Temporal sequence of major biochemical events during Blood Bank storage of packed red blood cells

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Background. We used sensitive spectroscopic techniques to measure changes in Band 3 oligomeric state during storage of packed red blood cells (RBC); these changes were compared to metabolic changes, RBC morphology, cholesterol and membrane protein loss, phospholipid reorganisation of the RBC membrane, and peroxidation of membrane lipid. The aim of the study was to temporally sequence major biochemical events occurring during cold storage, in order to determine which changes may underlie the structural defects in stored RBC.

Materials and methods. Fifteen RBC units were collected from normal volunteers and stored under standard blood bank conditions; both metabolic changes and lipid parameters were measured by multiple novel assays including a new mass spectrometric measurement of isoprostane (lipid peroxidation) and flow cytometric assessment of CD47 expression. Band 3 oligomeric state was assessed by time-resolved phosphorescence anisotropy, and RBC morphology by microscopy of glutaraldehyde-fixed RBC.

Results. Extracellular pH decreased and extracellular potassium increased rapidly during cold storage. Band 3 on the RBC membrane aggregated into large oligomers early in the storage period and coincident with changes in RBC morphology. Membrane lipid changes, including loss of unesterified cholesterol, lipid peroxidation and expression of CD47, also changed early during the storage period. In contrast loss of acetylcholinesterase activity and haemolysis of RBC occurred late during storage.

Discussion. Our results demonstrate that changes in the macromolecular organisation of membrane proteins on the RBC occur early in storage and suggest that lipid peroxidation and/or oxidative damage to the membrane are responsible for irreversible morphological changes and loss of function during red cell storage.

Keywords: red blood cell, storage lesion, Band 3.

Introduction

During blood bank cold storage, red cells progressively lose deformability and elasticity due to irreversible changes in the cell membrane[1]. Morphologically this is observed as progressive irreversible formation of sphero-echinocytes[2]. These changes in deformability, elasticity and morphology underlie the poor *in vivo* survival of transfused red cells[1]. Recent studies have demonstrated that there may be a correlation between the number of days of cold storage of packed red blood cells (PRBC) and adverse outcomes, including mortality, among transfused patients[3,4], presumably due to structural or functional changes in red blood cells (RBC) that occur during storage. This phenomenon has been referred to as the red cell storage lesion[1,4].

Theories to explain the underlying biochemical mechanism of the red cell storage lesion include changes in spectrin-protein 4.1-actin interactions[1,5], spectrin oxidation[1,6], lipid peroxidation[4], spontaneous lipid loss in the form of vesicles[1], and phospholipid asymmetry due to ATP depletion or ineffective ATP utilisation at low pH[7]. Several studies have identified mechanisms to reduce loss of membrane lipid in the

form of vesicles during cold storage[8-12], however, they do not prevent morphological changes in the red cell or decreased deformability during storage[8]. Oxygen removal or depletion results in maintenance of ATP levels and improved 24 hour post-transfusion *in vivo* survival of RBC[13], although it is not clear to what extent anaerobic storage prevents morphological or biochemical changes. Until the primary mechanisms of RBC membrane structural changes are understood, it will be difficult to identify storage conditions that will prevent irreversible membrane changes leading to loss of function of transfused red cells.

We previously described an experimental approach that allows sensitive detection of changes in the organisation of cytoskeletal membrane proteins on intact red cell membranes during blood bank storage[14]. In the present study we extend our findings to temporally define cold storage-induced changes in RBC morphology, membrane protein oligomeric state, cholesterol and lipid loss, phospholipid reorganisation of the RBC membrane as measured by flow cytometry, and lipid peroxidation as measured by a sensitive and specific mass spectrometric assay. The results help to define important biochemical mechanisms of the storage lesion by differentiating early and late biochemical events during cold storage of PRBC.

Materials and methods

Preparation of labelled red blood cells

Fifteen normal volunteers were solicited to donate one standard unit of whole blood collected in a Pall Corporation Leukotrap® WB 500 mL collection bag set containing 70 mL CP2D (Pall Corporation, East Hills, NY, USA). Within 8 hours the whole blood was passed through an in-line leucoreduction filter and centrifuged at 4,000 rpm for 5 minutes in a Sorvall RC3BP centrifuge equipped with a HBB-6 rotor. Plasma was expressed after centrifugation and AS-3 solution (Nutricel, Haemonetics, Union, SC, USA) containing citrate, phosphate, dextrose, adenine and sodium chloride added to produce leucoreduced PRBC units of 300-395 mL having a haematocrit between 45-55% according to institutional blood bank procedures. PRBC units were stored in a monitored blood bank refrigerator at 4-6 °C for the duration of the experiment.

On days 0, 7, 14, 21, and 42 of PRBC storage, 26 mL of the contents of the storage bag were removed for experiments using a sterile connecting device from the PRBC units. RBC were labelled with eosin-5-maleimide (EMA) (Invitrogen, Carlsbad, CA, USA) under conditions previously demonstrated to result in specific (>80%) labelling of Band 3 in the RBC membrane[15] at one extracellular exposed lysine residue, lysine-430[16]. Labelled RBC ghosts were then lysed in 20 volumes of 5 mM sodium phosphate, pH 7.4 (phosphate buffer) and prepared for spectroscopy (see below) as described previously[14]. The study design was approved by the Mayo Clinic Institutional Review Board.

Metabolic experiments and morphology of packed red blood cells

After thorough mixing, PRBC aliquots were injected into a Radiometer ABL 725 blood gas analyser (Radiometer Medical A/S, Brønshøj Denmark) for pH and potassium measurements. Lactate was measured on the same PRBC aliquot using a Lactate Pro (ARKRAY, Kyoto, Japan) whole blood lactate device whose results were previously demonstrated to correlate well with those of laboratory reference methods[17]. For light microscopy of glutaraldehyde-fixed PRBC, 25% glutaraldehyde (Sigma-Aldrich, St Louis, MO, USA) was diluted into AS-3 solution to obtain 1% glutaraldehyde in AS-3. Fifty microlitres of PRBC were fixed in 1 mL of 1% glutaraldehyde in AS-3 solution and blood smears prepared and stained with Wright-Giemsa. Five hundred cells were counted for each PRBC sample under a 100x oil immersion objective. RBC morphology was scored using a scale similar to that described by Immerman *et al.*: I-normal RBC, II-few bumps with central pallor, III-echinocytes, IV-sphero-echinocytes (loss of central pallor, smaller and more dense), V-spherocytes[18]. A sixth category, non-specific changes, was added to account for schistocytes, fragments and other abnormalities. Light microscopy performed in this manner has been demonstrated to be highly concordant with morphological classification by either phase contrast or electron microscopy[18]. For purposes of this analysis the percent of abnormal cells, defined as percent RBC in categories III-VI, is presented.

Lipid loss and haemoglobin measurement

Red cell acetylcholinesterase activity has been used as a marker for lipid vesicle loss from the RBC membrane because acetylcholinesterase activity is found only in RBC membranes and in lipid vesicles lost from the membrane. Furthermore, loss

of acetylcholinesterase activity has been shown to be linearly correlated with loss of phospholipid in the form of vesicles during cold storage[6,12]. The acetylcholinesterase activity of the RBC pellet after centrifugation at 1,865 x *g*[14] was determined by a modification of the technique described by Ellman[19]. The ratio of esterified to unesterified cholesterol in the supernatant was determined by measuring supernatant esterified and unesterified cholesterol on a Hitachi 911 analyser (Roche Diagnostics, Indianapolis, IN, USA) using reagents provided by Wako Chemicals (Richmond, VA, USA). Haemoglobin concentration in the RBC supernatant was measured using a HemoCue plasma/low haemoglobin photometer (HemoCue AB, Ängelholm, Sweden).

Lipid peroxidation (8-isoprostane $F_{2\alpha}$)

For RBC supernatant samples 1 mL of RBC supernatant was added to 2 mL of 15% (1.75 M) KOH and incubated at 38 °C for 1 hour. Extra RBC supernatant from stored samples was spiked with 0, 200, 500, 1,000 and 2,000 pg/mL of 8-isoprostane $F_{2\alpha}$ (Cayman Chemical, Ann Arbor, MI, USA) for use as calibrators. Next, 1 mL of calibrator and 3 mL of RBC sample in KOH were added to a Waters Oasis mixed-mode cation exchange MCX column (Milford, MA, USA) after the columns had been conditioned with 1 mL MeOH and 1 mL distilled water. The columns were washed twice with 2 mL of distilled water, and eluted with 2 mL MeOH + 0.1% formic acid. The methanol was collected in a glass tube, and dried down in a Zymark Turbovap (Caliper LifeSciences, Hopkinton, MA, USA) at 50 °C and 15 PSI. Methanol (250 µL) was added to each tube and vortexed to resuspend the pellet giving a 4X concentration of the sample. To each resuspended pellet, 5 µL of d4-deuterium labelled 8-isoprostane $F_{2\alpha}$ (Cayman Chemical) was added for a final concentration of 1,000 pg/mL in a glass vial. Forty-five microlitres of sample were loaded under distilled water +0.1 % formic acid onto a ThermoFisher Scientific Cyclone MAX turboflow column (Waltham, MA, USA) and eluted with MeOH +0.1% NH_4OH. From the turboflow clean-up, the sample passed to a Waters Xbridge 2.1 mm x 50 mm C18 column. Separation was achieved with 50/50 MeOH/acetone +0.1% NH_4OH, then 8-isoprostane $F_{2\alpha}$ passed to an Applied Biosystems API 5000 mass spectrometer (Foster City, CA, USA) for detection. Ions analysed were 353.3/193.1 for 8-isoprostane $F_{2\alpha}$ and 357.3/197.1 for d4-8-isoprostane $F_{2\alpha}$. Concentrations were calculated from the calibration curve using a linear fit and 1/x weighting. The R value of the curve was 0.98, with a y-intercept of 0.08.

CD47 flow cytometry

CD47 surface antigen was measured using a flow cytometry kit from BD Biosciences (San Jose, CA, USA) according to manufacturer's instructions. Analysis was performed with Cell Quest Pro Software (BD Biosciences). A total of 25,000 events were acquired using forward versus side scatter. An R1 gate was then set around the main population of interest on the forward versus side scatter plot and, using the R1 gate histograms of IgG phycoerythrin (PE) and CD47 PE, average mean fluorescence intensity was determined for each sample.

Time-resolved phosphorescence anisotropy experiments

The optical laser system and data acquisition for time-resolved phosphorescence anisotropy (TPA) experiments has been described previously[20,21]. Anisotropy is similar to light polarisation and allows detection and differentiation of membrane protein oligomeric states based on relative rotational mobility of oligomers, aggregates and complexes. The TPA decay, r(t), is given by $r(t) = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$; where I_{VV} and I_{VH} are obtained by signal-averaging the time-dependent decay in anisotropy following 300 vertically polarised laser pulses, with a single detector and emission polariser that alternates between the vertical (I_{VV}) and horizontal (I_{VH}) positions every 300 pulses. After each laser pulse, the signal [either $I_{VV}(t)$ or $I_{VH}(t)$] was digitised for a period of at least 10 ms, and the laser pulse repetition rate was set at 70 Hz. A typical r(t) acquisition, therefore, required 10-15 minutes to record and average 100 loops, or cycles, of 600 pulses (300 in each orientation). G is an instrumental correction factor[20,21]. An enzymatic oxygen removal system[21] was used and data collected at 37 °C after incubation for 10 minutes to allow oxygen removal and temperature equilibration.

TPA data were fitted by a sum of two exponentials plus a constant[14], and normalised to the initial (r_0) anisotropy such that:

Eq. 1: $r(t)/r_0 = A_1 \exp(-t/\varphi_1) + A_2 \exp(-t/\varphi_2) + A_\infty$

where amplitude 1 (A_1) and φ_1 have previously

been shown to represent the relative fraction and rotational correlation time of small Band 3 oligomers, amplitude 2 (A_2) and φ_2 the relative fraction and rotational correlation time of large Band 3 oligomers and aggregates, and A_∞ (r_∞/r_0) the relative fraction of Band 3 immobilised (tethered) to the membrane via ankyrin and spectrin[22]. Previous studies established that approximately 25% of Band 3 exists as dimers and small Band 3 oligomers; while approximately 50% of Band 3 is composed of large oligomers and aggregates of Band 3 that are not motionally restricted by the cytoskeletal network. The remainder of the Band 3 in the membrane is immobile on the time scale of phosphorescence anisotropy and represents Band 3 attached to the cytoskeletal network via ankyrin and spectrin. We previously demonstrated that TPA performed in this manner is capable of detecting Band 3 aggregation during blood bank cold storage[14].

Results

Metabolic changes and red blood cell morphology during cold storage

Extracellular pH decreased and potassium increased rapidly with cold storage of PRBC (Table I), as observed previously[10,14,23]. RBC morphology changed rapidly, with an increase from nearly zero to 9.5% abnormal cells (echinocytes, sphero-echinocytes, spherocytes and other abnormal cells) at day 7 (Table I). Lactate also increased rapidly as seen in previous experiments[9]. Changes in RBC morphology, pH, potassium, and lactate were statistically significant at day 7 compared to day 0 (Table I). In contrast supernatant free haemoglobin, a marker of RBC haemolysis, remained unchanged until day 14 and became statistically significantly increased only at day 21 (Table I). Other investigators have also found that the percent of haemolysed RBC increases slowly over several weeks of cold storage[9,10]. Thus metabolic changes occur early and are associated temporally with RBC morphological changes; while haemolysis occurs late during storage and is probably the end result of biochemical and morphological changes leading to membrane instability.

Lipid vesicle and unesterified cholesterol loss

Acetylcholinesterase activity of the red cell pellet has been used as a marker of lipid vesiculation during PRBC storage, correlating well with the extent of lipid vesicle loss from the RBC membrane[6,12]. A trend towards decreasing acetylcholinesterase activity was seen at day 21, which became statistically significant only at day 42 (Table II). This is consistent with results of our previous study showing decreased acetylcholinesterase activity at day 28 of storage[14], and one previous study that found slow release of acetylcholinesterase activity over 2-8 weeks of PRBC storage[6]. Changes in the percent of unesterified cholesterol in the supernatant could be detected as early as day 7 of cold storage (Table II). Because cholesterol in the red cell membrane is unesterified cholesterol[24], while cholesterol derived from residual volunteer plasma is largely esterified[25], an increase in the percent of unesterified cholesterol in the PRBC supernatant indicates a loss of cholesterol from the RBC membrane which occurs early during storage.

Lipid peroxidation during cold storage

Previous studies of lipid peroxidation relied on measurement of malondialdehyde by the thiobarbituric acid reaction to assess lipid peroxidation[26-28]. However this assay is susceptible to interference from formation of aldehydes other than malondialdehyde, leading to falsely increased results[29]. Malondialdehyde is also produced during the measurement reaction, and this

Table I - Mean (± standard deviation) value of metabolic measurements and percent of abnormal cells (defined as echinocytes, sphero-echinocytes and spherocytes observed by morphological examination) for 15 PRBC units sampled at days 0, 7, 14, 21 and 42 of cold storage.

	Day 0	Day 7	Day 14	Day 21	Day 42
% abnormal cells	0.01%	*9.5%	*14.6%	*13.8%	*16.2%
pH	6.88±0.06	*6.67±0.02	*6.60±0.03	*6.52±0.05	*6.37±0.06
Potassium (mmol/L)	2.2±0.4	*14.4±0.8	*22.8±2.5	*29.9±2.9	*44.2±3.5
Lactate (mmol/L)	1.4±0.3	*6.2±1.0	*9.8±1.2	*11.6±1.1	*12.6±0.5
Supernatant free haemoglobin (mg/dL)	20±21	18±16	31±26	*79±75	*88±54

*Statistically significant difference from baseline (day 0) measurement, P <0.05 Tukey's one-way ANOVA

Table II - Mean (± standard deviation) value of amplitudes from TPA decay fitting (A_1, A_2, A_∞), biochemical measurements, and mean cell fluorescence from flow cytometric measurement of CD47 expression for 15 PRBC units sampled at days 0, 7, 14, 21 and 42 of cold storage.

	Day 0	Day 7	Day 14	Day 21	Day 42
A_1	0.27 ± 0.02	*0.20 ± 0.02	*0.20 ± 0.02	*0.19 ± 0.02	*0.18 ± 0.01
A_2	0.07 ± 0.02	*0.16 ± 0.02	*0.17 ± 0.02	*0.19 ± 0.02	*0.20 ± 0.03
A_∞	0.57 ± 0.04	0.55 ± 0.04	0.57 ± 0.05	0.57 ± 0.03	0.54 ± 0.03
8-isoprostane (pg/mL) in PRBC supernatant	136 ± 105	198 ± 89	*246 ± 86	*351 ± 138	*376 ± 104
% free cholesterol in PRBC supernatant	25.5 ± 1.1	*29.6 ± 2.6	*30.8 ± 2.4	*30.9 ± 3.4	*32.8 ± 4.1
RBC AchE (U/g Hb) in PRBC pellet	57.9 ± 6.6	58.9 ± 5.5	57.6 ± 6.3	53.5 ± 7.2	*47.9 ± 8.6
CD47	347 ± 26	328 ± 24	*313 ± 27	*319 ± 23	357 ± 29

*Statistically significant difference from baseline (day 0) measurement, P <0.05 Tukey's one-way ANOVA. RBC AchE: red blood cell acetylcholinesterase in units (U) per gram haemoglobin.

production can be amplified or inhibited by substances found in biological samples[29]. In contrast 8-isoprostane $F_{2\alpha}$ (8-isoprostane) is a specific product of the non-enzymatic peroxidation of arachidonic acid and has been shown to possess adverse biological activities, and as such has been used as a reliable indicator of both lipid peroxidation and oxidative stress[30]. We measured 8-isoprostane in PRBC supernatant by a sensitive and specific mass spectrometric assay. A trend towards increased 8-isoprostane in PRBC supernatant can be seen at day 7, which reached statistical significance at day 14 (Table II).

CD47 flow cytometry

CD47 is an integrin-associated surface glycoprotein and is a potent RBC membrane marker for self, preventing RBC removal by the spleen[31,32]. One study found that CD47 expression by flow cytometry decreased by day 14 of cold storage (first time-point measured in that study) and slowly decreased further until day 42[32]. Another study looked at earlier times during storage (4 and 10 days) but found that CD47 expression decreased relatively late during cold storage (day 24)[33]. We found that CD47 expression showed a trend toward decreased values by day 7 which reached statistical significance by day 14 (Table II). Because the previous study looking at earlier times during storage used only three PRBC units for experiments[33], earlier detection in our study may be a function of greater statistical power to detect differences.

Both our study and one previous investigation[33] noted a paradoxical increase in CD47 detected by flow cytometry late during storage. The authors of the previous study found increased CD47 antigen at day 31 (compared to day 24) by both ELISA and flow cytometry[33], and speculated that residual CD47 after day 24 was due to cytoskeletal-bound molecules that are not prone to release. It is possible that late during storage residual CD47 undergoes proteolytic cleavage, oxidation, and/or conformational changes secondary to phospholipid loss or rearrangement, resulting in exposure of additional CD47 antigen. This phenomenon deserves further investigation. Together with early loss of unesterified cholesterol from the PRBC membrane and appearance of 8-isoprostane in the PRBC supernatant, changes in CD47 exposure during the first 2 weeks indicate that significant changes in membrane lipid composition and structure occur early during cold storage of PRBC.

Changes in Band 3 oligomeric complexes during cold storage

TPA decays of EMA-labelled red cell ghosts were fit to a sum of two exponentials plus a constant (Eq. 1), representing the relative fraction of dimers and small oligomers (A_1), large (A_2) Band 3 aggregates, and immobile (A_∞) Band 3 oligomers tethered to the cytoskeletal network via spectrin and ankyrin.

Averaged anisotropy decays for the 15 PRBC units sampled at days 0, 7 and 14 are shown in Figure 1. Averaged decays for samples taken on days 7 and 14 were normalised to the initial anisotropy (r_0) on day 0 for ease of comparison. When normalised, the initial anisotropy (r_0) and residual anisotropy (r_∞) are nearly identical over 14 days of storage; indicating that the fraction of immobile Band 3 tethered to the membrane cytoskeleton (A_∞ in Table II) does not change. The amplitude of the most rapid motion (small oligomers,

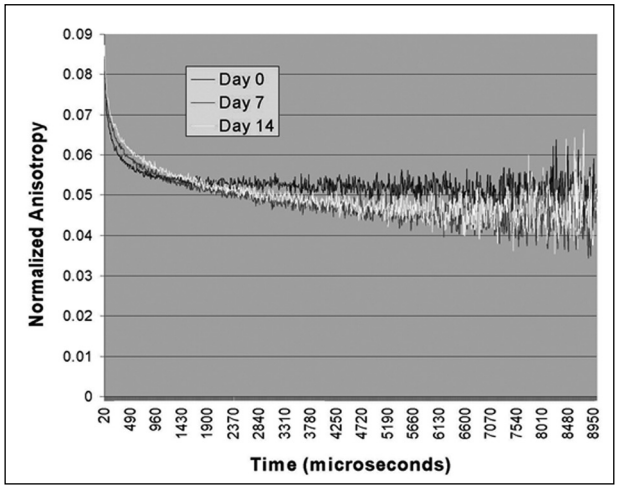

Figure 1 - Averaged time-resolved phosphorescence anisotropy (TPA) decays of EMA-labelled RBC determined by averaging 15 individual TPA decays from 15 PRBC units sampled on days 0, 7 and 14 of cold storage. For sake of comparison the averaged decays at days 7 and 14 are normalised to the initial anisotropy (r_0) at day 0. There is no change in normalised residual anisotropy (r_∞/r_0) over 14 days of cold storage. However, there is less anisotropy decay in the first ~1,500 microseconds on days 7 and 14 compared to on day 0.

A_1 in Eq. 1) decreased by day 7 of cold storage, which can be seen in Figure 1 as less decay in anisotropy over the first ~1,500 μs. The amplitude of the slower motion increased by day 7 of cold storage, which can be observed by more decay between 1,500-10,000 μs (Figure 1). The same effect (loss of rapid motion and gain of slower motion) continued at day 14 (Figure 1) and at later times of cold storage (not shown).

Fitting of the individual TPA decays, as described previously[21], demonstrated a rapid (200-250 μs) motion associated with small oligomers of Band 3, a slower motion (~4,000 μs) assigned to large Band 3 aggregates not tethered to the membrane cytoskeleton, and a residual anisotropy (A_∞) associated with the fraction of Band 3 tethered to the membrane cytoskeleton[14]. The amplitude (relative fraction) of small, mobile oligomers (A_1 in Eq. 1) decreased significantly by day 7 of cold storage (Table II). The amplitude (relative fraction, A_2) of large Band 3 aggregates showed a parallel increase by day 7 of storage; while the relative fraction (A_∞) of immobilised Band 3 did not change over 42 days of cold storage (Table II). Our results demonstrate that early during cold storage the equilibrium of small and large Band 3 oligomers is shifted toward large aggregates without affecting the population of immobile Band 3 tethered to the RBC membrane via spectrin and ankyrin.

Comparison of biochemical, morphological and biophysical changes

In order to compare changes in PRBC structure (percent of morphologically abnormal cells), Band 3 aggregation (A_2) and biochemical changes we plotted percent maximal change for these parameters as a function of number of days of cold storage (Figure 2). Percent maximal change is defined as the difference between the mean values at days 7, 14 and 21 and baseline (day 0) values, divided by the maximal change (difference between mean values at day 42 and baseline). Band 3 aggregation (A_2), percent abnormal cells, and supernatant unesterified cholesterol increased rapidly such that over 50% of the maximal change (total change observed over 42 days of cold storage) occurred by day 7 (Figure 2). pH and 8-isoprostane achieved 50%

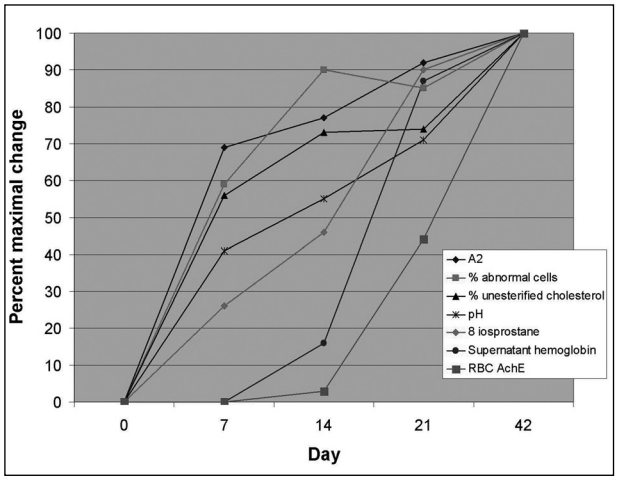

Figure 2 - Percent maximal change, defined as percent change from baseline (day 0) compared to maximal effect observed at day 42 of cold storage. To determine percent maximal change the mean value on day 0 was used as the baseline, and the difference between the mean values on days 7, 14 and 21 and baseline were divided by the maximal change (difference between mean values on day 42 and baseline). Mean values for these parameters can be found in Tables I and II. The percent maximal change in A_2, percent abnormal cells, percent unesterified cholesterol, pH, 8-isoprostane, supernatant free haemoglobin, and RBC acetylcholinesterase (RBC AchE) are plotted.

maximal change by approximately day 14 of cold storage; while supernatant free haemoglobin and PRBC acetylcholinesterase activity did not change at all until after day 7 and did not achieve 50% maximal change until day 21 or later (Figure 2). This provides further evidence for the temporal association between Band 3 aggregation, changes in RBC morphology, lipid peroxidation and release of unesterified cholesterol from the membrane; also further demonstrates that haemolysis (supernatant free haemoglobin) and release of protein-rich vesicles (acetylcholinesterase activity) occur later during PRBC cold storage.

Discussion

Band 3 aggregation has been shown to occur in a small number of RBC late during the storage period, although the studies in which this was demonstrated relied on relatively insensitive methods to detect Band 3 aggregation such as protein electrophoresis and indirect immunofluorescence[34,35]. One additional study observed aggregated Band 3 by electrophoresis at day 21 of cold storage[36]. In this study we have demonstrated early aggregation of Band 3 using sensitive spectroscopic techniques on intact RBC membranes and further shown that Band 3 aggregation is temporally related to RBC morphological changes (both occurring in the first 7 days of cold storage). It is unclear whether Band 3 aggregation has a causative role in RBC morphological changes, or whether the same underlying biochemical mechanisms induce both morphological changes and Band 3 aggregation. Because Band 3 clustering is postulated to be a primary mechanism of targeting RBC for removal from circulation[37,38], it is likely that the aggregation of Band 3 that occurs early during cold storage limits post-transfusion survival of RBC. Thus further understanding the mechanism of Band 3 aggregation may lead to improved storage conditions for PRBC.

Damage to the cytoskeletal network via spectrin oxidation or defects in spectrin-protein 4.1-actin complex formation have been postulated to lead to defects in RBC structure during storage[5,6]. However defects in the cytoskeletal network associated with hereditary haemolytic anaemias have been shown to dissociate immobile Band 3 complexes resulting in an increased fraction of mobile Band 3 oligomers in the membrane[39,40]. Partial trypsin digestion of the cytoskeletal network also results in an increased fraction of mobile Band 3 oligomers[41]. In contrast we observed an increase in the fraction of aggregated (less mobile) Band 3 oligomers as a function of storage time. Our results do not provide evidence for cytoskeletal defects similar to those observed in hereditary haemolytic anaemias occurring during cold storage of PRBC.

In our previous study, in which we used only five PRBC units, we did not detect loss of unesterified cholesterol from the RBC membrane until day 28 of cold storage[14]. In contrast, during this study we used 15 PRBC units and found that changes in the percent of unesterified cholesterol in the supernatant could be detected as early as day 7 of cold storage (Table II). We observed significant loss of unesterified cholesterol from PRBC which preceded changes in acetylcholinesterase activity in the RBC membrane. In fact loss of unesterified cholesterol was temporally associated with RBC morphological changes and Band 3 aggregation, while loss of acetylcholinesterase activity clearly lagged behind morphological changes occurring only after 21 days of storage (Table II). One previous study found that protein content of RBC vesicles shed from PRBC increased over the storage period, with aggregated Band 3 being found in vesicles only towards the end of the storage period (43 days)[42]. These investigators speculated that formation of lipid rafts in RBC membranes might lead to vesiculation during cold storage[42]. Coupled with the finding that specific end-products of lipid peroxidation can be observed in PRBC supernatant early during storage (Table II), our findings raise the possibility that lipid peroxidation may lead to membrane damage early during cold storage of PRBC, resulting in loss of vesicles relatively enriched in lipid and cholesterol.

One study found that antioxidants reduced anti-Band 3 antibody binding during cold storage (an indirect measure of aggregation)[43]. This suggests that oxidative processes play a role in early biochemical changes to the RBC membrane including both early cholesterol loss and Band 3 aggregation. The finding that oxygen depletion reduces Band 3 fragmentation as measured by two-dimensional gel electrophoresis provides further evidence for the role of oxidative damage in Band 3 aggregation[44]. A very recent study demonstrated increased protein fragmentation by day 14 and accumulation of oxidized lipid

and protein products between days 14-21 of cold storage, providing further evidence of early oxidative changes to the RBC membrane during blood bank storage[45]. In future studies we will determine whether antioxidants and/or oxygen depletion are effective in preventing lipid peroxidation, cholesterol loss, and/or morphological changes that occur early during PRBC storage.

Study limitations

We compared the temporal sequence of biochemical events to help predict which events or mechanisms might be involved in RBC morphological and structural changes during storage. We cannot assess the relative importance of these diverse biochemical changes. For example, a small amount of cholesterol loss from the membrane could have greater consequences than a large amount of Band 3 aggregation. However this temporal sequence of biochemical and RBC morphological changes identified events occurring early during storage which are probably related to underlying structural changes in the RBC membrane.

Conclusions

We compared the time scale of RBC morphological defects, Band 3 aggregation, cholesterol and acetylcholinesterase loss from RBC membranes, lipid peroxidation, phospholipid reorganisation of the RBC membrane (CD47 expression), and metabolic changes during cold storage of PRBC. Band 3 aggregation, cholesterol loss from the RBC membrane and defects in RBC morphology occurred early (in the first 7 days) during PRBC storage. Lipid peroxidation, phospholipid reorganisation of the membrane, and changes in extracellular pH, lactate and potassium also occurred in the first 7-14 days of cold storage. In contrast loss of acetylcholinesterase activity and haemolysis of RBC occurred relatively late (14-21 days) during the storage period. These results suggest that oxidative processes are responsible for early protein aggregation, cholesterol loss, and/or membrane phospholipid reorganisation which leads to structural RBC changes during cold storage. Loss of protein-rich vesicles and haemolysis occur later and are probably the result of ongoing membrane damage. Band 3 aggregation, which begins early during storage and continues throughout the storage period, may play a role in reduced post-transfusion survival of PRBC.

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The Authors declare no conflicts of interest.

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