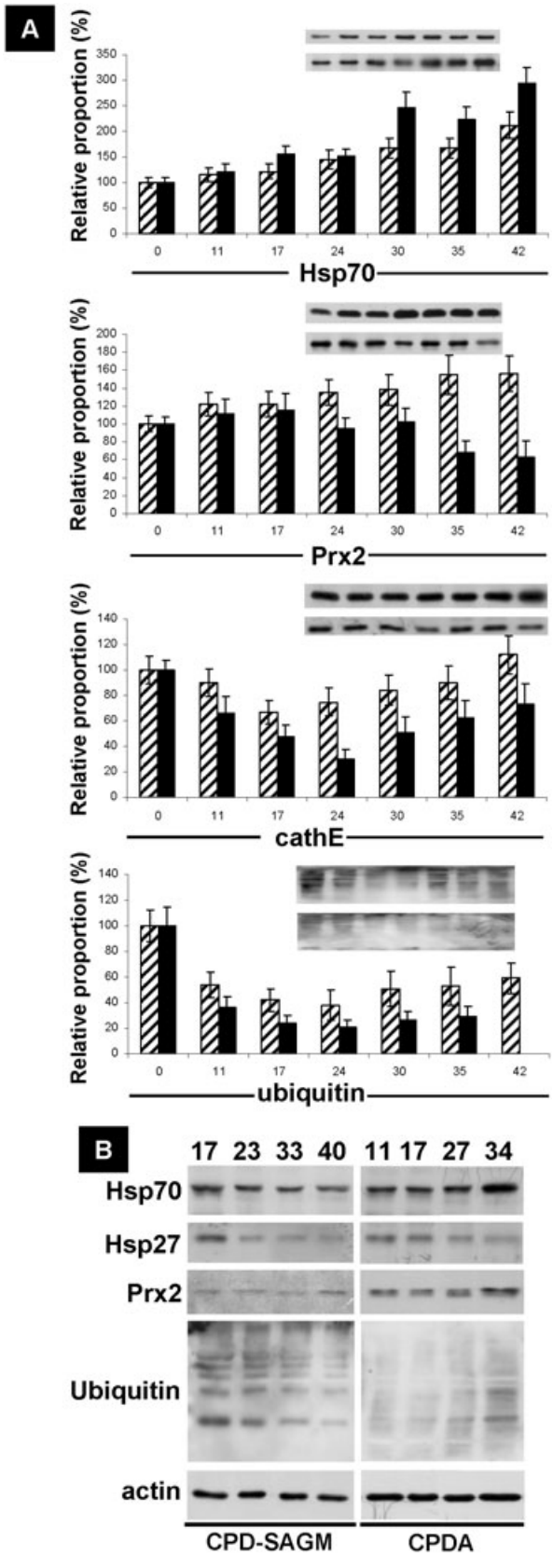

Fig. 5. Membrane expression and vesicle participation of cellular stress and proteasome proteins during the storage of RBCs in CPD-SAGM or CPDA. (A) Densitometry analysis of immunoblots of RBC ghosts (insets) from CPD-SAGM– (▨) and CPDA- (■) stored RBCs, probed for Hsp70, Prx2, cathepsin E (cathE), and ubiquitin. The data are the relative proportion (%) of each protein to the total membrane proteins per day of storage and final normalization to the controls, namely nonstored samples or samples stored for a short period (of 1-3 days). Error bars demonstrate the SD among donors (n = 12 and n = 10 for CPD-SAGM and CPDA, respectively). (B) Immunoblot analysis of representative vesicle preparations showing the participation of Hsp70, Hsp27, Prx2, and ubiquitin into the vesicles collected from CPD-SAGM or CPDA units of RBCs. Actin was utilized as loading control. The duration of storage is indicated to days at the top of blot figures.

(Fig. 5A). Once again, the decrease was sharper in the membranes of CPDA-stored RBCs compared to the CPD-SAGM ones (Fig. 5A). The vesicles contained a quite different range of ubiquitinylated proteins in comparison with that of ghosts (Fig. 5B). Furthermore, the variation in the total content of ubiquitinylated proteins in the vesicular extracts (Fig. 5B) was opposite to the one shown in ghosts.

Fas-related signaling proteins

The relative proportion of Fas in the membrane of stored RBCs was decreased at the end of the storage period (Figs. 6A and 6B). Furthermore, HMW bands of nonreducible Fas oligomers were detected after the 17th or 24th day of storage in CPD-SAGM (Fig. 6A). Their proportion varied between 4 and 13% of the total Fas protein in the CPD-SAGM samples (n = 12), while in the CPDA samples (Fig. 6A) the same proportion reached levels as high as 25% (8%-25%, n = 10). Through immunofluorescence microscopy, we detected a disturbed, aggregated distribution of Fas in the membrane of RBCs after prolonged storage, in contrast to the smooth, continuous peripheral distribution of Fas in control cells (Fig. 6C). The vesicles collected from the CPD-SAGM units contained detectable amounts of Fas and the following Fas-associated proteins: FADD, caspase 3 (Fig. 6A), and caspase 8. Pro–caspase 3 and FADD were also detected in the RBCs (Fig. 6C) but also in the membrane-free cytoplasm of all stored cells (Fig. 6A). Despite that observation, cleaved caspase 3 that is indicative of its activation is found in barely traceable amounts in the CPD-SAGM vesicle preparations by Western blotting analysis. To unequivocally confirm the probable caspase activation in CPD-SAGM–stored RBCs and vesicles, we employed in situ immunodetection methods. By means of immunofluorescence (Fig. 6C) or immunogold electron microscopy (Fig. 6D), where MoAbs that specifically detect the cleaved subunits of activated caspases were applied, we scouted activated caspases in a relatively small proportion of both RBCs and released vesicles, especially after a long storage period. In many cases, the signal representing activated caspases was located near the cell and vesicle membrane (double arrows in Figs. 6C and 6D).

DISCUSSION

Variation in protein carbonylation index and membrane vesiculation

The increased protein carbonylation of RBCs as a result of storage time in CPD-SAGM corroborates previous reports, suggesting that oxidation is a main phenomenon in storage lesions and that the cytoskeleton consists as the most sensitive and early target of this process.[19,21,26] The decline of the oxidative index at the end of the storage period could be partially attributed to the hemolysis/microvesiculation of the more severely damaged RBCs. Furthermore, this study reveals a rather important difference between the two storage mediums regarding both the extent and the rate of protein carbonylation. The carbonylation levels of cytoplasmic (currently reported for the first time), membrane, and especially of cytoskeletal components were lower than those reported in CPDA-stored RBCs[19] and appeared later on during the storage period. Considering the fact the oxidation accelerates the aging and the removal of RBCs,[2,3] this certain finding was the first mark of dissimilarity in the cell aging potential of cells stored in each condition.

The more effective management of oxidative stress in the case of CPD-SAGM RBCs is probably connected to the observed lower levels of membrane vesiculation compared to the CPDA-stored RBCs,[18,27,28] as previously suggested.[10,18,29] Apart from representing a prominent feature of RBC senescence,[1] the storage-associated vesiculation is a powerful contributor to the adverse effects of the transfusions.[1,14] The sharp increase in the relative proportion of carbonylated cytoskeletal proteins at the end of the storage period is probably associated with the low participation of cytoskeleton components into the released vesicles.[18]

The magnitude of spectrin- and Band 3–associated modifications under storage in CPD-SAGM

Surface remodeling of stored RBCs has been associated with clustering/proteolysis of Band 3, crosslinking of Hb, and increased binding of Hb and autologous IgG.[19,20,25] These modifications induce disorganizing effects on the membrane and furthermore, constitute effective signals in molecular pathways, leading to the erythrophagocytosis of senescent RBCs.[2,3,30] It is now well documented that a range of oxidative processes underlie them in both ex vivo storage[26] and normal RBC senescence.[30]

This study, documenting milder expression of those aging markers in RBCs under CPD-SAGM storage conditions compared to the CPDA ones, further confirms the aforementioned association. The comparative analysis of protein aggregation status and reducibility suggests that CPDA storage conditions favor the protein aggregation and oxidation more than CPD-SAGM conditions do. It seems that storage of RBCs on CPD-SAGM is more relevant to the in vivo RBC aging over the storage in CPDA, due to the slower stimulation of the recognition signaling pathway that triggers the erythrophagocytosis. The RBC cytoskeleton remodeling under storage in CPD-SAGM is also featured by protein aggregation and fragmentation events. The high-affinity association of Hb with spectrin, which is considered as a credible RBC aging marker,[2] seems to be a very early membrane modification storage effect, in similarity with the storage in CPDA medium.[19]

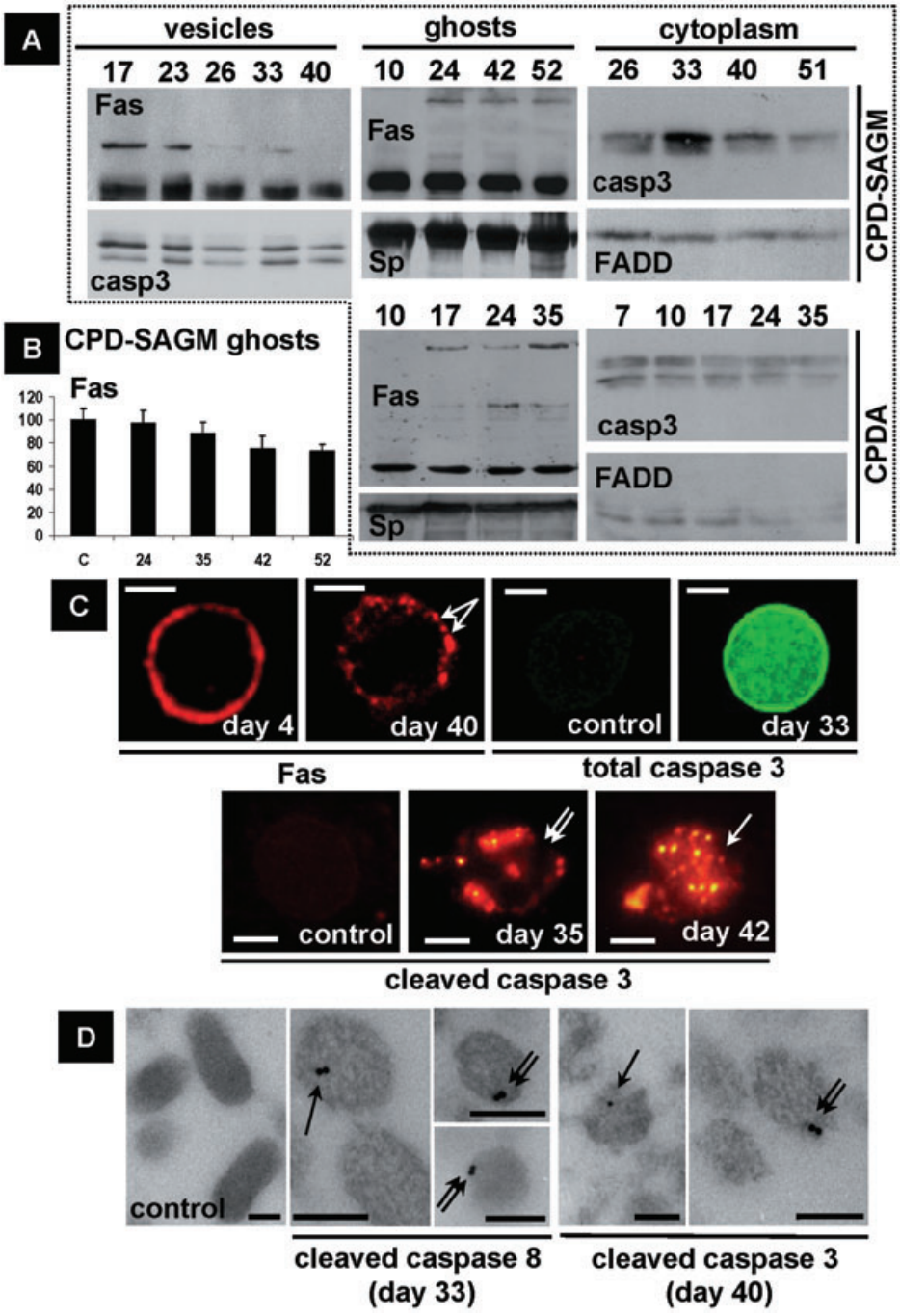

Fig. 6. Modifications in Fas-related signaling proteins in RBCs stored in CPD-SAGM or CPDA. (A) Representative immunoblot analysis of RBC vesicle, ghost, and cytoplasm preparations with antibodies specific for Fas, caspase 3 (total and activated), and FADD (spectrin was applied as loading control). (B) Densitometry analysis of Fas in the membrane of RBCs stored in CPD-SAGM. Protein levels were quantified as percentage of total per day of storage and final normalization to the controls (% of controls). Error bars = the SDs of the tested units (n = 12). (C) Fluorescent micrographs of RBCs stored in CPD-SAGM after immunostaining for Fas or caspase 3 (total and cleaved). A proportion of RBCs stored for a long period exhibited a punctuated staining pattern of Fas (arrows). The signal of cleaved caspase 3 was either dispersed into the cytoplasm (single arrow) or concentrated on the rim (double arrows) of the cells. No immunoreactivity was observed in the control parallel experiments, where the primary antibody was either omitted or replaced by preimmune serum. Bars = 3 μm. (D) Representative immunogold electron micrographs of vesicles showing the dual localization of cleaved caspase 8 and caspase 3 either in the interior (single arrow) or in the periphery of the vesicles. In control experiments (as in C) no gold particles were detected. Bars = 0.1 μm.

This association is probably driven by oxidative effects, in the context of both currently (i.e., carbonylation index of cytoskeleton proteins) and previously reported proteomic data.[26]

Variation in lipid raft–associated proteins under storage in CPD-SAGM

Normal in vivo RBC aging is further related to the loss of lipid raft proteins.[31] In continuance with previous studies in stored RBCs,[18,20,25,32] our data verified the storage-associated loss and selective exocytosis of lipid raft proteins. The sorting of nonreducible and probably oxidized stomatin oligomers into the released vesicles (Fig. 4C) further supports the suggested role of vesiculation in the disposal of modified/damaged proteins.[18,25] The movement of Ca^{2+}-dependent raft-associated proteins synexin and sorcin[33] to the membrane of CPD-SAGM cells in similarity with that reported in the CPDA-stored RBCs[18,20] suggests a storage-induced activation of Ca^{2+}-driven pathways, independently of the storage medium of choice. This is of further importance for the elucidation of the mechanisms that underlie the storage lesions,[13] since increased Ca^{2+} levels and permeability are tightly associated with RBC aging,[2] oxidative stress, microvesiculation, and stimulation of signaling pathways leading to "eryptosis."[4,5]

Effect of storage on the membrane recruitment and vesiculation of Hsps

Hsps are ubiquitous proteins that exert important biologic functions in molecular chaperoning as well as in promoting cell survival in response to stress.[34] In this study, we report a storage time–dependent increase in the membrane recruitment of Hsp70 that is detected quite early during the storage period. The duration of storage is associated to the generation of modified proteins, needing assistance by chaperones. Gudi and Gupta[35] referred that the stress-responding increase in the membrane affinity of Hsp70 involves specific interactions with the partially damaged cytoskeleton proteins, to protect and stabilize them. Accordingly, the early membrane recruitment of Hsp70 in the stored RBCs (Fig. 5A) could be proportionally driven by the early oxidative damage of cytoskeleton proteins (Figs. 1A and 3A). In the same context, the greater membrane binding of Hsp70 in the CPDA-stored RBCs compared to the age-matched CPD-SAGM–stored RBCs was probably a cellular defense response to the comparably increased oxidative insults of the membrane proteins, as has been shown in sickle RBCs.[31] The membrane delivery of Hsps in stored RBCs might be further connected to the storage-induced changes in the lipid raft proteins[36] and/or to their secretion through vesiculation, shown by the present and previous reports.[25]

Effect of storage on the membrane association of Prx2

Prx2 is an antioxidant enzyme.[37] Despite the fact that it is a preferential cytosolic protein, it binds to the RBC membrane, especially in the presence of increased reactive oxygen species and free intracellular calcium.[31,38] Storage-dependent changes in the membrane association of Prx have been shown in RBCs.[25] We currently report an increase over storage time in both the immunoblotted band of Prx2 and the electrophoretic Band 8 that has been previously described as Prx2.[39] A similar increase in Band 8 (containing Prx2, sorcin, and probably other proteins) has been reported in the RBC membrane from anemic patients exhibiting signs of protein oxidative insults.[22,40,41]

Prx2 has been shown to bind to Hb to prevent its oxidation,[42] either as a hydrogen peroxide scavenger or as a chaperone-like protein.[37] Our results, showing gradually increasing Prx2 in the CPD-SAGM–stored RBC membranes that contain fewer amounts of denatured Hb compared to the CPDA ones, support this view. The difference in the membrane association of Prx2 observed in the RBCs stored in the two media could also be attributed to the differential preservation of putative linker proteins, like the stomatin.[43]

Membrane expression of cathepsin E during storage

A progressive membrane release of cathepsin E as a soluble, activated enzyme has been shown on in vitro aged and oxidatively damaged RBCs.[44] The release has been found to be processed through membrane protein damage and breakdown, resulting from increased binding of Hb. In that context, the different levels of membrane-associated cathepsin between the CPD-SAGM– and CPDA-stored RBCs might be partially justified. Increased activation of cathepsin E has been found in RBCs treated with peroxynitrite, in association with a range of RBC senescence– and apoptosis-associated changes[45] and with the degradation of Band 3.[46]

Reduction in membrane protein ubiquitinylation and exocytosis of ubiquitin conjugates

Stored RBCs, as previously reported, are characterized by a progressive decrease in the total number of membrane-associated proteasome system proteins.[25] We now provide evidence for decreased membrane protein ubiquitination during storage, especially in the CPDA-stored RBCs. This is important in light of previous studies on sickle[47] and senescent[48] RBCs showing that ubiquitination and ubiquitinating activity of spectrin is a critical regulator of cell deformability.

According to previous studies reporting the modulation of protein ubiquitinylation by the cellular redox

status,[47,49] the observed reduction might probably be the result of diminished ubiquitination capacity of stored RBCs. On the other hand, the progressively increased release of ubiquitinated protein–containing vesicles (Fig. 5B) support the view of a more effective removal of ubiquitin conjugates after prolonged storage. The composition of the vesicles in ubiquitinated protein components suggests either the participation of cytoplasmic proteins and/or the sorting of specific membrane and cytoskeleton ubiquitinated proteins for exocytosis.

Fas-related signaling proteins in CPD-SAGM–stored RBCs

Prolonged storage of RBCs in CPDA has been associated with Fas-associated signaling proteins modifications and exocytosis.[18,20] According to both current (Fig. 6A) and previously reported data on CPDA storage,[18] the death-signaling oligomerization of Fas and the activation of caspases were less prominent in the case of CPD-SAGM age-matched cells. This finding suggests a controlled responsive activation of the Fas-associated cascade, related or unrelated to apoptosis, in RBCs exhibiting more severe and extended storage lesions, probably driven by the management of the oxidative stress.[8-10,45,50]

Moreover, we gave circumstantial evidence for the potential localization of activated caspases at submembrane sites on both cells and vesicles. This unexpected result resembles the recently reported "capping" of microscopy signals for PS and activated caspases in senescent RBCs.[11] A reasonable interpretation could be that either the caspase 3 activation or its subsequent action(s) might be occurring near by the membrane. In light of recent reports documenting the effect of Hb oxidation to the activation of caspase 3,[50] the detection of cleaved caspase 3 in the membrane areas of stored RBCs containing great amounts of crosslinked oxidized/denatured Hb cannot be excluded. Moreover, recent accumulating research data confirmed the direct effect of activated caspases on membrane proteins, resulting in the fragmentation of spectrin[6] and the cleavage[7] or clustering[10] of Band 3. In that context, our findings showing caspase activation in cells mostly presented with modifications in Band 3 and cytoskeleton proteins suggest the targeting of stimulated caspases on substrate RBC membrane components or the reverse effect of membrane and cytoskeleton remodeling on caspase activation, as already previously assumed.[11] Activation of caspases in aging or damaged RBCs could induce their erythrophagocytosis representing a safer way of cell clearance. On the other hand, the presumptive caspase-induced protein modifications might be associated with permanent remodeling of the membrane cytoskeleton that interfere with both the mechanical properties of the membrane[9] and the extent of vesiculation.[10]

Concluding remarks

In the long course of successive improvements in RBC storage solutions, ASs like SAG have been developed to provide additional volume and nutrients for longer storage and better flow of RBCs.[51] Addition of mannitol to SAG effectively reduce the hemolysis[52] and vesiculation, compared to CPDA-1,[27,28,51] probably because mannitol works as a free radical scavenger and membrane stabilizer.[53]

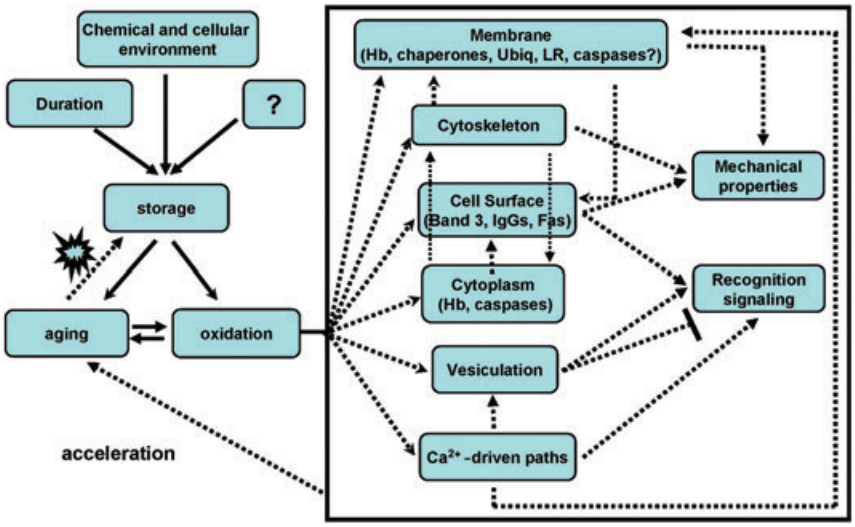

Fig. 7. Schematic representation of a model functionally connecting RBCs storage to oxidative injury and aging profile. Cell aging and protein oxidation are two main, intrinsic variables (indicated by solid arrows) of the storage lesion, interworking between each other throughout the storage period. A range of membrane, cytoplasm, and cytoskeleton modifications (enclosed by solid borders) are probably (probability is demonstrated by dashed arrows) associated with the more-or-less efficient management of the oxidative stress, that is sequentially influenced by the length, the chemical environment (i.e., AS or plasma), the cellular environment (i.e., WBCs), and probably other variables of the storage (denoted by a question mark). These modifications, which potentially interact with each other, have detrimental effects on the membrane/cellular deformability and induce (or temporarily inhibit in the case of vesiculation) the immunologic recognition of the carrier cells, establishing a population of untimely aged RBCs and biologically responding vesicles. This web of correlations, which apparently represents only a part of the overall picture, probably contributes to the configuration of the "storage lesion" cellular phenotype.

This study reports that RBCs stored in CPD-SAGM exhibited lower protein oxidative damage compared to RBCs stored in CPDA. That is probably associated with 1) the differential membrane expression of cellular stress markers like the molecular chaperones and proteasome proteins and 2) the slower and less conspicuous stimulation of common signaling pathways that trigger the recognition and erythrophagocytosis. These observations are indicative of comparatively improved cell senescence and, consequently, storage lesion profiles. The findings of the study were summarized in the schematic model of Fig. 7. At least a part of the observed modifications is anticipated to be functionally mediated by the residual white blood cells of the nonleukoreduced units. The leakage of free radicals and other biologic response modifiers from WBCs probably increases the demands on the RBCs antioxidant capacity and influence their apoptosis-like program. As a result, the changes observed in this study during storage of RBCs in SAGM and CPDA plasma might be different if leukoreduced RBCs were studied.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

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