Molecular Defect in the Membrane Skeleton of Blood Bank-stored Red Cells

Abnormal Spectrin-Protein 4.1-Actin Complex Formation

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Abstract

During liquid preservation under blood bank conditions, red cell membranes inexorably undergo damage that decreases erythrocyte survival after transfusion. Accordingly, we have surveyed membrane skeletal protein interactions during storage.

We uncovered a decrease in the in vitro formation of spectrin-actin complex in the absence (50%) or presence (60%) of protein 4.1. Actual formation of the spectrin-actin-protein 4.1 complex fell in a linear fashion during the storage period. This fall in spectrin-actin interaction tightly correlated with the decline in total red cell phospholipid (R = 0.9932) measured simultaneously. This decrement of spectrin-actin association could be restored to >70% of normal values by preincubation of stored spectrin with 50 mM dithiothreitol.

This storage injury to spectrin-actin interaction might weaken the membrane skeleton and lead to decreased red cell survival. In vitro reversability of the damage by reducing agents suggests a possible new direction for prolonging the shelf life of stored blood.

Introduction

During liquid preservation under standard blood bank conditions, red cells undergo a series of reversible and irreversible changes that lead to a failure of stored erythrocytes to circulate after transfusion. Although it is generally accepted that the posttransfusion survival of preserved red cells is related to the status of red cell membrane function (1), most studies have focused on the complex relationship between the metabolic status of the red cell and membrane function (1, 2). Preservation of red cell ATP levels significantly lengthened the storage time of erythrocytes. Nonetheless, the correlation of erythrocyte ATP levels with survival after transfusion is valid only on extremely depleted cells (3, 4), and preservation of ATP levels by perturbation of anticoagulant solutions does not block the storage lesion (5). Better correlations are observed between posttransfusion survival and several measures of red cell surface-area-to-volume ratio (observance of red cell shape change, osmotic fragility, or ektacytometry) (2, 6, 7). These changes are presumably related to the steady spontaneous loss of membrane material known to occur throughout the storage period. Similar membrane loss and alteration in red cell surface-area-to-volume relationships have been demonstrated in several congenital hemolytic anemias and occur as a result of deficiency of, or qualitative changes in, the red cell membrane proteins that comprise the membrane skeleton (8).

The red cell membrane skeleton, a filamentous meshwork of proteins lining the inner membrane surface, likely has the major role in the maintenance of structural stability of the red cell membrane in addition to its influence on red cell shape, flexibility, endocytosis, and lipid organization (9). The skeleton is composed predominantly of four peripheral membrane proteins: spectrin, actin, protein 4.1, and ankyrin. Spectrin, the major red cell membrane protein, is a long, unusually flexible heterodimer that participates in the three major protein-protein interactions in the membrane skeleton (10).

Spectrin associates with itself, head to head, to form tetramers (10) and perhaps higher order oligomers (11). The opposite end of the molecule binds short filaments of actin, thus completing lateral connections within the skeleton. This association of spectrin with actin is greatly enhanced by the interaction of protein 4.1 with spectrin, near the spectrin-actin binding site (12, 13). This protein laminate is set into the membrane by the action of ankyrin, which attaches to spectrin and to the cytoplasmic exposure of protein 3 (14).

In 1979 Schrier et al. demonstrated an abnormality of red cell endocytosis in stored erythrocytes, further indicting the membrane skeleton as a site of injury during preservation (15). However, the degree of inhibition of endocytosis did not correlate with red cell survival (16). No other consistent changes in the amount or function of red cell membrane proteins have been demonstrated during storage (7). Using recently developed assays, we systematically examined the quantity and function of the membrane skeletal proteins in red cells during storage under blood bank conditions. We detected an acquired decrease in the association of spectrin with actin which temporally correlates with the loss of lipid throughout the storage period.

Methods

Source and storage of blood. For our initial studies, 30 ml of blood was drawn into 4 ml of citrate phosphate dextrose solution (trisodium citrate, 26.3 g; citric acid, 3.27 g; sodium dihydrogen phosphate, 2.22 g; and dextrose, 25.50 g in 1 liter H_2O; CPD) from volunteers and used immediately (fresh) and compared with blood that had been drawn into CPD polyvinyl chloride bags (Travenol Geventech Diagnostics, Cambridge, MA) and stored for 6 wk (old, stored, or outdated). For kinetic studies, blood was collected in CPD polyvinyl chloride bags from compensated donors and stored at 4°C in a blood bank refrigerator while 30-ml samples were obtained at various times during the storage interval through a sterile port. As needed to provide enough samples of uniform red cell content for multiple analyses at each time point, blood of com-

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patible type, collected as above, was pooled in 1,000-ml polyvinyl chloride bags and then returned to separate 500-ml polyvinyl chloride bags.

Preparation of membranes and membrane proteins. Erythrocyte membranes were produced by hypotonic hemolysis (17). Spectrin dimer was extracted from prepared membranes by exposure to low ionic strength buffer at 37°C and purified by gel filtration chromatography (17). If the spectrin was not immediately assayed, sodium azide 0.2% was added to the extracts and the tubes containing them were flushed with nitrogen gas, tightly capped, and kept at 4°C. Normal spectrin could be kept free of injury up to 7 d when stored in this way. Spectrin-depleted inside-out vesicles (IOVs)[1], required for assay of rebinding of spectrin to membranes, were separated from low ionic strength extracts of red cell membranes by sedimentation (18). Protein 4.1 was isolated either by the procedure of Tyler et al. (19) or Becker et al. (20).

The function of protein 4.1 did not differ by the method of preparation (20). We consistently used our purified protein 4.1 within 1 wk of preparation. Rabbit muscle filamentous actin (F-actin) was prepared by the method of Spudich and Watt (21). Previous studies have demonstrated that erythrocyte spectrin interacts equally well with red cell or rabbit muscle actin (22). Spectrin was radioiodinated with Bolton-Hunter reagent (New England Nuclear, Boston, MA) (19).

Assay of membrane protein composition. SDS polyacrylamide gel electrophoresis (PAGE) was performed by the method of Steck when protein loads 50 μg or less were applied to the gels (23). For larger loads, the Laemmli technique was used (24). To observe the formation of disulfide-sensitive protein aggregates, we omitted dithiothreitol (DTT) from the solubilizing solution. Staining of carbohydrate-containing proteins was performed using the periodic acid Schiff reagent (23). Gels were scanned by densitometry. Quantitative analysis of membrane protein composition was achieved by a pyridine elution technique (25).

Assay of membrane skeleton protein interactions. The association of spectrin dimers to form tetramers was followed by non-denaturing gel electrophoresis (26). We analyzed the initial amount and ratio of spectrin species in low temperature (4°C), low ionic strength extracts, an indication of the proportion of such species on the membrane. In addition, we observed in these extracts the conversion of spectrin tetramer to dimer during 6 h of incubation at 30°C (26).

The association of spectrin dimer to spectrin-depleted IOVs was analyzed by the method of Goodman and Weidner (18). In one experiment comparing the rebinding of fresh spectrin with fresh and outdated IOVs, the homogeneity of the sidedness of vesicles was demonstrated as previously described (27).

Analysis of the interaction of spectrin and F-actin was performed by microassay (28). This procedure has recently been simplified (20). The incubation conditions remain the same. 60 μl of the incubation mixture are centrifuged over a 200-μl cushion of 5% sucrose in incubation buffer in polyethylene microcentrifuge tubes at 48,000 *g* for 2 h. The tubes are frozen and cut 7 mm from the bottom of the tube and the F-actin pellets, containing bound ^{125}I-spectrin with or without protein 4.1, are assayed for radioactivity. In our initial experiments comparing the association of fresh and outdated spectrin with F-actin, DTT was present at 0.5 mM in the final incubation mixture. Performing these experiments without DTT did not change the absolute value of fresh or old spectrin association. In addition, in a single experiment (data not shown), protein 4.1 from outdated blood performed with equivalent function in enhancement of spectrin–actin–4.1 complex formation. Later in our studies we wished to examine whether the demonstrated diminished capacity of outdated spectrin to bind actin was related to spectrin sulfhydryl oxidation. Accordingly, we incubated fresh and outdated spectrin (0.6 mg/ml) in a buffer containing 150 mM sodium chloride, 10 mM Tris HCl, 0.1 mM EDTA, and 50 mM DTT for 6 h at 4°C and dialyzed 1 ml of this spectrin solution against four changes (average 6 h between each change) of 4,000 ml of buffer without DTT under a nitrogen seal.

1. *Abbreviations used in this paper:* DTT, dithiothreitol; F-actin, filamentous actin; IOVs, inside-out vesicles; PAGE, polyacrylamide gel electrophoresis.

Quantitation of total red cell membrane phospholipids. Care was taken to acid wash all glassware before processing. Red cell membrane phospholipid was extracted using the method of Rose and Oklander (29). After isopropanol–chloroform extraction, inorganic phosphorus was removed by exposing the extract to 0.5 M KCl (5:1, vol:vol) and discarding the aqueous layer. The latter procedure was performed three times to ensure complete removal. The procedure of Bottcher et al. (30) was used to quantitate organic phosphorus.

Other techniques. Protein concentration was estimated according to the method of Lowry et al. (31).

Results

Membranes from stored red cells have normal membrane protein composition. SDS PAGE of red cell membranes from fresh and outdated blood did not reveal any differences in membrane protein composition (Fig. 1 *A*). We did not observe enhanced formation of high molecular weight protein aggregates, even when membranes were solubilized without DTT before SDS PAGE (Fig. 1 *B*). Fig. 2 illustrates the results of densitometric quantitation of membrane proteins. The only consistent difference between fresh and outdated membranes was the increased amount of globin on the membranes ($P < 0.01$). This finding has been observed before in hemolytic anemia and ATP-depleted cells as well as in stored red cells (2, 32, 33). Pyridine elution of Coomasie blue–stained gels was performed to better quantitate the relationships of the membrane skeletal proteins. The ratios of spectrin, ankyrin, protein 4.1, and actin to protein 3 did not differ between the two groups (Table I).

The spectrin self-interaction is unchanged during storage. We examined spectrin–spectrin interaction by analysis of the amount and proportion of spectrin dimer, tetramer, and oligomer in cold (4°C) low ionic strength extracts of ghosts. The extraction was efficient (>96%) for both fresh and outdated ghosts and the extracts had indistinguishable elution patterns on gel filtration chromatography. Fig. 1 *C* depicts the effect of non-denaturing gel electrophoresis at 0°C on fresh and outdated spectrin extracts.

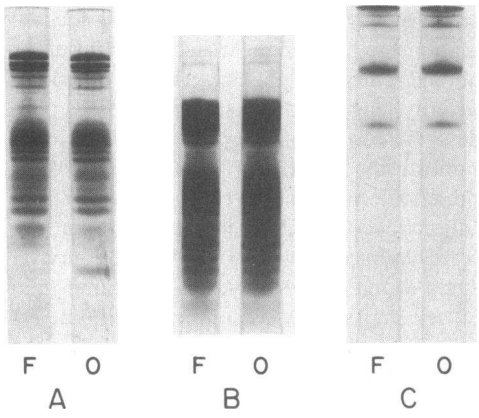

Figure 1. PAGE of membranes (*A* and *B*) or low ionic strength 4°C spectrin extracts (*C*) from red cells, freshly drawn (*F*) or stored for 6 wk (0) in CPD. In *A*, 50 μg of membrane protein were loaded on SDS gels. The only difference noted is an increase in globin in the stored ghosts. Higher loads (~250 μg) were placed on gels without DTT in the solubilizing solution and run using the Laemmli system. (*B*) No difference in high molecular weight aggregates is seen, nor are there proteolytic fragments observed. (*C*) PAGE without SDS of spectrin extracts shows no difference in proportion of spectrin oligomer (*top*), tetramer (*bold band*), or dimer (*bottom band*).

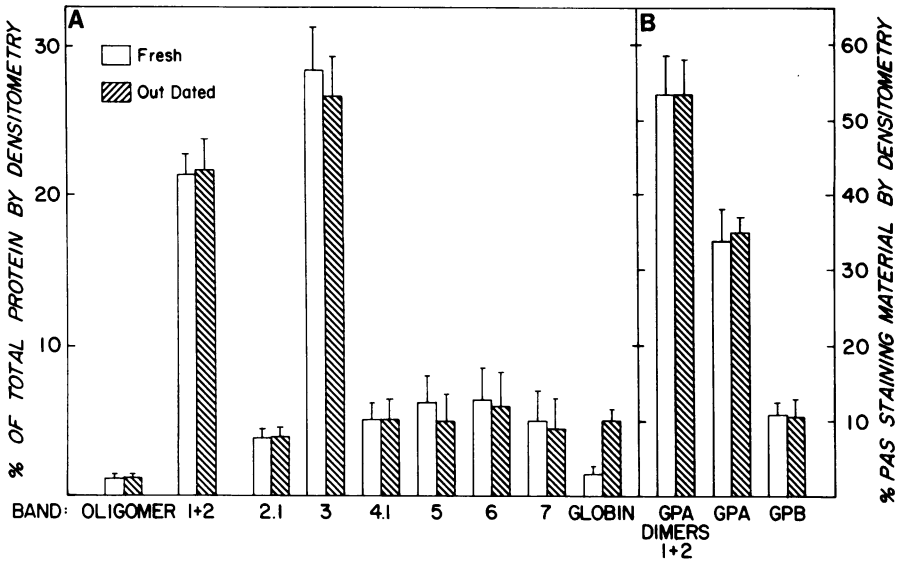

Figure 2. Results of densitometry performed on SDS PAGE gels loaded with 50 μg of membrane proteins from red cells freshly drawn (*open bars*) and stored for 6 wk in CPD (*hatched bars*). Data in *A* is derived from Coomasie blue-stained gels. Data in *B* is derived from periodic acid Schiff staining for carbohydrate. Error bars demonstrate the standard deviation.

There is no difference in the proportion or amount of spectrin dimer, tetramer, or oligomers in these extracts. Conversion of tetramer to dimer at 30°C over 6 h was identical for the two groups (63±8%, fresh; 65±10%, outdated. $n = 5$).

The rebinding of spectrin to spectrin-stripped IOVs is unchanged during storage. We used the rebinding of fresh and outdated spectrin to spectrin-stripped IOVs as a measure of the ability of spectrin to bind to ankyrin. Fig. 3 *A* shows that there was no loss of spectrin–ankyrin binding capacity in three separate experiments. We then incubated fresh spectrin with fresh and outdated IOVs to compare the availability of spectrin membrane-binding sites. In this experiment, uniformity of the sidedness of IOVs between the two groups was verified by chymotryptic digestion of surface proteins (data not shown). There was no difference between fresh and old vesicles in their capacity to rebind fresh spectrin (Fig. 3 *B*).

The capacity of spectrin to bind F-actin is diminished during storage. Fig. 4 demonstrates that there was a significant decrement in the ability of outdated spectrin to associate with F-actin. At the saturation point (spectrin concentration = 0.6 mg/ml) (26), spectrin–actin association was decreased 50% in the absence of protein 4.1 and 60% in its presence. The actual formation of spectrin–actin–protein 4.1 complex (determined by subtracting spectrin bound in the absence of protein 4.1 from that bound in its presence) was decreased 70%, which demonstrates damage to the spectrin–protein 4.1 interaction (19, 28) as well.

For the spectrin lesion to be considered significant, it must correlate with known changes in posttransfusion survival over time. One would expect a slow decrease during the first 3 wk of storage (the official outdate time of CPD blood where posttransfusion survival would remain >70%), with a more precipitous decline thereafter. We did not perform simultaneous survivals to calculate an actual correlation coefficient. Nonetheless, curves depicting the fall in spectrin–actin interaction over storage time, shown in Fig. 5 *A*, are certainly consistent with the expected pattern. The actual in vitro formation of the spectrin–actin–protein 4.1 complex falls in a linear fashion from the onset of storage (Fig. 5 *B*). This pattern is different from the changes in red cell ATP levels which show a complex non-linear decrease during the first weeks of storage (3).

The loss of total red cell membrane phospholipid during storage correlates with the spectrin–actin lesion. In two experiments we concurrently measured red cell membrane phospholipid loss and the loss of spectrin–actin binding function over a 6-wk period (Fig. 6). Our results for loss of lipid membrane material are similar in pattern but somewhat less in quantity than those published by Haradin et al. for cells stored in acid citrate dextrose

Table I. Proportion of Membrane Proteins in Freshly Drawn Red Cells and Those Stored for 6 wk

Protein Ratios	Fresh	Outdated
	n = 16	*n = 16*
Sp/Band 3	0.91±0.03	0.94±0.04
Ankyrin/Band 3	0.15±0.02	0.16±0.03
4.1/Band 3	0.25±0.03	0.23±0.02
Actin/Band 3	0.22±0.02	0.21±0.03

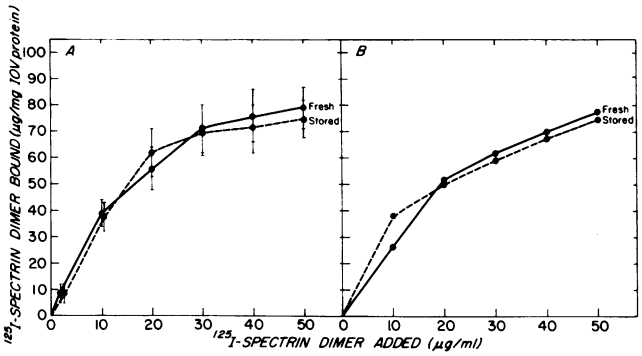

Figure 3. Results of rebinding of ^{125}I-spectrin to spectrin stripped inside out vesicles. *A*, the rebinding of spectrin extracted from freshly drawn red cells (solid line) and that of spectrin drawn from stored red cells (dashed line) to fresh IOVs. *B*, the rebinding of fresh spectrin to fresh and stored IOVs. Error bars demonstrate the standard deviation. ^{125}I-spectrin dimer was incubated for 90 min at 0°C in a 0.225-ml volume containing 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM NaN_3, 1.0 mM DTT, 1 mM $MgCl_2$, and 10 mM $NaPO_4$ (ph 7.5) with IOV protein. There is no difference between fresh and stored spectrins or IOVs.

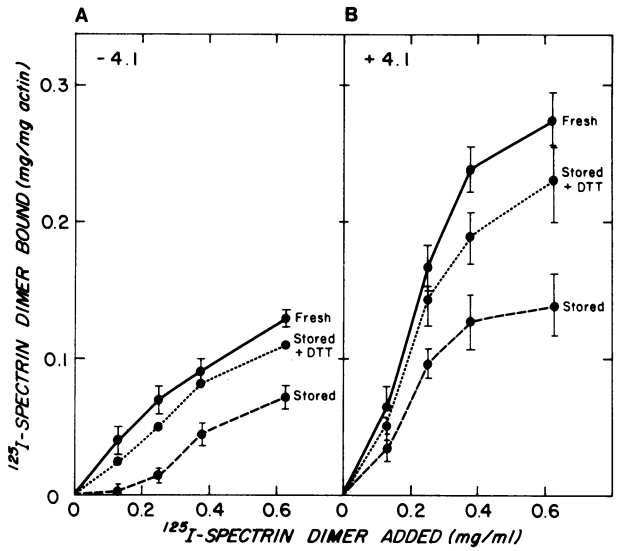

Figure 4. The binding of ^{125}I-spectrin dimer to F-actin in the presence (*A*) or absence (*B*) of protein 4.1. Error bars demonstrate the standard deviation. In each experiment, various concentrations of ^{125}I-spectrin (5,000 cpm/μmol) were incubated with F-actin (0.3 mg/ml) in the presence or absence of protein 4.1 (0.075 mg/ml) in a buffer with the following composition: 156 mM NaCl, 7.5 mM KCL, 10 mM Tris HCL, 3.8 mM Na_2HPO_4, 0.5 mM DTT, 0.1 mM adenosine triphosphate, 0.75 mM Mg_2Cl_3, 0.01 mM $CaCl_2$, 0.04 mM EDTA, and 4.4 mg of bovine serum albumin. The mixture was incubated at room temperature for 60 min and the spectrin-actin complex pelleted through a 20% sucrose cushion by centrifugation. Solid lines, spectrin extracted from fresh red cells; dashed lines, from stored red cells; dotted lines, from stored spectrin per incubation with 50 mM DTT. There is a deficit of spectrin-actin complex formation in stored spectrin, which is reversible with DTT.

solution (2). In Fig. 6 *B*, the relationship between phospholipid loss and the in vitro formation of the spectrin-actin-protein 4.1 complex is illustrated. The strong correlation (R = 0.9932) suggests a role for the spectrin damage of storage in the serious preservation injury of lipid loss.

The spectrin storage lesion can be reversed in vitro by reducing agents. Previously, we have shown that the capacity of spectrin

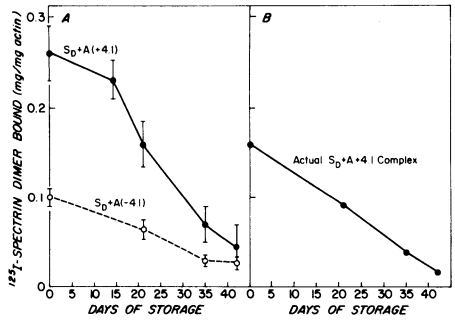

Figure 5. Spectrin-actin complex formation measured at different days into the storage period of red cells in CPD. Spectrin-actin complex formation was performed as described in Fig. 4. (*A*) The results of complex formation in the presence (solid line) or absence (dashed lines) of protein 4.1. Error bars demonstrate the standard deviation. (*B*) The spectrin-actin-protein 4.1 complex formation (obtained by subtracting lower curve from upper curve in *A*). Spectrin-actin complex formation decreases with time in storage.

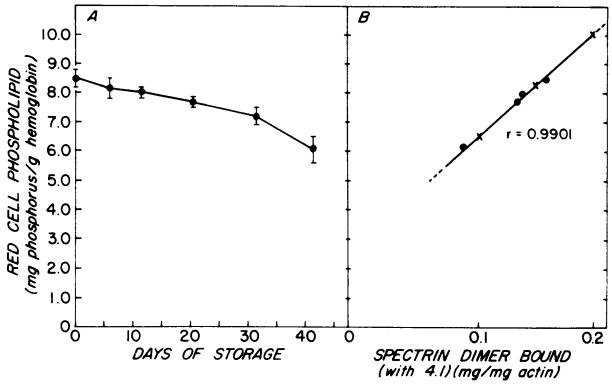

Figure 6. Phospholipid loss and its relationship to spectrin-actin-protein 4.1 complex formation. (*A*) the amount of red cell phospholipid remaining in red cells after given periods of time. Error bars demonstrate the standard deviation. (*B*) a graph of spectrin-actin complex formation on the horizontal axis vs. phospholipid loss on the vertical axis. The line shown is a linear regression analysis of the points depicted. There is a striking correlation between red cell lipid loss and spectrin-actin complex formation.

to bind protein 4.1 and actin could be disrupted by oxidation (28). We thus preincubated spectrin from outdated blood with an excess of DTT and obtained a significant improvement of the amount of spectrin-actin complex formed in both the presence and absence of protein 4.1 (Fig. 4). In both cases, >70% of the capacity of spectrin to bind actin was returned.

Discussion

The survival of transfused red cells has long been thought to relate to the integrity and structure of the preserved red cell membrane (1, 2). This is reflected in the fact that the most consistent predictors of posttransfusion survival are parameters of membrane shape and deformability (2, 6, 7). Clearly, there is a crucial relationship of ATP levels to survival as well, because the poor posttransfusion performance of ATP-depleted cells (levels 50% of normal) can be dramatically reversed by repletion of ATP in cells. This "rejuvenation" of red cell survival is accompanied, however, by major improvements in membrane-related functions, including shape, filterability, and whole blood viscosity (2). In addition, despite maintenance of ATP at even supernormal levels, red cells in liquid storage undergo an inexorable loss of posttransfusion survival (5).

Current understanding of red cell membrane structure and function assigns the major role for shape, integrity, and deformability to the red cell membrane skeleton. The proteins that comprise the skeleton (spectrin, actin, protein 4.1, and protein 2.1) can be quantitated and their relationships studied in vitro. Accordingly, we hypothesized that an acquired injury to the membrane skeleton—independent of the reversible injury of ATP depletion—might contribute to the loss of posttransfusion survival of stored red cells. Previous investigations of membranes from stored red cells have demonstrated changes in non-skeletal proteins (increased protein 4.5 and hemoglobin) (32) but no major qualitative changes in skeletal proteins (15, 33, 34). Of interest is the previous work of Schrier et al. (15). They demonstrated a significant loss of primaquine-induced endocytosis during the storage period (15). Although the lesion did not correlate with posttransfusion survival (16), this finding was a clear demonstration of a membrane skeletal defect in storage.

We systematically surveyed the membrane skeletal proteins and their interactions in red cells exposed to typical blood bank storage conditions. In our study, we detected a progressive loss of the ability of spectrin to bind F-actin in the presence or absence of protein 4.1, while other protein relationships did not change. We may analyze the significance of this finding by examination of: (*a*) the relationship between our in vitro assay and the in vivo situation; (*b*) storage-related changes in red cell phospholipid loss and membrane function; and (*c*) similarities between our acquired spectrin damage and congenital or in vitro fabricated spectrin damage.

We performed our kinetic storage experiments at a spectrin concentration of 0.6 mg/ml. In actual fact, the spectrin concentration on the surface of the membrane may be as high as 200 mg/ml (9). Thus, this assay can only be considered a qualitative reflection of loss of protein function.

The effect of other compensatory or mitigating skeletal protein relationships may explain the fact that red cells at 42 d of storage, which have 12% of spectrin–4.1 binding capacity, appear structurally sound. Red cells from the congenital hemolytic anemia with a similar molecular defect have little increased mechanical instability (35) despite a 40% decrement in spectrin–actin–protein 4.1 complex formation. It would appear that fragmentation and mechanical instability may be the exclusive province of recently named horizontal defects (e.g., failure of spectrin dimer–dimer interaction), whereas vertical defects, such as the defective spectrin–protein 4.1 interaction observed in the congenitally defective or injured spectrin, lead to spontaneous surface area loss (36).

The acquired spectrin damage of storage was observed as early as our first assay point (at 2 wk) and continued to fall even beyond 6 wk of storage. Our data indicates, in addition, correlation between loss of total phospholipid from stored red cells and the decrease in spectrin–actin–protein 4.1 complex formed in vitro. This relationship provides a potential connection between the spectrin storage injury and the changes in red cell surface-area–to-volume ratio that characterize and perhaps create the preservation injury.

Our experiments suggest, but do not prove, that the loss of spectrin function is responsible for the lipid loss observed. However, alterations of spectrin interactions within the membrane skeleton are consistently associated with shortened red cell lifespan and hemolytic anemia. Of special interest are those defects that lead to spontaneous surface area loss. These include quantitative spectrin deficiency (25), failure of spectrin–spectrin interaction (26, 36), protein 4.1 deficiency (37) and decreased spectrin–actin–protein 4.1 interaction (28, 38).

Of these, the spectrin–protein 4.1 lesion that interferes with spectrin–actin association most closely resembles the acquired storage lesion. This inherited abnormal spectrin causes the clinical phenotype of hereditary spherocytosis, a condition often considered as a model of the storage lesion (39). Of note is the fact that, just as in the acquired spectrin damage, the inherited spectrin defect can be reversed in vitro by preincubation with reducing agents. In addition, the capacity of normal spectrin to bind to protein 4.1 can be eliminated by oxidation of a single disulfide bond (37).

Three other species of evidence are consistent with a relationship of this oxidative lesion to posttransfusion survival. First, the correlation coefficient between spectrin–actin–4.1 complex formation and posttransfusion survival in CPD (40) is 0.8650. That this is not as strong a relationship as the one between complex formation and lipid loss, reflects, in our opinion, the multifactorial nature of the storage lesion.

In addition, the results of two experiments examining storage of blood under anaerobic conditions are of interest. In one study of vesiculation, storage in a nitrogen environment decreased the amount of vesicles formed (41). Hogman et al. showed that red cell fluidity (measured by filtration) was significantly improved after 28 d of anaerobic storage under blood bank conditions (42).

What might be the sources of oxidation, especially of spectrin in the storage period? External to the red cell there are ample sources of free radicals or other oxidizing agents (degenerating white cells, metals, and exposure to light and agitation). As there is little evidence of lipid peroxidation during storage (43), it would be unlikely that the peripheral membrane proteins on the inner membrane surface could be oxidized selectively by an external agent. The more likely sources of spectrin oxidation are on the cytoplasmic side of the membrane. Hebbel has shown that membranes from patients with homozygous sickle cell disease are capable of generating superoxide, peroxide, and hydroxyl radical in proportion to the amount of hemichrome bound to the membrane (44). Platt et al. have demonstrated a spectrin injury remarkably similar to the storage lesion in a patient with hemoglobin Nottingham (45). They postulate that this spectrin lesion, which interferes with spectrin–protein 4.1 interaction and is reversible with reducing agents, results from oxidation by breakdown products of this unstable hemoglobin. Our data show that stored red cells accumulate hemoglobin on the membrane as well. Accordingly, it is possible that hemoglobin breakdown products may be responsible for oxidation of membrane proteins in storage.

There is a decrement in the ability of spectrin from stored blood to bind actin. Whether this observation is directly linked to posttransfusion survival remains to be examined. It is likely that the storage lesion is the result of many injuries to the red cell membrane and the link between ATP depletion effects and membrane skeletal function remains to be explored. In this regard, analysis of the effect of boosting and preserving ATP levels as is now common in blood bank storage (e.g., CPD-A, Adsol, etc.) requires examination in our system. The effect of antioxidants added to the storage milieu, experiments currently underway, may also reveal the ultimate importance of this phenomenon.

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