RBC-derived vesicles during storage: ultrastructure, protein composition, oxidation, and signaling components

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BACKGROUND: Red cells (RBCs) lose membrane in vivo, under certain conditions in vitro, and during the ex vivo storage of whole blood, by releasing vesicles. The vesiculation of the RBCs is a part of the storage lesion. The protein composition of the vesicles generated during storage of banked RBCs has not been studied in detail.

STUDY DESIGN AND METHODS: Vesicles were isolated from the plasma of nonleukoreduced RBC units in citrate-phosphate-dextrose-adenine, at eight time points of the storage period and shortly afterward. The degree of vesiculation, ultrastructure, oxidation status, and protein composition of the vesicles were evaluated by means of electron microscopy and immunoblotting. RBCs and ghost membranes were investigated as controls.

RESULTS: The total protein content of the vesicle fraction and the size of the vesicles increased but their structural integrity decreased over time. The oxidation index of the vesicles released up to Day 21 of storage was greater than that of the membrane ghosts of the corresponding intact RBCs. The vesicles contain aggregated hemoglobin, band 3, and lipid raft proteins, including flotillins. They also contain Fas, FADD, procaspases 3 and 8, caspase 8 and caspase 3 cleavage products (after the 10th day), CD47 (after the 17th day), and immunoglobulin G.

CONCLUSION: These data indicate that the vesicles released during storage of RBCs contain lipid raft proteins and oxidized or reactive signaling components commonly associated with the senescent RBCs. Vesiculation during storage of RBCs may enable the RBC to shed altered or harmful material.

Red cells (RBCs) lose membrane in vivo,[1-3] after ATP depletion or rise of cytosolic Ca^{2+} in vitro,[4,5] and during ex vivo whole blood storage,[6] by the blebbing of vesicles from the spicules of cells that have undergone echinocytic transformation. The membranes of both microvesicles and nanovesicles shed from RBCs after Ca^{2+}-induced vesiculation contain lipid rafts.[4] In vivo, the RBC-derived microvesicles expose phosphatidylserine and are rapidly removed from the circulation by means of scavenger receptors of Kupffer cells.[2]

Microvesicles generated during the storage of banked RBC units are enriched in hemoglobin (Hb) and acetylcholinesterase and contain lipids, band 3 protein, glycophorin A (GpA), and actin but are essentially free of spectrin and ankyrin.[7] The vesiculation contributes to irreversible cell shape and membrane changes. The resulting dense, poorly deformable spherocytes are quickly removed from the circulation when transfused.[8] Improvements in blood storage conditions that lead to increased in vivo survival also result in reduced accumulation of microvesicles.[9] Oreskovic and colleagues[10] showed that

ABBREVIATIONS: GpA = glycophorin A; GpC = glycophorin C; MW = molecular weight.

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vesicles shed by RBCs during storage express blood group antigens. Thus, the investigation of accumulation and composition as well as the potentially pathogenic effects of microvesicles generated during blood product processing and storage is of primary interest for transfusion medicine.[11]

During the past decade, a general consensus has been established on the mechanisms by which senescent RBCs are removed from the circulation. Although the identity of those mechanisms is still a subject of discussion, there is evidence that RBC aging leads to the binding of autologous immunoglobulin G (IgG) followed by recognition and phagocytosis mainly by Kupffer cells in the liver. Removal may be promoted by the exposure of phosphatidylserine at the RBC surface and by the appearance of senescent RBC-specific antigens, probably originated either from aggregated or from proteolyzed band 3. In these theories, there is a central role for oxidation as a causative event.[12] Recently a consensus has emerged that RBC aging is a form of apoptosis that is concentrated in the cell membrane.[12] At least a part of the apoptotic machinery active in nucleated cells exists in mature RBCs and plays a role in regulating RBC clearance from the circulation.[13] RBCs suffer protein-oxidative damages during storage[14-16] and progressively exhibit most of the "aging" events that are parts of signaling pathways leading to erythrophagocytosis, namely, the activation of caspases, aggregation of band 3, and membrane binding of denatured Hb and IgG.[17]

Our previous studies on the remodeling of the RBC membrane during storage of RBC units[17] gave us reasons to suspect the participation of lipid raft proteins into the released vesicles. In this study, we report that vesicles released during storage of RBC units vary in structure, protein composition, and oxidative index. They contain lipid raft proteins as well as phagocytosis-inducing and apoptosis-related signaling molecules.

MATERIALS AND METHODS

Collection and processing of blood

Whole blood (450 ± 50 mL) from 21 eligible young blood donors was collected in citrate-phosphate-dextrose-adenine (CPDA) double-pack container systems. Most of the plasma was removed and RBCs (260 ± 40 mL) were produced (hematocrit [Hct] 74.20 ± 2.70%, RBC count $8.25 \times 10^{12} \pm 0.34 \times 10^{12}$/L, Hb level 23.60 ± 1.15 g/dL, mean cell volume 89.60 ± 2.01 fL, mean corpuscular Hb concentration 29.10 ± 0.81 pg, mean corpuscular Hb concentration 32.50 ± 0.23 g/dL, red cell distribution width 15.90 ± 0.21%, WBC count $9.50 \times 10^9 \pm 1.10 \times 10^9$/L, PLT count $485.50 \times 10^9 \pm 78.81 \times 10^9$/L). Leukoreduction was not performed. The units were stored at 4°C according to the standard banking procedures for the storage period of 35 days and 1 week afterward. The bags were fitted with a sterile sampling-site coupler (MacoPharma, Mouvaux, France), and aliquots were withdrawn by use of a 19-gauge needle at regular time points. The sequential sampling of the units results in progressive volume decrease; therefore, the experimental units might not represent accurately the intact units under normal banking conditions. To diminish any sampling effect that might compromise the results, the 21 examined units were separated as following: 19 units were used for the biochemical analysis and sampled in two groups for Days 1, 11, 21, and 34 and 7, 17, 27, and 45, respectively, while 2 units were used for the electron microscopy analysis and sampled for Days 1 and 35 of storage. Furthermore, 4 unsampled outdated units of RBCs were also tested to evaluate the effect of multisampling, but no significant differences were detected (in total vesicular protein content, structure, and protein composition, data not shown). 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.

Isolation of vesicles and white ghosts

The vesicles were isolated from the plasma of the RBCs bags by high-speed centrifugation as previously described.[18] Briefly, a centrifuged Hct from each unit was done and 55-mL aliquots were removed and centrifuged at $2000 \times g$ at 4°C. The supernatant was centrifuged once again to ensure the absence of any RBCs and immediately filtered through sterile 0.8-µm pore size syringe-driven nitrocellulose filter units (Millipore, Carrigtwohill, County Cork, Ireland). The supernatant was ultracentrifuged at $37{,}000 \times g$ at 4°C for 1 hour, and the pellet of vesicles was resuspended in PBS and ultracentrifuged twice under the same conditions. Light- and dark-field microscopy disclosed no residual RBCs or RBC membranes (data not shown). After the addition of protease inhibitors mix (protease inhibitor cocktail, Sigma, St Louis, MO), the protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Quantification of the extent of vesiculation was based on the total protein content of the vesicle suspensions and expressed relative to the volume of the RBC unit taking into account the volume of the sampled bags, the volume of the supernatant, and the variation in the Hct (M. O'Leary, written communication, Hoxworth Blood Center, Cincinnati, OH, 2006). The samples of the first days of donation (0-2 days) did not yield sufficient vesicle material for biochemical analysis; hence the sampling of most units began after the first week of storage. RBC membranes prepared by hypotonic lysis and largely devoid of Hb (white ghosts) were isolated from RBCs as previously described.[17]

Electrophoresis and immunoblotting analysis

Equal amounts (7 μg) of total protein of vesicle and ghost membranes fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (0.72 mol/L 2-mercaptoethanol) or nonreducing conditions. Coomassie brilliant blue R-250 and silver staining were used to stain the protein bands. Immunoblotting analysis was performed as previously described.[19] Primary monoclonal antibodies (MoAbs) and polyclonal antibodies for the following proteins were used: band 3, GpA, glycophorin C (GpC), spectrin, Gs-α, actin, and anti-human IgG horseradish peroxidase (HRP)-conjugated (Sigma, Munich, Germany); sorcin (Zymed Laboratories, San Francisco, CA); flotillin-1, flotillin-2, synexin, and FADD (BD Biosciences, San Diego, CA); aquaporin-1 (AbD Serotec, Oxford, UK); caspase 8 and caspase 3 that detect both the full-length caspases and the cleaved fragments, as well as cleaved caspase 8 (Cell Signaling Technology, Beverley, MA); Fas/CD95 and CD47 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-Hb A (Europa Bioproducts, Cambridge, UK). Protein 4.1R and pallidin (4.2) antiserum samples were 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 used were anti-rabbit (GE Healthcare Amersham, 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 immunoblots was performed by lengthwise scanning densitometry using an image-processing program (Gel Analyzer v.1.0, Biosure, Athens, Greece). Individual protein levels were quantified as percentage of total blotted proteins per day of storage. This relative proportion, further normalized to the total protein content of the vesicle fraction (mg of vesicular protein/mL of RBCs), is presented in the figures, as the mean and the standard deviations (SDs) of the units examined each day of storage.

Estimation of protein-oxidative modification in vesicles

Equal amounts of total vesicular protein collected from the units of RBCs at different time points of storage 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.[15] For quantification purposes, the oxidative index was used, that is, the relative percentage of the densitometric values of the oxyblot bands to the total protein bands per day. The results are expressed as the mean oxidative index, normalized for both the extent of vesiculation (i.e., total vesicular protein/volume unit of RBCs) and the oxidative index of the corresponding ghost membranes (equal amounts of total protein).

Conventional electron microscopy and immunogold localization

Fresh aliquots of RBCs and vesicles were prepared from two, nonsampled RBC units stored for either 1 or 35 days. The electron microscopy analysis was performed as described elsewhere.[20] For conventional electron microscopy, the vesicles were fixed with 4 percent paraformaldehyde (Polysciences, Inc., Warrington, PA), 2.5 percent glutaraldehyde (Serva, Heidelberg, Germany) in 0.1 mol per L sodium cacodylate buffer, pH 7.4, and embedded in epoxy resins. For immunoelectron microscopic studies, RBCs and vesicles were fixed in 4 percent paraformaldehyde, 0.1 percent glutaraldehyde in PBS, pH 7.2, and embedded in unicryl acrylic resin (British Biocell International, Cardiff, Wales, UK). For electron microscopy immunolabeling, the grids were blocked with bovine serum albumin and incubated with MoAb against human Hb (Immuno-rx, Augusta, GA) and 10- or 15-nm gold-conjugated probes (British Biocell International). Routine procedures were applied as controls (data not shown) to demonstrate the specificity of the antibody used.[20] Sections were viewed in an electron microscope (Model EM 900, Zeiss, Oberkochen, Germany) at 80-kV accelerating voltage.

Statistical analysis

The data on the vesicles' size were analyzed by Student's t test. Differences with p values smaller than 0.05 were considered significant. Calculations on the data were performed with computer software (Excel, Microsoft Corp., Redmond, WA).

RESULTS

Ultrastructure and origin of the vesicles

The RBC vesicles analyzed by electron microscopy (Fig. 1A; n = 300 vesicles in five experiments per sample) showed an apparent size ranging from 50 to 210 nm (mean, 150 nm) in diameter, as described previously.[3-5] According to the size estimation and the statistical analysis, we concluded that microvesicles (and not

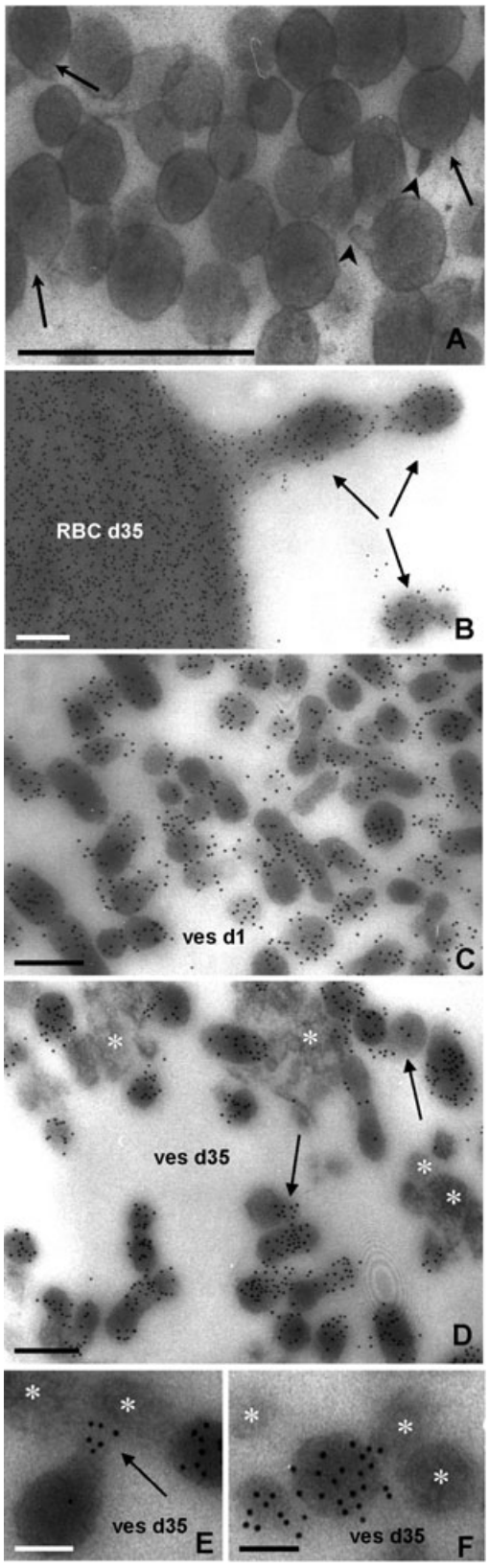

Fig. 1. Conventional and immunogold electron microscopy of RBCs and vesicles released after short (1 day, C) or prolonged (35 days, the rest of images) storage in CPDA. (A) Ultrastructural appearance of a microvesicle preparation collected after prolonged storage. Of particular interest, the degeneration of the membrane that is characterized by pedicles and protrusions (arrowheads) or gaps resulting in the shedding of the enclosed material (arrows; bar, 0.5 μm). (B) Dense gold particles representing Hb in the main RBC body and the released vesicles (arrows) in a 35-day (d35) RBC preparation (bar, 0.2 μm). (C) Hb labeling of vesicles (ves) released from RBCs after 1 day (d1) in storage. Of special note, the fine membrane structure and the positive labeling of all particles (bar, 0.2 μm). (D-F) Hb labeling of vesicles collected from a unit stored for 35 days (d35). The asterisks denote the absence of gold particles in degenerated vesicles and the arrows signify vesicles being in the process of shedding Hb (bars, 0.2, 0.1, and 0.1 μm, respectively).

nanovesicles) were mainly harvested. The vesicles released in the supernatant after a short banking period exhibited a significant difference ($p < 0.003$) in size (mean, 137 ± 76 nm; 50-nm minimum) compared to those released at the end of the storage period (mean, 160 ± 47 nm; 77-nm minimum). Vesicles collected the last day of storage exhibited the previously reported "tail" structures[5,6] (arrowheads in Fig. 1A) and membrane gaps through which the encapsulated material seems to be shed (arrows in Fig. 1A). In other cases, the vesicles are partly or fully degenerated, showing hazy membrane outlines and diffused vesicular material.

The isolated vesicles released after short (Fig. 1C) or long (Figs. 1D-1F) storage were labeled with Hb-specific gold particles in electron microscopy immunogold in situ assays, in similarity with their paternal RBCs (Fig. 1B), confirming that they were RBC-derived. Despite that, a proportion of vesicles collected after long storage was not labeled for Hb (asterisks in Figs. 1D-1F). The majority of them exhibited morphologic signs of degeneration (Fig. 1F). Considering that we did not use any WBC- or PLT-specific markers, we cannot exclude the possibility that the unlabeled vesicles are WBC- or PLT-derived, although we detected Hb-specific gold particles in the material shed by many vesicles stored for long in CPDA (arrows in Figs. 1D and 1E).

Total vesicular protein content, electrophoresis pattern, and expression of the main RBC proteins

As expected, the vesicular protein content was progressively increased over time. Compared to RBCs stored for 7 days (9 μg/mL of RBCs), there was a two-, three-, and five-fold increase in the vesicular protein content when RBCs were stored for 17, 34, and 45 days, respectively (22, 28, and 44 μg/mL).

To learn about the protein composition of the vesicles, we compared their protein patterns to those of the RBC membrane ghosts of the same units by SDS-PAGE. Figure 2A shows a representative electrophoresis pattern of the vesicles, signifying 1) variation in the composition of the vesicles released at different storage times (arrowheads in Fig. 2A) and 2) great amounts of protein bands that are not main components of the SDS-PAGE pattern of the RBC membrane ghosts (Fig. 2A, Lane 1). Hb and bands with MW lower than 70 kDa appeared as the main electrophoretic bands in vesicles.

Subsequent immunoblotting analysis of vesicle preparations (Fig. 2B) revealed selectivity toward the participation of various RBC proteins into the vesicles in relation to the duration of storage. More specifically, the vesicles did not contain pallidin and were essentially free of spectrin, 4.1R (data not shown), and GpC (Fig. 2B), since only traces of these proteins were detected in the preparations of the first days of storage. The Hb was present in vesicles as both monomeric and heavily aggregated bands. Furthermore, toward the end of the storage period, the high-MW bands of Hb were found in greater amounts than those of monomers (arrowheads in Fig. 2B). Actin, band 3 (Figs. 2B and 2C), and high-MW bands corresponding to band 3 aggregates (arrowheads in Fig. 2B) were also detected in increasing amounts toward the end of the storage period. The major integral protein GpA was found in progressively decreasing amount (Fig. 2B).

The presence of lipid raft proteins

Recurrent immunoblot data showed the presence of a wide range of membrane-associated and cytoplasmic lipid raft-associated proteins in the vesicles (Fig. 3A). In particular, we detected increasing amounts of the membrane-associated proteins Gs-α, stomatin, flotillin-1, and flotillin-2 (Fig. 3B) and of the cytoplasmic synexin and sorcin. Furthermore, the vesicles seem to be enriched in stomatin but depleted in flotillins compared to the RBC membranes of origin (Fig. 3A). The vesicles derived from RBCs after long storage were further characterized by high-MW bands exhibiting stomatin and synexin immunoreactivity (arrowheads in Fig 3A). On the other hand, no aquaporin-1 was detected in vesicle preparations.

Phagocytosis-related molecules

IgG-specific bands were detected by immunoblotting analysis in vesicles both under reducing (data not shown) and under nonreducing conditions (Fig. 4A), beginning from the first days of storage. Compared to identical quantities of loaded ghosts extracted from RBCs stored for 10 days, the vesicles contain multifold amounts of endogenous IgG molecules, especially until the 30th day. Furthermore, we tested the accumulated vesicles for the

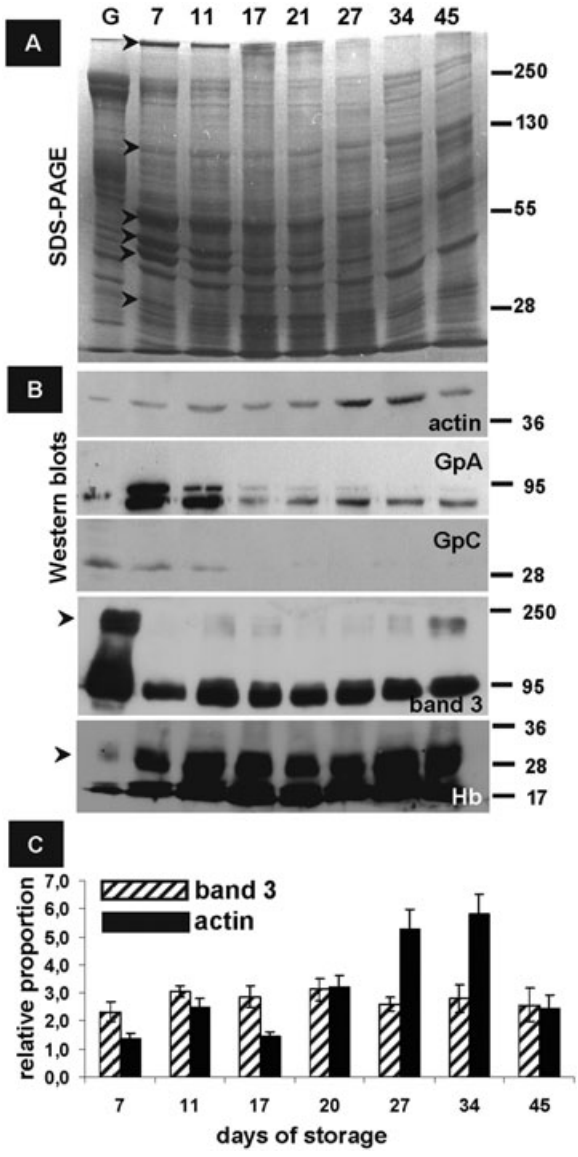

Fig. 2. SDS-PAGE and immunoblotting analysis of the vesicles for the detection of the main RBC proteins. (A) SDS-PAGE analysis of vesicles. Protein bands that show sharp variation during the storage period are denoted by arrowheads. (B) Western blot analysis of a representative vesicle preparation using anti-human–specific antibodies for the major integral (GpA, GpC, band 3), cytoskeletal (actin), and cytoplasmic (Hb) RBC proteins. Of special note, the bands of aggregated band 3 and Hb (arrowheads). The duration of storage is indicated in days at the top of the blot figure. The running of the MW markers is shown in kDa (right side). Ghost preparations from RBC units stored either for 10 days (Hb blot) or for 43 days (other blots) in CPDA are included for comparison in the first lane (G). (C) Quantitation of the band 3 and actin immunoblots. The data are the mean values of the relative proportion (%) of each protein to the total vesicular proteins and the respective SDs of the tested blood units, estimated as presented under Materials and Methods.

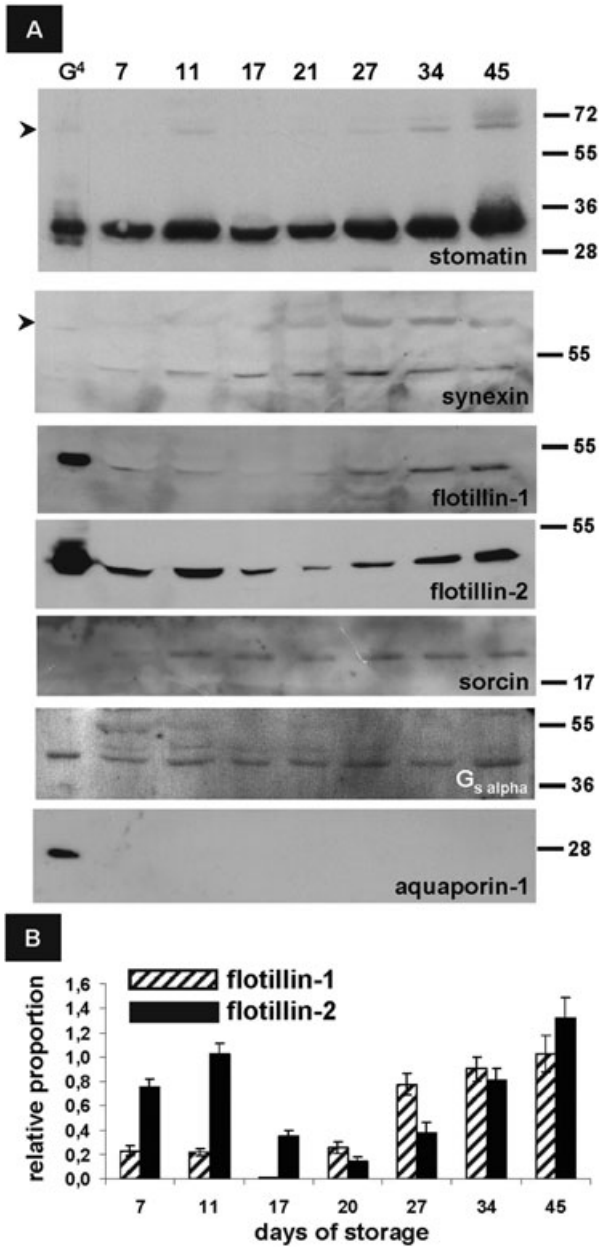

Fig. 3. The variation of the major RBC lipid raft proteins in the vesicles over the storage period. (A) Western blot analysis of a representative vesicle preparation with antibodies specific for the major RBC lipid raft–associated proteins and markers. Oligomeric high-MW bands of stomatin and synexin gradually increasing with storage are denoted by arrowheads. The duration of storage is indicated in days at the top of blot figures. The running of the MW markers is shown in kDa (right side). A ghost preparation from a RBC unit stored for 4 days in CPDA is included for comparison in the first lane (G4). (B) Quantitation of the flotillins immunoblots. The data are the mean values of the relative proportion (%) of each protein to the total vesicular proteins and the respective SDs of the tested blood units, estimated as presented under Materials and Methods.

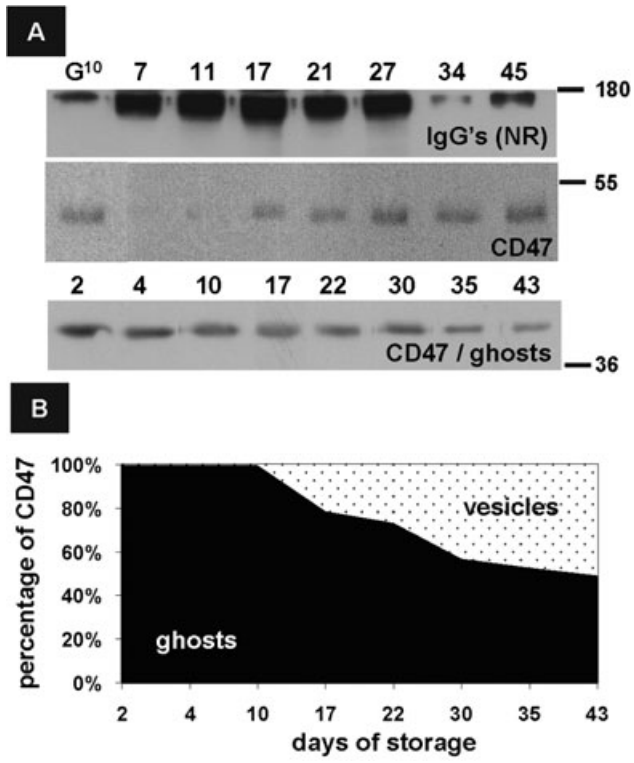

Fig. 4. IgG and CD47 expression in the vesicles during storage. (A) Western blot analysis of a representative vesicle preparation using anti-human–specific antibodies for IgG under non-reducing conditions (NR) and CD47 phagocytosis-related molecules. A ghost preparation from a RBC unit stored for 10 days in CPDA in the first lane of the first two blots (G10) and a representative immunoblot analysis of the CD47 in ghosts for the whole period of storage are included for comparison purposes. The duration of storage is indicated in days at the top of the blot figure. The running of the MW markers is shown in kDa (right side). (B) Graph representing the results of the quantitative immunoblotting analysis for the distribution of CD47 in ghosts and vesicles (% of total CD47).

presence of the critical RBC antigenic marker CD47 that exhibits an inhibitory role in macrophage activation.[21] Our experiments verified the progressive loss of CD47 from the RBC membrane during storage (Fig. 4A) and more importantly they showed for the first time that at least a proportion of the CD47 membrane marker is exocytosed through vesiculation from 2 weeks (Figs. 4A and 4B).

Fas-related signaling molecules

By means of immunoblotting, we detected the consistent presence of Fas/CD95, FADD, and full-length caspase 3 and caspase 8 in the vesicles throughout the storage period (Fig. 5). The vesicles contain many Fas/CD95-immunopositive bands (arrowheads in Fas immunoblot) of probably aggregated or proteolyzed protein, while in

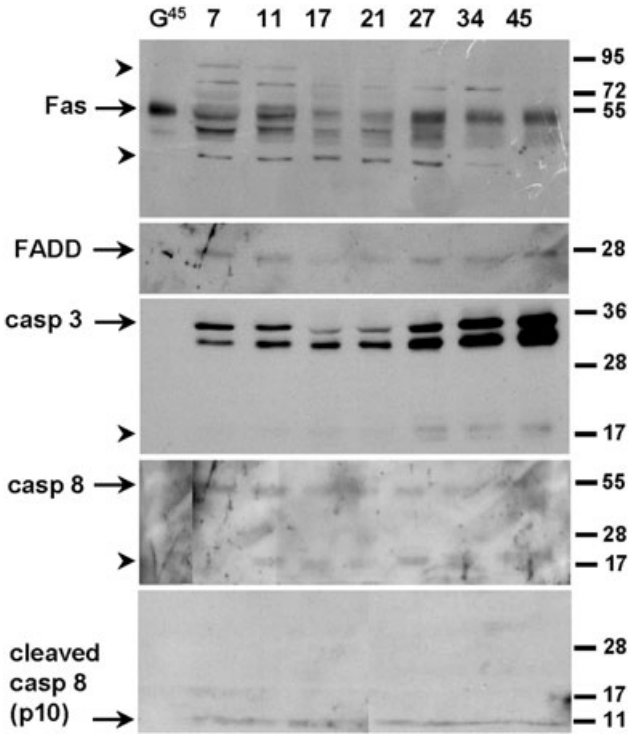

Fig. 5. Fas-related signaling molecules in vesicles. Western blot analysis of a representative vesicle preparation using antibodies specific for Fas/CD95, FADD, caspase 3, caspase 8, and cleaved caspase 8 (detecting the small subunit of caspase 8 resulting from cleavage at Asp384). The aberrant bands of Fas and those corresponding to the cleavage products of caspase 3 and caspase 8 are shown by arrowheads. The duration of storage is indicated in days at the top of the blot figure and the running of the MW in kDa (right side). A ghost preparation from an outdated unit is included for comparison in the first lane (45 days in CPDA, G45).

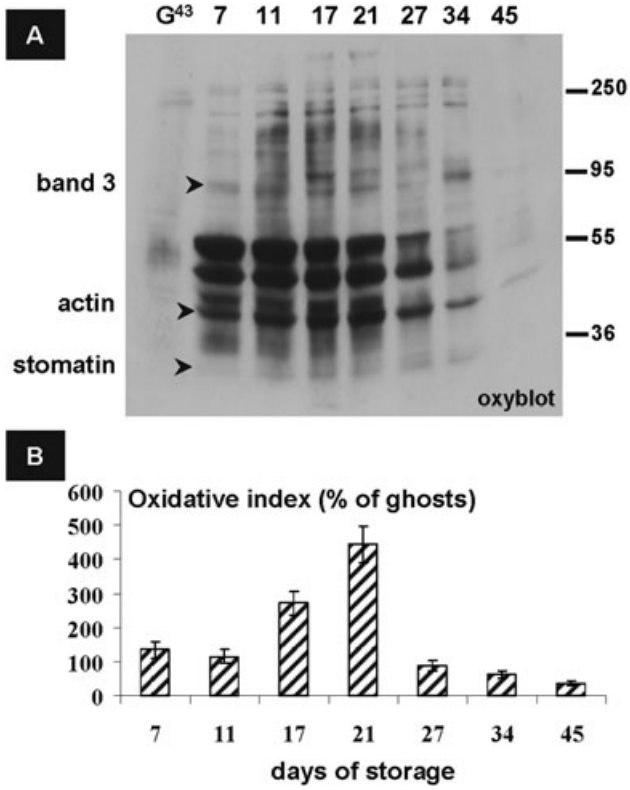

Fig. 6. Oxyblot analysis and oxidative index of the vesicles collected in storage. (A) A representative oxyblot analysis, that is, immunoblot analysis of vesicles stained with the anti-DNP antibody. The arrowheads demonstrate the carbonylated bands of some major RBC membrane proteins (left side). The duration of storage is indicated in days at the top of figures. The running of the MW markers is shown in kDa (right side). A ghost preparation from an outdated unit is included for comparison in the first lane (43 days in CPDA, G43). (B) The estimation of the oxidative index, that is, the percentage of carbonylated vesicular bands per day after normalization to the oxidation index of the corresponding RBC membranes. The data are the mean values of the oxidation index and the respective SDs of the tested blood units, estimated as presented under Materials and Methods.

the control ghosts' membranes from outdated samples only the anticipated band of Fas was detected. The low-MW cleavage products of caspase 8 and caspase 3 were increasingly detected in the vesicles from 10 days (arrowheads in caspases immunoblots). In accordance with these results, by using a MoAb that specifically detects the small subunit of caspase 8 (p10, 10 kDa) resulting from the cleavage of activated caspase 8 at Asp384 after CD95-related apoptosis induction, we showed the presence of the active subunit in the released vesicles. In the ghosts' membranes extracted from outdated stored RBCs, no caspase-specific band was detected (Fig. 5).

Oxidative index of the vesicular proteins

To ascertain the components of the vesicles affected by protein oxidation, we assayed for the production of carbonyl groups, a credible biomarker of protein oxidative stress,[22] by means of immunoblot (Fig. 6A). As reference values, we used the oxidative index of the corresponding ghost membranes. We found that the protein-oxidative indexes of vesicles stored up to 21 days were significantly greater compared to those of the corresponding membranes of origin (Fig. 6B). After that period of storage, the oxidative index of the vesicles exhibited a dramatic decline, probably attributed to the depletion of a range of heavily carbonylated proteins from vesicles. These proteins that are not major components of the electrophoretic pattern of the ghost membrane show a gradual decrease in their amount in vesicles (Fig. 2A). After the oxyblot membranes were stripped and reprobed with RBC-specific antibodies, some of the oxidized protein

bands have been identified as actin, band 3, and stomatin (Fig. 6A, arrowheads).

DISCUSSION

In this report, we show that the duration of storage of RBCs affects the quantity of the total vesicular protein collected in the residual plasma, the size, the structure, the oxidation status, and the protein composition of the vesicles.

Structural degeneration of the vesicles

We provide direct evidence for storage-related changes concerning the size and the structure of the vesicles. The latter are manifested by morphologic signs of degeneration (such as the membrane gaps) and the shedding of the enclosed Hb-positive material, as demonstrated by in situ microscopic immunolocalization. It is reasonable to assume that the vesicles suffer gradual structural deterioration, considering that they are depleted of cytoskeletal components, and furthermore they accumulate oxidized and damaged proteins, as presently reported. In the absence of the effective elimination machinery operating in vivo,[1,2] the "maturing" vesicles probably undergo various degradation reactions and detrimental effects originating not only from the stored RBCs but also from the residual WBCs and PLTs present in the units.[23,24] The latter have been shown to contribute to the RBC storage lesion by releasing bioactive substances during degradation. It has been suggested that microvesiculation contributes more to the increase in the plasma Hb occurring during storage than free Hb.[25] Based on our microscopic data, it seems probable that the plasma Hb found in prolonged liquid storage is not so effectively enclosed into vesicles, but it can be released from them.

Degree of membrane vesiculation

In accordance with previous reports on CPDA-stored whole blood,[6] we documented a continuing release of Hb-containing vesicles. In the context of findings associating the degree of vesiculation with the storage conditions and in vivo survival,[9,26] this study points out the 2 weeks of storage in CPDA as a critical time point regarding the possible detrimental effects of accumulated vesicles on the posttransfusion performance. Our data concerning the time-dependent variation in the rate of total vesicular protein accumulation are consistent with previous studies reporting that the deformability of RBCs begins to decline during the second week of storage.[27]

Expression of major RBC membrane proteins into vesicles

The expression of GpA in the vesicles is higher in the first 10 days of storage exhibiting the reverse pattern to the other membrane proteins investigated. This is in agreement with previous studies showing that the GpA leaves the RBC membrane mainly during the first 10 days of banking.[17] Furthermore, this study adds strength to the notion that cytoskeleton connectivity is a factor involved in the sorting of RBC membrane proteins into the vesicles.[28,29] The RBC vesicles were virtually depleted not only of spectrin, as previously shown,[6,9] but also of skeletal 4.1R and 4.2. In a similar way, they contain more GpA (Fig. 4) compared to the skeleton-associated[30] GpC. The partial depletion of vesicles in band 3 in combination with the total absence of 4.2 protein that is required for normal band 3-cytoskeleton linkage[31] suggests that the vesicular band 3 probably concerns the subpopulation of the protein that is not skeleton-associated.

Despite that, the vesicles contain increasing amounts of actin, that is, the only cytoskeletal protein found in the supernatant of stored RBCs,[32] and traces of skeletal or skeleton-associated proteins (spectrin, 4.1R, and GpC). These vesicular proteins represent components that either have lost their connection to the RBC skeleton as a result of the storage lesion or were associated with components that are sorted into the vesicles. In support, it has been shown that many RBC skeletal proteins can be partly associated with the erythrocyte lipid raft components.[33]

Phagocytosis-related signals and protein oxidation

Previous studies reported higher cross-linkability for band 3 and glycophorins on vesicles than on intact RBCs[34] and preferential binding of autologous IgG to vesicles shed from outdated blood and ATP-depleted RBCs.[35] Furthermore, Willekens and coworkers[1] have shown a similarity between the Hb composition of RBC vesicles shed in vivo and old RBCs. Our study showed that the band 3, IgG, and Hb composition of the vesicles closely resembles that of RBCs stored for long periods in CPDA.[16,17] These findings suggest that the vesicles 1) represent a microenvironment of high oxidative stress or of gathering oxidatively damaged proteins and 2) have the same mechanism of recognition with their parent[17] and/or senescent RBCs,[12,36,37] including the binding of denatured Hb to the membrane, clustering of band 3, binding of autoantibodies, and phagocytosis mediated by scavenger receptors. In agreement with our findings, a study on the fate of RBC-derived vesicles in vivo demonstrated their rapid elimination from the circulation that is mediated by scavenger receptors.[2]

Moreover, it appears that the vesicles contain more than one phagocytosis-related signal. It has been documented that the RBC membrane loses CD47 markers during storage;[38] however, the gathering of CD47 into vesicles is shown for the first time and is supported by studies showing increasing amounts of CD47 in the super-

natants of RBCs after 14 days of storage.[39] The effectiveness of the clearance could be related to both the type and the amount of signals exposed on vesicular surface; however, this assumption needs further examination.

Although the protein oxidation seems to be a part of the RBC aging[12] and storage lesion,[15,16] there was not an established connection between oxidation and vesiculation during storage, apart from a study reporting the influential role of spectrin oxidation on the extent of storage-related vesiculation.[40] Our study provides further indications that the two processes (protein oxidation and vesiculation) are linked. The extensive oxidative modifications in a large group of RBC proteins found presently in vesicles are expected to influence either directly[4] or indirectly (through the cytoskeleton connectivity)[29] the protein sorting into them. Furthermore, the gathering of oxidative RBC proteins into vesicles suggests that the vesiculation further operates as an effective way of eliminating damaged proteins produced by the storage-related oxidative stress in RBC units.

Raft markers in vesicles

Our previous findings on the remodeling of the RBC membrane during storage of RBCs showed that the major membrane-associated proteins of the RBC lipid rafts stomatin and flotillins progressively leave the membrane while the cytoplasmic synexin and sorcin become gradually membrane-associated.[17] The currently presented protein composition of vesicles verified the observations on stored RBCs. Furthermore, the detection of aggregated and oxidized bands of synexin and stomatin in the long-storage preparations of vesicles suggests that a proportion of vesicular raft proteins may be structurally and/or functionally disorganized. The presence of flotillins points out a difference with the reported segregation of RBC membrane proteins during calcium-induced vesiculation,[5] but it is consistent with the protein composition of vesicles collected from the medium of subcultured human RBCs and reticulocytes in vitro.[41]

Lipid rafts have already been described as key elements in the vesiculation of the reticulocyte[41] and RBC membrane when there is an increase in cytosolic Ca^{2+}.[4] The membranes of vesicles contain lipid rafts[4] and a growing body of evidence suggests that they might be involved in vesicle formation and structure as well as in setting up sorting platforms to concentrate proteins and effectors destined for extracellular secretion. Although we did not demonstrate the presence of organized lipid raft platforms on the membranes of vesicles released during storage and hence we do not know what proportion of the detected proteins are raft-associated in situ, the participation of a wide range of raft markers into them, and especially of stomatin, which is a central raft-scaffolding protein in RBCs,[33] is at least suggestive of their involvement in the vesiculation of stored cells and the generated particles.

Fas-associated signaling molecules

The prolonged storage of RBCs is associated with caspase activation and Fas oligomerization.[17] The protein composition of vesicles reveals that they contain Fas-associated signaling components in amounts proportional to the length of storage. The demonstrated coexistence of a variety of lipid raft- and Fas-associated proteins into the released vesicles suggests a structural and/or functional interaction between the two protein groups during RBC vesiculation in storage, inevitably recalling the established localization of Fas-FADD-caspase 8-FasL complex into the lipid raft microdomains in old and oxidized RBCs containing activated caspase 8 and caspase 3.[13]

In spite of not assaying the released vesicles for caspase activity, the demonstrated cleavage bands of caspases 8 and 3 that are consistently associated with caspase activation after Fas-related induction of apoptosis favor the assumption that the vesicles contain activated caspases. Emerging data suggest the involvement of vesiculation in apoptosis[42] and the presence of apoptosis-related proteins in vesicles.[43] At least a proportion of the vesicles expose phosphatidylserine[2,44] and exhibit caspase 3—like protease activity, as shown in vesicles released from reticulocytes.[41] According to recent studies, the oxidative stress renders the RBCs susceptible to phagocytic recognition through the activation of caspases that drives membrane changes, like the aggregation of band 3.[45] Band 3 has been documented to be a substrate of activated caspases in oxidatively stressed[46] and aged RBCs.[47] Our novel findings commit substantiating data for the existence of Fas-dependent signaling components inside the vesicles released during storage and circumstantial evidence for the action of a Fas-FADD-caspase 8-caspase 3 complex. These proteins might be sorted into the vesicles either as activated complex components or as inactivated (and afterward partly in situ activated) molecules. The role of death receptors and caspases on the mature RBC life span is largely unknown. The currently presented data add validity to the notion that they are likely to play a physiologic role in the mature human RBC. The caspases may be involved in the process of membrane vesiculation mediating processes other than apoptosis. Nevertheless, the molecular mechanisms that drive the construction of signaling complexes, the possible link with the rafts or the oxidation stress, and the consequences of caspase activation in RBC aging and death events—if any—need further studies.

In conclusion, the monitoring of the storage-induced vesiculation reveals variation in the quantity, the structure, and the protein composition of the released vesicles over time. This variation might be attributed to several

factors, including the interference of the residual WBCs and PLTs of the units[23,24] and the changes in the physiologic status of the origin RBCs that accompany their ex vivo aging[1,48] and "storage lesions."[15-17] More studies are needed to identify whether the reported variation refers homogenously to the vesicles released after a period of storage or whether they are heterogeneous in terms of composition. Our results support the concept that the release of vesicles containing harmful and high signaling potential components may prevent the premature removal of otherwise functional cells, as previously suggested.[11,12]

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