Storage-dependent remodeling of the red blood cell membrane is associated with increased immunoglobulin G binding, lipid raft rearrangement, and caspase activation

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BACKGROUND: The elucidation of the storage lesion is important for the improvement of red blood cell (RBC) storage. Ex vivo storage is also a model system for studying cell-signaling events in the senescence and programmed cell death of RBCs. The membrane hosts critical steps in these mechanisms and undergoes widespread remodeling over the storage period.

STUDY DESIGN AND METHODS: Fresh and CPDA-stored RBCs from 21 blood donors were evaluated as whole cells, membrane ghosts, and cytoskeletons by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblotting, immunofluorescence microscopy, and in situ assays. Band 3 content, immunoglobulin G (IgG) content, specific protein movement to and from the membrane, and caspase system activation were measured.

RESULTS: During storage, Band 3 protein was aggregated and its content decreased as did the content of several lipid raft–related proteins. IgG binding to the membrane increased. Sorcin and synexin moved from the cytosol to the membrane, stomatin and flotillins left the membrane, the Fas protein was oligomerized, and caspase was activated.

CONCLUSION: The remodeling of the RBC membrane during storage includes loss and oxidative cross-linking of Band 3 as well as IgG binding. This process occurs with lipid raft development and loss and is probably driven by caspase activation. Oxidative injury appears to be an important driver of RBC aging during storage.

Red blood cells (RBC) are increasingly recognized to participate in cell-to-cell signaling and to regulate their own destruction. The RBC membrane is often the key mediator in these signaling mechanisms. Subtle modifications of RBC surface topography, including the disruption of phospholipid asymmetry[1] and the clustering of Band 3, are effective signals in molecular pathways leading to the clearance of senescent, oxidized, or pathological RBC by phagocytosis.[2] The cross-linking of Band 3 that provides a recognition site for autologous immunoglobulin G (IgG) and complement components is often associated with the denaturation of hemoglobin (Hb).[3] Phosphatidylserine (PS) externalization and erythrophagocytosis are also the result of apoptosislike death programs operating in mature RBCs in response to environmental signals or in various diseases.[4] This programmed cell death

ABBREVIATIONS: GpA = glycophorin A; HMW = high molecular weight; MW = molecular weight; PCD = programmed cell death; PS = phosphatidylserine; Red = sulforhodamine; SLP-2 = stomatinlike protein 2; VAD-FMK = valine-alanine-aspartic acid fluoro-methyl-ketone.

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(PCD) might be either Ca^{2+}/calpain- or Fas/caspase-dependent.[5,6]

Complex membrane signaling events in the RBC rely heavily on proteins resident in the lipid rafts. These membrane microdomains are enriched with glycosylphosphatidylinositol-linked proteins, stomatin (Band 7.2b), flotillins, and Band 3.[7,8] The cytoskeletal stomatinlike protein 2 (SLP-2) is thought to have a regulatory role in their organization.[9] The vesicles released upon Ca^{2+} treatment of RBC are enriched with lipid raft–associated proteins and Hb, with stomatin and synexin/sorcin being the main components of the released microvesicles and nanovesicles, respectively.[10]

The biochemical changes occur in RBC during storage are viewed as processes undermining RBC viability and reducing the effectiveness of transfusions.[11,12] RBCs lose lipids and membrane through vesiculation. They become rigid and demonstrate reduced oxygen offloading. The suspending fluid exhibits free Hb, biologically active lipids, and negatively charged microvesicles. The exovesicles are shed by the spicules of cells that have undergone echinocytic transformation after surface remodeling.[13,14] The membrane of RBCs is further characterized by disturbed phospholipid asymmetry and modifications of Band 3.[12,15] In addition, the atrophy of the endogenous antioxidant defense systems results in oxidative damage to membrane proteins, phospholipids,[16-18] and Hb when a standard oxidant stress is provided.[19]

Some of these changes are irreversible and when passing a critical threshold, effectively mark cells for destruction, either inside the storage unit or at a short time after transfusion. Examples include spherocytosis, externalization of PS, and a decrease in CD47.[12] In either case, they are undesirable cells: they fail to correct anemia due to low viability, they have a potential immunomodulatory impact, and furthermore in some cases they could up regulate cellular apoptosis or necrosis during storage.[12]

The idea of PCD of RBCs remains controversial.[20] We investigated the expression pattern of potential cell-signaling changes in RBC membrane over storage. We submit evidence for: 1) Band 3 aggregation in situ in the membrane in association with increased IgG binding, 2) gradual reduction in several integral lipid raft–associated proteins, 3) membrane translocation of cytosolic synexin and sorcin, 4) membrane receptor Fas oligomerization, and finally 5) activation of caspases after prolonged storage.

MATERIALS AND METHODS

Collection and processing of blood

Whole blood (450 ± 45 mL) from 21 eligible young blood donors was collected in citrate phosphate dextrose adenine (CPDA) double-pack container systems. After centrifugation, most of the plasma was removed and RBCs were produced (final hematocrit level, 70%-75%). Leukoreduction was not performed. The units were stored at 4°C for the whole storage period of 35 days and 1 week after expiration time and sampled in sequential time intervals of 2 to 5 days, beginning from the day of donation (Day 0). Each bag was fitted with a sterile sampling-site coupler (MacoPharma, Mouvaux, France) and mixed gently. Aliquots (6 mL) of RBCs were withdrawn through the sampling site at 4°C by use of a 19-gauge needle and attached syringe, to avoid any mechanical damage to the cells. As controls (C), samples of Days 0 to 2 of the 21 RBC units, together with RBCs freshly prepared from blood samples of 21 subjects of matching age, were used. The nonstored blood samples were collected in ethylenediaminetetraacetate and heparin anticoagulants–containing tubes. In every experiment both groups of controls were included in equal number to that of tested CPDA units. Owing to the fact that no difference was found between them in any of the experiments performed, they have consistently been referred to as "controls" throughout the text. The study gained approval from the local ethics committee and informed consent was obtained from all subjects.

Preparation of membrane ghosts and cytoskeletons

White ghosts (total RBC membrane without Hb) were prepared by hypotonic lysis of RBC in phosphate buffer as previously described,[21] containing 0.3 mmol per L phenylmethylsulfonyl fluoride or 0.2 mmol per L phenylmethylsulfonyl fluoride, 4 μg per mL leupeptin, 0.5 mg per mL di-isopropylfluorophosphate to the lysis buffer to inhibit protease activity. The membrane skeleton of RBCs is a structure experimentally defined as the insoluble residue remaining after extraction of the RBC membrane with nonionic detergents, like Triton X-100. The RBC skeleton is organized in a two-dimensional hexagonal network predominantly composed of spectrin, actin, and protein 4.1R, along with adaptor proteins like ankyrin, protein 4.2, p55, protein 4.9, adducin, tropomyosin, myosin, and tropomodulin. Membrane skeletons were prepared from the washed ghosts of stored and fresh samples by Triton X-100 extraction as previously described.[22] Protein concentration was assayed with the Bradford protein assay reagent with bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA).

Electrophoresis and Western blotting

Isolated ghost membranes and extracted cytoskeletons were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (0.72 mol/L 2-mercaptoethanol) and nonreducing conditions (without 2-mercaptoethanol) with the discon-

tinuous buffer system of Laemmli and homogenous 11 percent or 5 to 15 percent gradient slab gels. Equal amounts of protein were loaded per track of each gel. The gels were stained with Coomassie blue R-250 (10 µg/track) or with periodic acid-Schiff reagent (80 µg/track) for the detection of glycophorins and analyzed by lengthwise scanning densitometry with an image-processing program (Gel Analyzer v.1.0, Biosure, Athens, Greece). The electrophoretic protein bands were quantified in units of intensity, and the relative amount of each band was given as a percentage of total. The points in the graphs represent the mean values among the 21 tested blood donors, that is, the ratio of each band to the sum of the main bands revealed after gel staining (proportion%).

For the Western blot analysis, the proteins (10 µg of ghosts, except where otherwise stated) were electrophoretically transferred to nitrocellulose membranes and probed with monoclonal antibodies (MoAbs) to Band 3 (Sigma, Munich, Germany), sorcin (Zymed Laboratories, San Francisco, CA; 40 µg of ghosts), flotillin-1, flotillin-2, SLP-2 (20 µg of cytoskeletons), synexin, and Fas (35 µg of ghosts; all from BD Biosciences, San Diego, CA) and polyclonal anti-human IgG (horseradish peroxidase [HRP]–conjugated, Sigma), with standard immunoblotting techniques as previously described.[23] After the application of species-specific HRP-conjugated secondary antibodies (anti-rabbit [Amersham-Pharmacia Biotechnology, Piscataway, NJ] at 1:8,000 and anti-mouse [Dako, Denmark] at 1:10,000), appropriately diluted in blocking solution, the immunoblots were developed with an enhanced chemiluminescence reagent kit (Amersham Biosciences, Piscataway, NJ). Reference antibodies against RBC membrane proteins were used as internal loading controls (anti-spectrin and anti-actin polyclonal antibodies from Sigma). Immunoblotted protein bands were quantified by scanning densitometry and presented as means compared to control samples, sample total protein, or internal reference proteins as noted (relative proportion). The molecular weight (MW) definition of unknown bands was compared against a lane of MW protein standards (Fermentas, Hanover, MD). The immunoblots shown are derived from different representative donors.

Indirect immunofluorescence assays

Human RBCs either fresh or stored in CPDA were allowed to adhere to 0.1 percent poly-L-lysine–coated glass coverslips for 30 minutes at room temperature. Immunofluorescence assays were performed as previously described,[6] with slight modifications. The cells were then fixed with 90 percent methanol in phosphate-buffered saline (PBS), pH 7.4 for 1 minute and permeabilized for 1 minute in the same solution containing 0.05 percent Triton X-100. After extensive washing and blocking in PBS containing 3 percent BSA and 0.1 percent Tween 20 for 30 minutes, the cells were probed with MoAbs to Band 3 (Sigma), sorcin (Zymed), synexin, and activated caspase 3 (BD Biosciences, 1:25-1:500, 30 min). After being washed with PBS containing 0.1 percent Tween 20, cells were incubated with secondary antibodies conjugated to fluorescein isothiocyanate (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), for 30 minutes. Finally, the slides were washed as above, mounted in antifading mounting medium and observed under a fluorescent microscope (Eclipse TE 2000-S, Nikon, Melville, NY). Routine procedures were applied to demonstrate the specificity of the 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, each using RBCs derived from different donors and an equal number of controls (fresh samples and those stored for 0-2 days). All the images were photographed with 100× objective and the same exposure time.

In situ assay for activated caspases

Activated caspases were detected with the sulforhodamine (Red)-valine-alanine-aspartic acid fluoromethyl-ketone (VAD-FMK) commercially available Pancaspase kit (Oncogene Research Products, San Diego, CA). In this protocol, the Red derivative of VAD-FMK, a potent chemically modified inhibitor of caspase activity, enters the living cells and binds irreversibly to active caspases, permitting the detection of activated caspases in situ in apoptotic cells. Cells were rinsed briefly with PBS and incubated immediately with the Red-VAD-FMK (1:500 in PBS, 30 min, 25°C in the dark). The slides were subsequently rinsed with 1× wash buffer three times for 5 minutes each, mounted in antifading mounting medium, and examined under a fluorescence microscope (Eclipse TE 2000-S, Nikon) with the appropriate rhodamine filter.

Statistical analysis

All quantitative data from SDS-PAGE and immunoblotting analysis are presented in the graphs as the mean ± standard deviation (SD). The mean of the 21 units examined was used to compare protein levels each day of storage against those presented by the reference samples (fresh samples or Storage Days 0-2). Protein deficiency or excess was considered significant whenever the mean value of any band was lower or higher than the mean value ±2× SD, respectively, presented by the reference samples (fresh samples and Storage Days 0-2) for the same protein.

RESULTS

Band 3 clustering and membrane-bound IgGs

Starting on Day 4, a small but important decrease in Band 3 protein (88%-94% of control values) was evident by SDS-PAGE analysis of ghosts isolated from stored RBCs in relation to the duration of storage (Fig. 1A). After 10 days of storage, high-molecular-weight (HMW) bands of 180 to 200 kDa were detected by immunoblotting with MoAb against Band 3 under reducing conditions (Fig. 1B). The HMW band was detected in increasing amounts toward the end of the storage period and it was basically resistant to increased concentrations of the reducing agent (data not shown) suggesting that it is stabilized by nonreducible linkages (such as amides or free radical-generated adducts, e.g., bityrosine). Subsequent analysis of Band 3 in situ by indirect immunofluorescence microscopy revealed the presence of Band 3 aggregates in 15 to 20 percent of RBCs that had been stored for 30 days in CPDA (Fig. 1F). Several cells exhibited membrane malformations like exvaginations and invaginations after prolonged storage (Fig. 1F).

To determine whether the aggregation of Band 3 was associated with increased IgG binding in stored RBCs, we tested the isolated ghost membranes for total endogenous IgG binding. Under reducing conditions, IgG-specific immunoblotted bands were detected in ghosts only after the 30th day of storage (Fig. 1C). To estimate the cross-linked membrane-bound endogenous IgGs, we repeated the experiment under nonreducing conditions. In that case, faint IgG bands were immunodetected in ghosts beginning from the 4th day of storage, yet in the last days they showed a sharp increase (Fig. 1D). For identical quantities of loaded ghosts (Fig. 1E), the control preparations (from fresh and stored RBCs) did not contain detectable quantities of either Band 3 oligomers (Fig. 1B) or IgGs (Figs. 1C and 1D).

Lipid raft–associated proteins in the RBC membrane during storage

Electrophoretic and immunoblotting data showed that the major integral proteins of the RBC lipid rafts flotillins and stomatin exhibited a statistically important elimination from the membrane during storage. While flotillin-1 was slightly decreased (10% below control) only in the last days of storage and especially (40% below control) in the outdated samples (Fig. 2A), the flotillin-2 showed a significant and progressive decrease (8%-28% below control value) at 10 days onward (Fig. 2B), accompanied by the appearance of an immunoreactive HMW band of 90 kDa (Fig. 2B, inset). The electrophoretic Band 7, which contains Band 7.2b of stomatin, exhibited a wider range gradual decrease from the 22nd to the last day of storage (30%-45% below control value; Fig. 2C). Densitometric

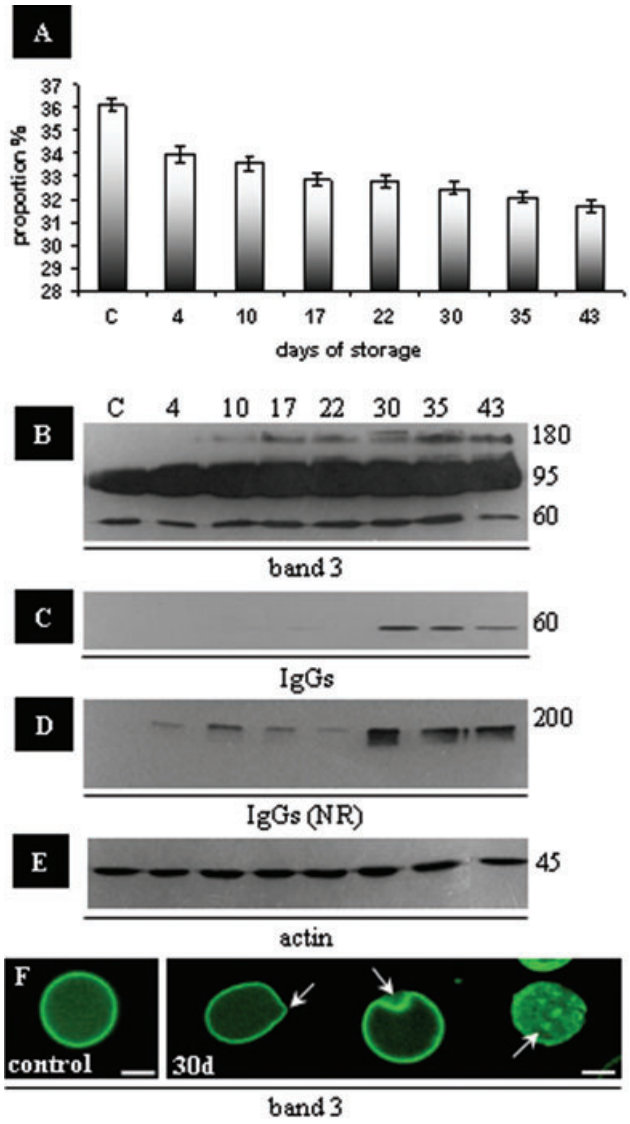

Fig. 1. Band 3 decrease and clustering and progressive binding of IgGs in the membranes of stored RBCs. (A) Densitometry analysis of Band 3 performed on SDS-PAGE gels. (B-E) Western blotting analysis of the ghosts with MoAb against Band 3 (B) or polyclonal antibody against human IgGs (C, D) under reducing or nonreducing (NR) conditions, as indicated. Blots were reprobed with anti-actin polyclonal antibody (E) to ensure equal loading of the gels. The duration of storage is indicated in days at the top of the blot figure. MW of the proteins is shown in kDa (right side). 60 kDa = the prospective main proteolytic fragment of Band 3. (F) Fluorescent micrographs of control RBC (left) or those stored for 30 days (right) after immunostaining with MoAb to Band 3 demonstrating the presence of protein clusters and membrane malformations (arrows) after prolonged storage in CPDA. Bars = 3 μm.

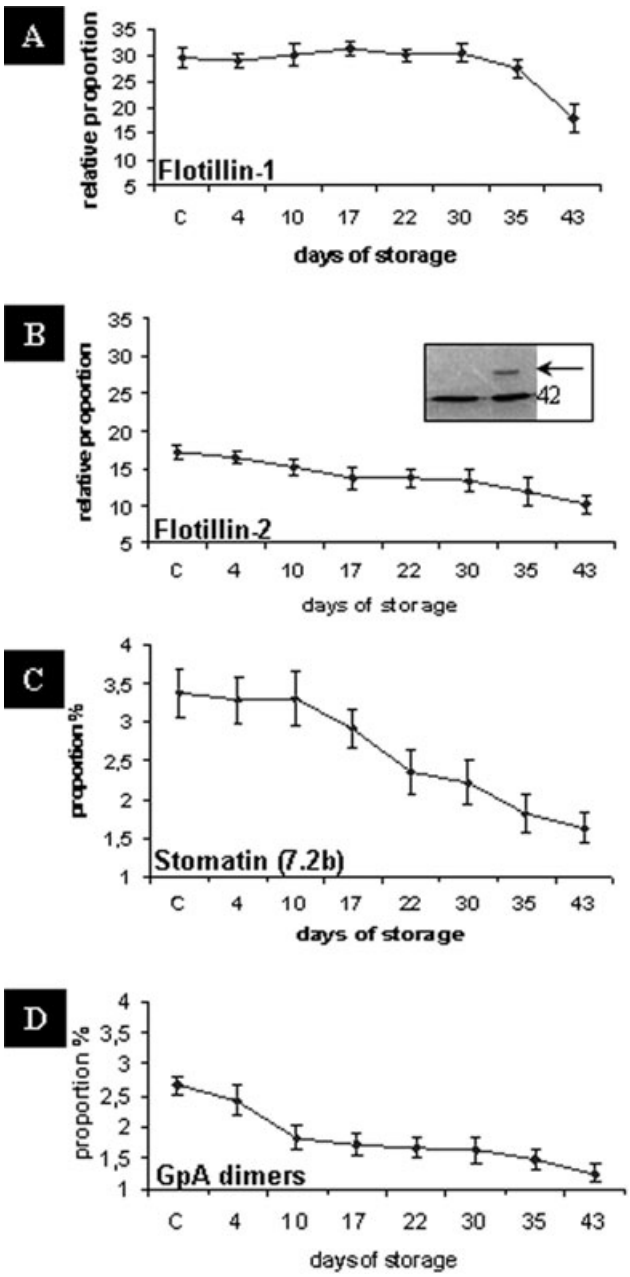

Fig. 2. The expression of the integral lipid raft–associated proteins stomatin, flotillins, and GpA in RBC membrane during storage. Densitometry analysis of flotillins (-1 and -2 in A and B, respectively) performed on enhanced chemiluminescence–developed immunoblotting films and stomatin along with GpA dimers (C and D, respectively) performed on SDS-PAGE gels. (Inset) Immunoblotting analysis performed in the ghosts of a representative donor for flotillin-2 after 2 and 30 days of RBC storage. The aberrant HMW band after prolonged storage is indicated by an arrow.

analysis of the membrane sialoglycoproteins after periodic acid-Schiff reagent staining of the gels revealed also a reduction (32%-45% below control values) in their total amount at 10 days onward (data not shown). The measured deficiency mainly concerns the glycophorin A (GpA) and B dimers (Fig. 2D for GpA2) and not the monomeric species of glycophorin A, C, and D that were unaffected by storage (data not shown).

Sorcin and synexin immunoreactivity was present in the membrane of stored cells after the 30th or 10th days of storage, respectively (Figs. 3A and 3B). Subsequent in situ analysis of those proteins in stored RBCs by indirect immunofluorescence microscopy was in line with the immunoblotting results. In control RBCs or those stored for a short period, sorcin and synexin exhibited a diffused, primarily cytoplasmic localization (Figs. 3E and 3F, respectively). In contrast, after prolonged storage a shift of the signal to the membrane was observed in a proportion of cells (below 10%), suggesting membrane localization of both proteins, either in the form of membrane spots (for sorcin, Fig. 3E) or as a membrane ring (for synexin, Fig. 3F). Finally, the lipid raft regulator SLP-2 showed a small but significant and progressive decrease in the cytoskeletons of stored RBCs, at 10 days onward. Only traces of SLP-2 were detected in the cytoskeletons of RBCs from the late storage period and from the outdated units (Fig. 3C).

Detection of active caspases and Fas oligomerization in RBC stored long-term in CPDA

By means of indirect immunofluorescence, we detected scattering RBCs that were immunopositive for active caspase 3 in units stored for 30 days in CPDA (Fig. 4A). Furthermore, by use of the fluorescent in situ pancaspase marker Red-VED-FMK, we verified the detection of activated caspases in apparently apoptotic RBCs that had been stored for 30 to 35 days in the unit (Fig. 4B). The fresh RBCs or those stored for short periods are completely devoid of any traceable signal of caspase activity.

To examine the implication of the Fas membrane receptor in the caspase-dependent activation of apoptosis, we examined the membranes of the RBCs stored for 30 to 35 days in CPDA for the presence of Fas. Under reducing conditions, the monomeric band of membrane Fas (48 kDa) was decreased in stored cells compared to the controls (Fig. 4C, left blot). In contrast, under nonreducing conditions (Fig. 4C, middle blot), a double-HMW band (100-200 kDa) of cross-linked Fas could be seen in the membranes of RBCs stored for a long period. In all the units under examination, the membranes of unstored cells or those stored for a short period (up to 3 days) contained only traces or no Fas oligomers.

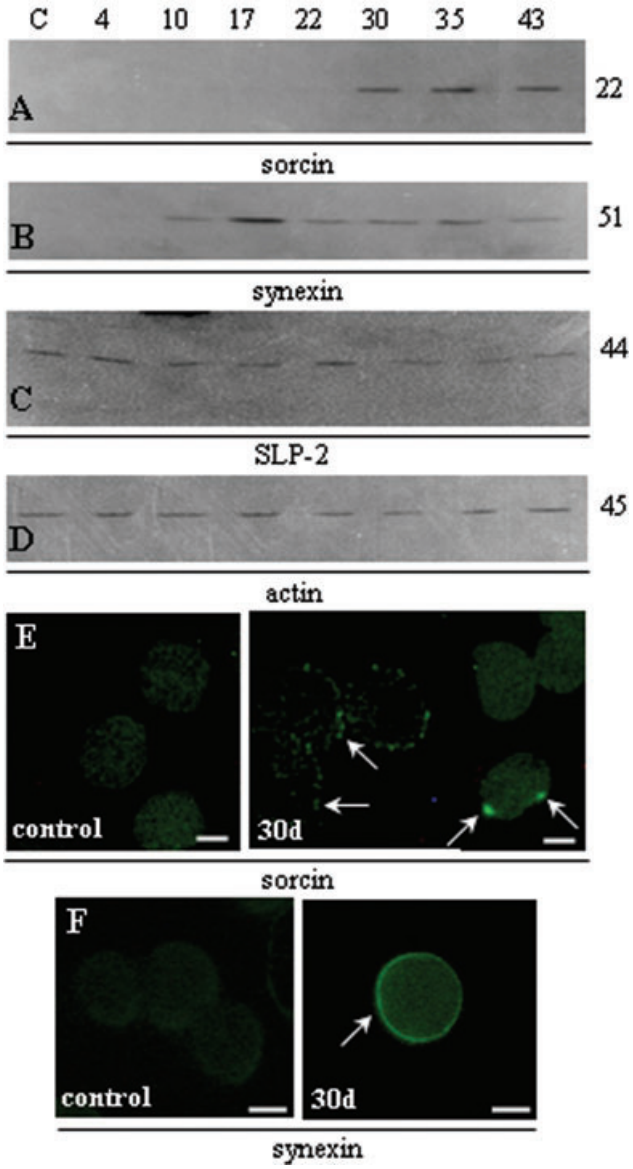

Fig. 3. Variation of cytosolic and peripheral lipid raft–associated proteins during storage: elimination of SLP-2 and translocation of sorcin and synexin to the membrane of stored RBC. (A, B) Western blot analysis of the ghosts with MoAbs against sorcin and synexin under reducing conditions. (C) Immunoblotting analysis of cytoskeletons with SLP-2–specific MoAb. Blots were reprobed with anti-actin antibody (D) to ensure equal loading of the gels. The duration of storage is indicated in days at the top of the blot figure. MW of the proteins is shown in kDa (right side). (E, F) Fluorescent micrographs of control RBCs (left) or those stored for 30 days (right) after immunostaining with anti-sorcin or anti-synexin MoAbs, respectively, demonstrating the translocation of both proteins to the membrane (arrows) after prolonged storage in CPDA. Bars = 3 μm.

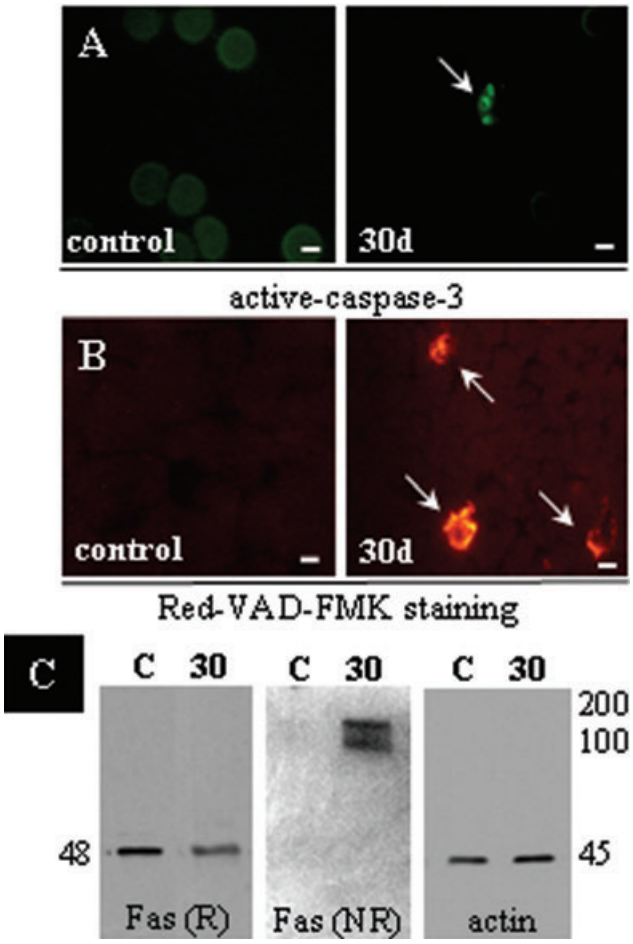

Fig. 4. Presence of an active caspase-based apoptotic pathway and expression of Fas in the membranes of stored RBC. (A) Fluorescent micrographs of fresh RBC (left) or those stored in CPDA for 30 days (right) after immunostaining with MoAb specific for active caspase 3. A positive signal was observed in a subpopulation of stored cells (arrow). (B) Fluorescent micrographs of fresh RBCs (left) or those stored in CPDA for 30 days (right) after their incubation with the Red-VAD-FMK staining reagent of the active caspase in situ assay. Strong staining signals reflecting the occurrence of active caspases were observed in a subpopulation of RBCs after prolonged storage in nonleukodepleted CPDA units (arrows). (C) Immunoblotting analysis of ghosts with MoAb against Fas under reducing (R) and nonreducing (NR) conditions. The RBCs were stored for 30 days in CPDA. Actin was used as an internal loading control. MW of the proteins is shown in kDa. Bars = 3 μm.

DISCUSSION

We looked for molecular modifications of the RBC membrane that serve as markers for processes associated with reduced function and survival.

Aggregation of Band 3

First, we demonstrated the progressive aggregation of Band 3 throughout the storage period. The modifications in Band 3 represent a permanent surface-remodeling event with disorganizing impact on the membrane. They have been associated with the oxidative susceptibility of stored RBCs[15] and the increased susceptibility to anti-Band 3 IgG autoantibody binding.[24] In senescent or oxidized RBCs, the aggregation of Band 3 is mostly due to oxidative insults and to the tight association of hemichromes with the cytoplasmic domain of the protein.[2,3] Therefore, it is reasonable to speculate that the clustering of Band 3 during storage is correlated with the withdrawal of the antioxidant RBC defense.[17]

IgG binding

The aggregation of Band 3 further represents a signaling modification leading to RBC phagocytosis, after autologous IgG and complement binding.[2] The rate of removal of transfused RBCs from the recipient's circulation can be delayed when the complement has been removed from the stored units.[25] Increased quantities of cell-bound total IgGs have been reported in stored RBCs after incubation with purified autologous IgGs.[26] Furthermore, enhanced susceptibility to anti-Band 3 IgG autoantibody binding, partially associated with decreased glutathione levels, has been estimated by enzyme immunoassay in RBCs isolated from stored blood.[23] In this study, we observed by direct immunoblotting the progressive increase in total endogenous IgGs in situ in the membrane of RBCs throughout the storage period. In harmony with previous reports,[23] the binding of IgGs was not absolutely disulfide-dependent, suggesting the additional contribution of other oxidative mechanisms to binding site generation. The reduction in the intensity of the IgG band in the membranes of the outdated samples might be explained in part by the progressively increased removal of membrane vesicles containing IgGs and fragment C3b of the complement.[27]

Partial loss of Band 3 and glycophorins

Microvesiculation has been correlated with membrane lipid asymmetry alterations associated with macrophage recognition.[28] Apart from lipids and Hb, the released exovesicles were found to contain several membrane proteins, including glycophorins and Band 3.[13,27,29] Consequently, continuing exovesiculation of stored cells leads to alteration of membrane composition,[30] with the membrane exhibiting a depletion of selectively sorted proteins that have gone to the vesicles released. The partial loss of Band 3 and glycophorins, shown in our samples, might be one instance.[15] Our finding regarding the selective loss of GpA2 argues strongly in favor of this type of elimination, consistent with previous studies showing asymmetric representation of glycophorins in the storage-induced released vesicles.[30] The decline in the expression of GpA could be additionally attributed to the detrimental effect of residual white blood cells (WBCs).[31]

Lipid raft–associated proteins

The lipid rafts participate in many signaling and sorting processes in RBC.[32] Therefore, their remodeling during storage shown in this study probably represents either disturbed or changed cell functions and an onset of signaling pathways.

The gradual reduction in SLP-2 in the cytoskeletons of stored RBCs seems to follow the loss of the integral lipid raft–associated proteins. This is consistent with previous studies suggesting a role for SLP-2 in the organization of lipid raft through the anchoring of stomatin or other membrane proteins to the cytoskeleton.[9] The disruption of this putative critical junction[33] might influence both the mechanical properties of RBC and the vesiculation process, because protein sorting during membrane vesiculation is regulated, in larger part, by interactions between integral proteins and the spectrin-based membrane skeleton.[34] The loss of SLP-2 might be caused by accumulative functional defects that affect its interaction with the cytoskeletons. In light of the ease with which SLP-2 can be oxidatively cross-linked to itself or to other proteins,[9] the above-mentioned defect could represent another storage-induced oxidative modification in membrane components, analogous to those previously seen in stored cells.[18]

The calcium-induced vesiculation of RBCs is a lipid raft–based process.[10] Although we did not analyze the exovesicles, the above-mentioned modifications in lipid rafts are probably suggestive of their involvement in the exovesiculation of stored cells. Increased Ca^{2+} levels and permeability have been implicated in the RBC storage lesion.[11,12] Furthermore, it has been shown that RBC aging is accompanied by an increase in cytosolic Ca^{2+} activity[35] and that oxidative stress could enhance Ca^{2+} entry.[5] Synexin and sorcin translocate to the membrane in a Ca^{2+}-dependent manner, playing a role in the signal transduction.[36] Based on these observations and on our results, it seems probable that storage triggers the translocation of synexin and sorcin from the cytosol to the membrane in a calcium-dependent manner. Although the involvement of another mechanism cannot be excluded, the reported changes do suggest an increase in Ca^{2+} levels during the middle and late periods of storage.

Apoptosis

The role of PCD in transfusion medicine has been considered minimal and restricted to the residual WBCs of RBC

units. The apoptosis of WBCs[37] and the release of biologic response modifiers might be associated with adverse effects on RBCs, apoptotic events in sensitive target cells, and posttransfusion immune response or suppression effects.[38] In recent years it has become evident that a part of the apoptotic machinery active in nucleated cells exists in mature RBCs.[39] Oxidative stress and RBC aging lead to stimulation of caspases 3 and 8; colocalization of Fas, Fas-L, FADD, and caspase 8 to rafts; and degradation of Band 3.[6,40,41]

The role of senescence and death mechanisms in stored RBCs is poorly understood. Although PS externalization has been shown in cases of in vitro RBC storage and vesiculation,[28] conflicting results have appeared in studies carried out in blood bank ex vivo conditions.[12,31,42] Older studies have asserted that the caspases of mature RBCs can be functionally active only in vitro and not in intact RBCs, either during prolonged storage or in response to various proapoptotic stimuli.[39] Our studies have shown that in conditions used for transfusion, the caspase 3 and probably other caspases can be activated in whole cells. The divergence between the two studies can be explained in part by the different conditions of storage and analysis followed. Indeed, in contrast to our study, Berg and coworkers[39] did not observe considerable surface expression of Fas.

Furthermore, we showed that the Fas undergoes some modifications, namely, reduced levels of surface expression and increased oligomerization. The death-signaling formation of high-order Fas oligomers has been reported in lysates of aged or oxidized RBCs after incubation with thiol-cleavable cross-linkers.[6] Although we did not describe in detail the nature of the observed Fas modifications in stored RBCs, it is interesting that their appearance coincides with that of activated caspases. The enhanced oligomerization of Fas might occur either through oxidation or through disturbances of lipid raft microdomains.[6] These findings deserve attention in the light of reports documenting the presence of functional soluble Fas ligand released by the residual WBCs in the cell supernatants after prolonged storage.[43]

The physiologic significance of an active PCD cascade in stored RBCs could be multifarious. It could play a role as a safe clearance mode for dysfunctional RBCs before detrimental hemolysis. In contrast, the apoptotic RBCs might as well represent a fraction of prematurely dead ex vivo cells that could affect sensitive target cells before and after their transfusion. Additional experiments on storage will now be needed to investigate the potential modulation of the PCD machinery through therapeutic intervention.

In conclusion, our findings indicate that the remodeling of the RBC membrane is associated with Band 3 loss and oxidative cross-linking, IgG binding, loss of lipid raft–associated components and the probable Ca^{2+}-dependent recruitment of others, modification of membrane-expressed Fas, and finally, caspase system activation. This study shows that several RBC signaling and alternative clearance pathways operate during storage. Comprehensive studies of these pathways in stored RBCs, however, still need to be undertaken.

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