On the Preservation of Contractile Proteins During Storage of Human Platelets

By Roger C. Lucas, John Lawrence, and Alfred Stracher

A study has been undertaken to determine the rate at which stored platelets lose their ability to respond to stimuli and to establish whether this decrease in function could be ascribed to the storage-induced proteolysis of prominent platelet proteins observed by others. Platelet concentrates were stored at 4°C and 25°C for up to 14 days, and their ability to secrete and aggregate in response to appropriate stimuli was determined at 6, 96, and 192 hr after venipuncture. At each time point the protein complement of the platelets was also monitored by SDS-polyacrylamide gel electrophoresis to assess the extent of intracellular protein degradation. Platelets from concentrates stored at either temperature exhibited a decreased ability to respond to stimuli as storage time increased. After 8 days of storage at 4°C and up to 9 days at 25°C, no proteolysis of major platelet proteins was observed; however, complete loss of platelet function was observed. This strongly indicates that a decrease in platelet function should not be causally linked to degraded contractile structural proteins and that extending the functional life of platelets during storage is still an attainable goal since proteolysis is not the inevitable result of short-term storage.

PLATELETS isolated from other blood cells and held in plasma as platelet-rich plasma (PRP) or as platelet concentrates have a clinically useful "shelf-life" of less than 72 hr. An increasing demand for transfusible platelets has stimulated efforts directed toward improving the hemostatic effectiveness of stored platelets, as well as extending the storage life of this extremely valuable blood resource. Whether or not these objectives are feasible has recently been called into question, for it has been reported that storage is associated with the proteolysis of the high molecular weight platelet contractile proteins, myosin and actin-binding protein (ABP). A recent report shows close to a 50% proteolysis of the platelet's ABP by the second day of storage at 22°C, and its complete disappearance by the fifth storage day. ABP is a significant platelet component, amounting to nearly 8% of the platelet's protein mass and upon interaction with another major platelet contractile protein, actin, is presumed to comprise the platelets cytoskeletal structure. Although no role for cytoskeletal structures have as yet been related to physiologic function for platelets, some involvement in platelet shape change seems obvious (e.g., disc to sphere, filopodia extension, etc.). Thus, it would be apparent that their degradation during storage should manifest itself as a decrease in platelet function and hence may be the cause of storage-induced platelet dysfunction.

Here we report a study designed to systematically test this hypothesis. Our study differs from those done previously in that during the storage period we monitored not only the platelets' protein composition, but in addition we measured the decline of two platelet functions, secretion and aggregation. This permits a determination as to whether a correlation between decreasing platelet function and proteolysis of the platelet's contractile components should in fact be drawn. In contrast to our earlier findings, we find no evidence to support the hypothesis that storage induces the proteolysis of these components, which then in turn results in platelet dysfunction.

MATERIALS AND METHODS

Whole blood was obtained from normal healthy volunteers who had signed an informed consent statement, and all investigations were conducted with the approval of our Health Science Review Committee on Investigation Involving Human Subjects in accordance with NIH guidelines. Platelet concentrates were prepared for us by arrangement with the New York Blood Center. One unit of whole blood was collected with CPD into a Fenwall 4R-1718 triple pack and the time of venipuncture noted and designated as time zero. The resulting platelet concentrate (7.5 × 10^10 platelets in 25 ml of plasma) was held undisturbed at room temperature for 0.5 hr after preparation, then transported to us in insulated containers (transportation time 1 hr, transportation temperature 18–22°C). Upon arrival, each bag of platelet concentrate was inspected, and if any clumping was detected in the bags, it was rejected for this storage study and diverted to another unrelated study.

Concentrates to be stored at 4°C were hung vertically in a refrigeration box (4°C ± 1°C), while those stored at 25°C were placed in a constant temperature room (25°C ± 2°C) affixed to a rotation device such that the long axis of the bag rotated in a plane perpendicular to that of the floor at one revolution per minute (rpm). Two hours before the function and protein complement of the stored platelets were to be monitored, 5 ml of concentrate was withdrawn from the appropriate bag, under sterile conditions, and centrifuged at 350 g for 20 min to remove any contaminating red cells and leukocytes. Platelets were then sedimented from the plasma at 1200 g for 20 min. The platelets were washed free of any

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contaminating plasma by gently resuspending the platelet pellet in 5 ml of 126 mM NaCl, 5 mM KCl, 0.3 mM EDTA, 5 mM glucose, 10 mM sodium phosphate pH 7.4, and then resedimenting them. This wash procedure was repeated twice more and the final platelet pellet (~200 μl) was resuspended with 2 vol of the above saline–wash solution. All of the above centrifugations of 4°C stored platelets were performed at 4°C; those for 25°C stored platelets were carried out at room temperature.

To test in vitro platelet function, 50 μl of the washed resuspended platelets at a concentration of approximately 2.5 × 10⁷ platelets/μl were added to a Chrono-Log Lumi-Aggregometer cuvette (Haverstown, Pa.) containing 4 mg of luciferin-luciferase (DuPont) and either 500 μl of autologous plasma or 500 μl of 135 mM NaCl, 25 mM Tris, 5 mM glucose, 4.3 mM MgSO₄ pH 7.4, and allowed to equilibrate with stirring at 37°C for 5 min before an appropriate stimulus was introduced. The progress curves of both secretion and aggregation were simultaneously recorded on the Lumi-Aggregometer as described by Feinman et al. ATP secretion was detected by the luminescence it produced in the luciferin–luciferase system and quantified by the addition of an ATP standard after each determination. Aggregation was measured in the usual fashion as the increase in light transmitted through a platelet suspension, setting the aggregometer limits such that transmittance through an unstimulated platelet suspension was recorded as 0% aggregation, and transmittance through the same media devoid of platelets was recorded as 100% aggregation. In plasma, 15 μg of collagen fibrils (Hormonchemie) or 153 nM γ-thrombin was the stimulus, whereas in saline the stimulus was 5 nM α-thrombin. Both species of thrombin were the generous gift of Dr. John W. Fenton III.

To assess the extent to which platelet proteins may have been degraded during storage, a 50-μl aliquot of the above washed, resuspended platelets (2 mg of protein) was injected into 1 ml of a 1% SDS, 1% beta-mercaptoethanol, 5 mM EGTA, 25 mM Tris- SO₄ pH 7.0 solution held at 100°C and maintained there for 5 min. Such a sample of total platelet protein solubilized in SDS was taken for each time point and held frozen (~20°C) until the term of the experiment, at which time all the samples were simultaneously electrophoresed to compare their protein composition. The details of the methods used for SDS-polyacrylamide gel electrophoresis and subsequent quantitative densitometry of these gels have previously been described by us, as have the methods used to identify platelet actin and ABP.

RESULTS

Before a correlation could be drawn between a storage-induced proteolysis of platelet contractile proteins and a storage-induced impairment of platelet function, it became necessary to establish the extent to which the platelets' response to stimuli in vitro decreased with increases in storage time. Both secretion and aggregation were monitored simultaneously. In order to insure that the decrease in the measured response was an indication of general platelet deterioration and was not due to the loss of a specific receptor site for a given stimulus, two different stimuli presumed to have different receptor sites were used.

<table>
<thead>
<tr>
<th>STORAGE TEMP</th>
<th>TESTED IN</th>
<th>4°C</th>
<th>25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLASMA</td>
<td>15 μg/ml COLLAGEN</td>
<td>153 nM γ-THROMBIN</td>
<td>5nMα-THROMBIN</td>
</tr>
<tr>
<td>SALINE</td>
<td></td>
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![Fig. 1] Aggregation and secretion response curves of platelets removed from concentrates stored at 4°C (A–C) and 25°C (D) and stimulated at 37°C with the agent indicated at 6, 96, 192 hr after venipuncture. The vertical bars to the right of each set of secretion curves are calibration bars used to quantitate the ATP secreted for the 6, 96, 192 hr curves, respectively. They show the luminescence produced when, after each secretion recording has been made, the platelet suspension is made an additional 2 μM (in curves A and B) or 10 μM (in curves C and D) in ATP.
Table 1. The Normalized Rates of Secretion and Aggregation

<table>
<thead>
<tr>
<th>Storage Temperature:</th>
<th>4°C</th>
<th>25°C</th>
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</thead>
<tbody>
<tr>
<td>Stimulus:</td>
<td>Collagen</td>
<td>γ-Thrombin</td>
</tr>
<tr>
<td>(conc)</td>
<td>(15 μg)</td>
<td>(153 nM)</td>
</tr>
<tr>
<td>Medium:</td>
<td>Plasma</td>
<td>Plasma</td>
</tr>
<tr>
<td>Storage Time (hr)</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>1.00 (51)</td>
<td>1.00 (70)</td>
</tr>
<tr>
<td>96</td>
<td>.28 (14)</td>
<td>.31 (22)</td>
</tr>
<tr>
<td>192</td>
<td>.19 (9.7)</td>
<td>.24 (16.5)</td>
</tr>
<tr>
<td>Aggregation Rates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.00 (9.4)</td>
<td>1.00 (8.4)</td>
</tr>
<tr>
<td>96</td>
<td>.15 (1.4)</td>
<td>.16 (1.5)</td>
</tr>
<tr>
<td>192</td>
<td>.04 (0.4)</td>
<td>.09 (0.8)</td>
</tr>
<tr>
<td>Secretion Rates</td>
<td></td>
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</table>

The initial rate of secretion and aggregation (data in parentheses) were computed from the curves in Fig. 1 and normalized by setting the 6-hr time point equal to unity.

employed: collagen and γ-thrombin. Further, to offset the possibility that there may be a build-up of substances in the plasma that would inhibit platelet function, the ability of washed platelets suspended in saline to respond to α-thrombin was also determined.

Figure 1 (A–C) shows typical secretion and aggregation response curves obtained from platelets stored at 4°C and subjected to three different stimulating conditions at 90-hr intervals. Figure 1D shows the response curves obtained from platelets, stored at 25°C, resuspended in saline and stimulated with α-thrombin. Several characteristics of the stored platelets' ability to respond to stimuli in vitro became apparent from examining these curves. The first and most obvious is that the platelets' response to stimuli decreases with increasing storage time. The second is that this decrease is observed with all three stimuli and in both plasma and saline, indicating that the decay is not stimulus specific nor the result of a build-up of inhibitor substances in the plasma. Third, platelets stored at 25°C become functionally impaired by these criteria far more rapidly than those stored at 4°C.

These data can be quantitated when the initial rates at which the secretion and aggregation reactions proceed at each of the storage times tested are computed and compared. The rates are calculated as the initial slope of the response curves presented in Fig. 1 (A–D) (resulting in the initial rate of secretion being expressed as μM ATP/min and that for aggregation as %/min) and are listed in parenthesis in Table 1. To facilitate a cross-comparison between the different stimulating conditions, the data have been normalized by setting the rate of the 6-hr time point for both platelet functions in each of the four stimulation groups equal to unity and expressing the rate determined at each subsequent time point as a fraction of the 6-hr rate. The table shows that when tested in plasma after 96 hr of storage at 4°C, the platelets aggregate at a rate approximately 30% of that
observed at 6 hr but their rate of secretion has declined to 15%. This result is even more dramatic in saline with α-thrombin as the stimulus. Here the rate of aggregation has declined to 44% and 19% by the 96th and 192nd hr of storage and the secretion rate has fallen to 5 and less than 1%.

Regardless of which of the two platelet functions are selected as a criterion, it is clear that by the eighth day platelets stored at 4°C show severe functional impairment and those held at 25°C are almost devoid of function. This established, it can now be determined whether or not this progressive decrease in platelet function can be correlated to a reduction or disappearance of major platelet proteins. As seen in Fig. 2, rupture of the platelets integrity in 1% Triton solutions without EGTA leads to a rapid disappearance of the two upper bands, ABP and band-2, with an increase in the amount of protein in the position of myosin. The proteolysis is essentially complete in 2 min. In the presence of EGTA or leupeptin, no such proteolysis is observed. This dependence of proteolysis on Ca²⁺ is no doubt due to the presence of a Ca²⁺-dependent protease present in platelets⁷,⁸ and first described in muscle.⁹

If platelets are stored intact as described for varying periods of time at either 4°C (Fig. 3) or 25°C (Fig. 4) and analyzed by SDS-PAGE, no indication of a loss of ABP is seen at either condition for at least 8 days at 4°C and 9 days at 25°C by densitometric analysis. At 11 days at 25°C, some loss of ABP (~10%) is observed. By 14 days, approximately 75% of the ABP is proteolyzed and a significant aggregation is noted in the bag. Analysis of this aggregate by SDS-PAGE shows complete disappearance of ABP and band-2 (see Fig. 4 Agg.). We have previously reported that ABP, myosin, and actin are present at a 1:1.5:4 weight ratio in freshly drawn platelets, and that taken together these three proteins amount to between 45% and 50% of the platelet’s total protein mass.⁶ No significant deviation from these values was detected in platelets that had been stored for up to 8 days at 4°C (10 bags assayed) or up to 9 days at 25°C (6 bags assayed).

DISCUSSION

The results presented here show that when platelet concentrates are prepared and stored under blood bank conditions, at either 4°C or 25°C, proteolysis of the platelet’s contractile-structural proteins does not occur even when held up to 9 days at 25°C. These results are at variance with a report that has noted a near complete proteolysis of ABP at shorter storage times than those employed here,⁷ as well as a report of the proteolysis of myosin.¹ In this study we have solubilized intact platelets directly in 1% SDS at 100°C to offset the proteolysis of high molecular weight proteins known to occur in platelet homogenates, as seen in Fig. 2.

The data also show that by the eighth day of storage, the rates at which platelets secrete and aggregate in response to in vitro stimuli have dropped to below 20% of their prestorage values. This observed decrease in function without a concomitant proteolysis of contractile proteins clearly negates the hypothesis that a degradation of these proteins is responsible for storage-induced platelet dysfunction.

It is not possible at this stage to rationalize the differences found earlier for myosin proteolysis and the results presented here. The major experimental difference is that in this study total platelet proteins are analyzed, whereas previously we isolated and puri-
fied the platelet myosin. Our myosin purification was carried out under conditions thought to minimize proteolysis but that was before recognition of the presence of a Ca$^{2+}$-dependent protease in platelets, and in retrospect, we probably had not inhibited this protease. In addition, greater care was made in this study to eliminate any small aggregates. It is thus possible that the previously reported proteolysis was the result of a Ca$^{2+}$-activated protease in a fraction of the total platelets that had spontaneously aggregated. Although no proteolysis of myosin or ABP was observed in fresh platelets in either our previous study or that by Robey et al., it would seem that some factor, perhaps greater susceptibility to aggregation, was responsible for the proteolysis seen in the stored samples.

While the platelets demonstrate a very reduced capacity to respond to stimuli by the eighth storage day, it can be inferred that their capacity to iono-regulate, at least with respect to Ca ions, continues. It has been reported that agents causing a rise in the platelet's intracellular Ca$^{2+}$ concentration result in a rapid proteolysis of the ABP. Since storage did not result in the proteolysis of this component, the data can be taken to imply that the platelet's ability to regulate its intracellular Ca$^{2+}$ is maintained through the eighth storage day.

In light of the remarkable resistance to proteolysis demonstrated by stored platelets in this study, finding conditions that would extend their functional life should be considered well within the realm of feasibility.

Fig. 4. SDS electrophoretic profiles of platelet protein isolated from concentrates that had been stored as described in the text at 25°C for the times indicated.
REFERENCES

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