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Further Studies on the Postmortem Changes of Red Blood Cells

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ABSTRACT. Postmortem changes in membrane components of red cells were investigated. The membrane components examined were sialic acid, protein subunits and lipids. Under ether anesthesia, rats were killed by stabbing in the breast to induce hemorrhage, after which blood was drawn from thorax. The observation period was limited to 18 hours after death, during which time changes in osmotic fragility and red cell shape are clearly observed.

The quantity of sialic acid declined with the passage of time during this early postmortem period. The value at 18 hours was about 75 per cent of that in normal cells.

The subunit patterns obtained by PAGE of membrane proteins of cells 18 hours after death were similar to those of normal red cells. In spite of the decrease in sialic acid content, PAS staining yielded no difference between normal and 18-hour-postmortem red cells, and it was thought that membrane proteins did not change markedly during this early postmortem period.

It was found that changes in lipid composition happened within 3 hours of death, and fragments were observed. The decrease in free cholesterol was most remarkable, and at 18 hours after death the cholesterol level fell to about 75 per cent of that of normal red cells. The ratio of free cholesterol to total phospholipid decreased from 0.44 to 0.36. These results suggest that a significant decrease in free cholesterol in red cell membrane may hold the key as to why changes in osmotic fragility and red cell shape occur during the early postmortem period.

Key words : osmotic fragility — postmortem changes — red cell membrane — rat

Soon after death, red cells in cadavers change in shape from discocytes to sphero-echinocytes, osmotic fragility increases, and, finally, the cells hemolyze. Previously, the author reported that observing this phenomenon by both coil planet centrifugation and scanning electron microscopy was of great value in estimating the time after death.[1-3)]

The approximate composition of the red cell membrane is 50 wt% protein, 40 wt% lipid and 10 wt% carbohydrate, and these components have been reported to take a vital part in osmotic fragility and morphology of red cells. Shinozuka *et al.* reported that nearly 30% of the bound sialic acid was released when hemolysis increased in stored blood.[4)] It has been proposed that the cytoskeletal network involving spectrin and actin may play an important

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role in determining red cell shape and deformability.[5,6] Alterations in the cholesterol concentration of red cells can significantly change cell morphology and function. Cooper *et al.* have shown that cholesterol-enriched red cells, i. e., spur cells, exhibit a broad and flat shape with increased surface area and irregular margins, whereas cholesterol-deficient cells have a spherocytic appearance and exhibit increased osmotic fragility as well as altered ion transport.[7] There exists considerable literature on the biochemical changes occurring in stored, aged and hereditary pathologic red cells, but little work has been done to study their postmortem changes occurring within cadavers.

The present study is aimed primarily at an analysis of the postmortem changes in the major membrane components, sialic acid, protein and lipid.

MATERIALS AND METHODS

Postmortem blood was drawn directly from the thoraxes of rats that were killed by stabbing in the breast as described previously.[3] Control samples were obtained by decapitating the animals.

Red cell ghosts were prepared by the procedure of Dodge *et al.*[8] using hypotonic phosphate buffer.

Sialic acid was determined according to the method of Warren[9] after one hour of hydrolysis of the ghosts in 0.1 N H_2SO_4 at 80°C. N-acetylneuraminic acid served as a standard.

For protein determination, the ghosts were dissolved in 1.0 N NaOH, and analyses were performed according to the method of Lowry *et al.*[10] with bovine serum albumin as the standard.

For polyacrylamide gel electrophoresis (PAGE), the ghosts were dissolved in 2% SDS, 2% β-mercaptoethanol, 2 mM EDTA, 80 mM dithiothreitol, 20% sucrose and 20 mM Tris-HCl (pH 8.0), and boiled for 3 min at 100°C. PAGE was performed according to the method of Fairbanks *et al.*[11] After electrophoresis, gels were stained for proteins with Coomassie brilliant blue R-250, and for glycoproteins (PAS staining). The stained gels thus obtained were then scanned with a Gilford spectrophotometer at 520 nm with a scan rate of 2 cm/min.

The examination of lipid components of red cell membrane was according to the method of Takemoto[12] as shown in Fig. 1. Lipids were extracted using isopropanol and chloroform from hemolysate prepared by adding distilled water to washed red cells (1 : 1, v/v).[13] Hematocrit values of washed packed red cells were measured prior to hemolysis. Aliquots of the lipid extract were evaporated, then dissolved in n-propanol, and used for determination of free cholesterol content. Free cholesterol values (mg/dl packed red cells) were determined by an enzymatic method using kits. The remainder of the lipid extract was evaporated to dryness, and then dissolved in Folch's solution[14] for examination of the change in each of the lipid components. An Iatroscan TH-10 analyzer (Iatron Laboratories, Tokyo, Japan) equipped with a chromatopack integrator (C-RIA, Shimadzu, Japan) was used for the TLC-FID analysis of lipids. The Folch's solution (2-5 μl) was spotted on a silica gel rod (chromarod, S-II), and developed with chloroform-methanol-water (70 : 35 : 3.2, v/v). After development, the rod was dried at 115°C for 5 min and scanned through a

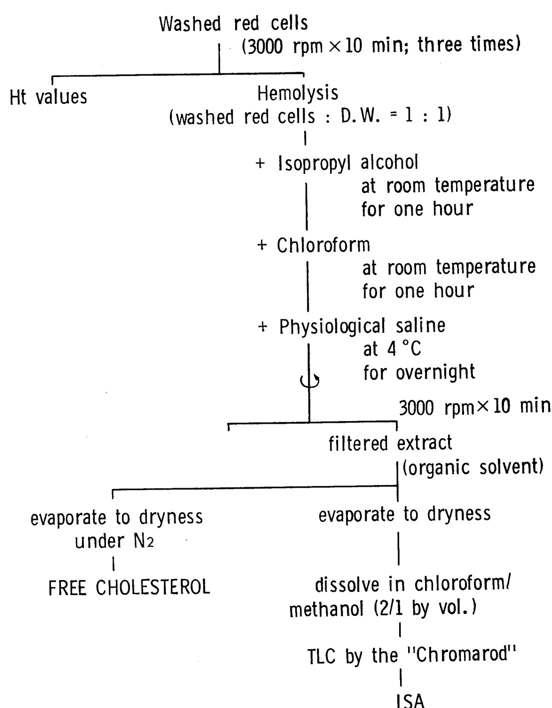

Fig. 1 Scheme of examination of lipid components in red cell membrane.

flame ionization detector (FID) under the following conditions : H_2 pressure, 1.1 kg/cm^2 ; air flow, 2000 ml/min ; scanning speed, 32 sec/scan ; chart speed, 120 mm/min. The peak areas obtained directly by the chromatopack refer to the percentage of ionized carbon atoms of the individual lipid components. Ionization depends on the number of carbons in the molecule, and the reactivity of the carbons differs with the chemical constitution of the molecule. Thus the coefficients for calculation of their amounts were determined from the relative responses of a given amount of each lipid, and the values of each lipid (mg/dl packed red cells) were calculated from free cholesterol values as described by Takemoto.[12)]

RESULTS

Postmortem changes in the quantity of sialic acid in red cell membrane are shown in Fig. 2. Sialic acid of control red cells was 67.1 nmole/mg protein, which is in good agreement with the published value.[15)] Sialic acid decreased with the passage of time during the early postmortem period, when an increase in osmotic fragility is observed.[3)] The value at 18 hours after death was 51.8 nmole/mg protein, which is about 75 per cent of that of control cells.

Fig. 3 shows electrophoretograms of membrane protein and sialoglycoprotein obtained by SDS-PAGE. When ghosts derived from normal red cells were electrophoresed on 1% SDS-polyacrylamide gels, ten protein bands and three glycoprotein bands were observed, as well as one band due to lipid. Ghosts

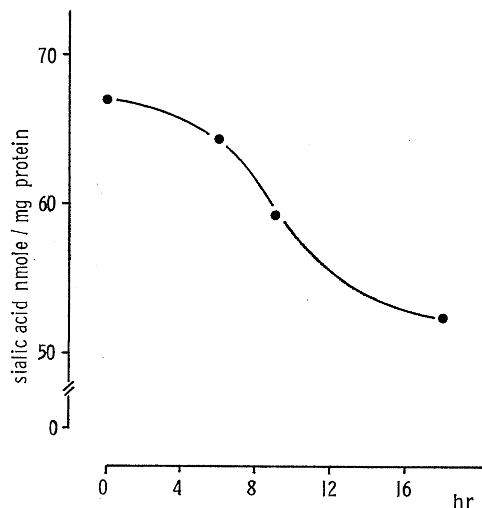

Fig. 2 Postmortem changes of sialic acid (—●—) in red cell membrane.

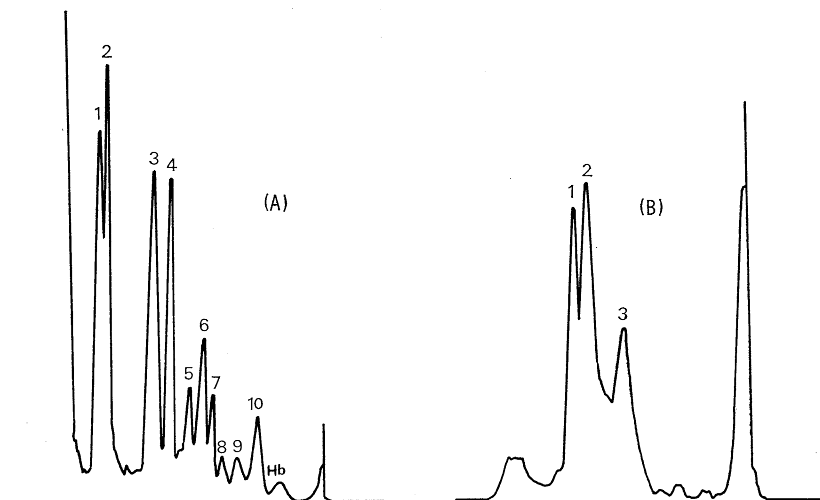

Fig. 3 Spectrophotometric scans of the polyacrylamide gels of red cell ghosts. (A) Coomassie brilliant blue, (B) PAS

from 18-hour-postmortem red cells yielded similar densitometric scans of Coomassie blue stained proteins, though a slight lowering of the ratio of band 9 against band 8 was observed. PAS staining revealed no difference between control ghosts and 18-hour-postmortem ones.

Fig. 4 shows a typical TLC-FID chromatogram obtained from the lipid extract of rat red cell membrane. Membrane lipids of normal red cells were separated into five distinct fractions : free cholesterol, phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl choline (PC) and sphingomyeline (SM). Free cholesterol migrated to near the solvent front, and the other components migrated in the order PE, PS, PC and SM, which was contiguous

with the origin. The ratio of SM in lipid components of rat red cell membrane was low as compared to that of human red cell membrane.[12] Postmortem changes in the percentage of ionized carbon atoms of the individual lipid components are shown in Table 1. It can be seen that lipid composition in red cell membrane changed within 3 hours of death, and after that time fragments which migrated less than SM in TLC were observed. As indicated in Fig. 5, the amounts of free cholesterol and PC decreased up to 18 hours after death, while other components such as PE, PS and SM showed minimal or no changes. Especially, free cholesterol progressively decreased with the lapse of time in

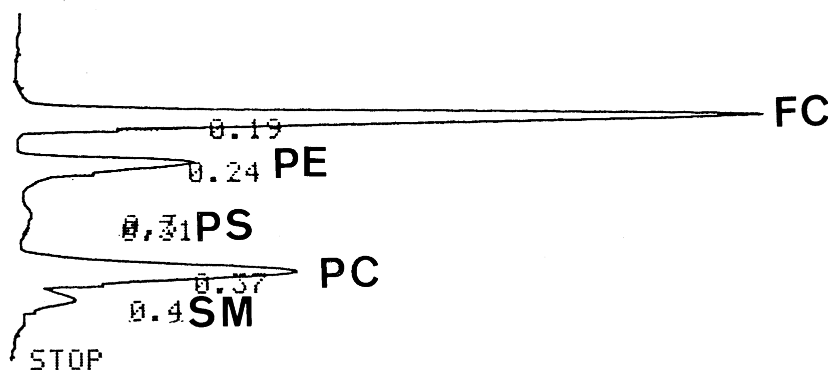

Fig. 4 TLC–FID chromatogram of the lipid extract from red cell membrane, FC ; Free Cholesterol, PE ; Phosphatidyl Ethanolamine, PS ; Phosphatidyl Serine, PC ; Phosphatidyl Choline, SM ; Sphingomyeline

TABLE 1 Postmortem changes of lipid composition (percent ratio) in red cell membrane.

		FC	PE	PS	PC	SM	Fragment
Normal		49.25±1.69	16.45±1.74	3.32±0.83	25.66±1.93	5.87±0.89	—
Postmortem	3H	47.99±2.14	18.89±1.57	3.11±0.47	23.30±2.28	5.67±0.70	0.87±0.44
	6H	47.66±2.01	16.27±0.85	3.41±0.59	25.58±1.24	5.91±0.61	2.04±0.31
	9H	43.69±1.99	17.77±1.39	3.92±0.69	26.88±1.28	6.17±0.80	1.94±0.44
	18H	43.35±1.79	18.01±0.78	3.49±0.38	27.62±2.37	7.04±1.15	1.93±0.39
	24H	44.53±1.92	17.15±1.00	3.84±0.57	26.30±1.15	6.66±0.49	2.10±0.27

FC; Free Cholesterol, PE; Phosphatidyl Ethanolamine, PS; Phosphatidyl Serine, PC; Phosphatidyl Choline, SM; Sphingomyeline

the early postmortem period from 141.6 mg/dl in normal red cell membrane to 100.2 mg/dl in 18-hour-postmortem membrane. The ratio of free cholesterol to total phospholipids decreased from 0.44 in control red cells to 0.36 in 18-hour-postmortem ones.

DISCUSSION

Red cells in cadavers hemolyze and develop a number of postmortem changes, and remarkable changes in the components of red cell membrane

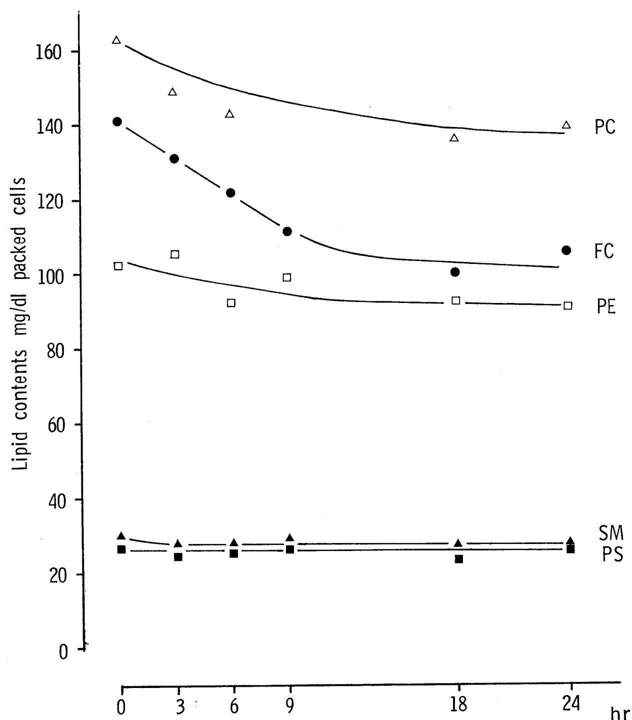

Fig. 5 Postmortem changes of lipid components in red cell membrane.

may be expected. From a biochemical point of view, the membrane is basically composed of lipids, proteins and carbohydrates which are found in red cell ghosts.

Sialic acid, which is located on the external surface, is reported to participate in virus receptor sites,[16] the M-N antigens[17] and surface charge.[18,19] The presence of sialic acid on the cell surface has been shown to be crucial for the survival of red cells in circulation, and its removal from red cells results in rapid removal of cells from the circulation.[20-22] In view of the noticeable relationship between osmotic fragility and sialic acid, Shinozuka *et al.*, with stored human red cells, observed that a significant release of bound sialic acid occurred simultaneously with an increase in hemolysis of stored red cells.[4] The observation of red cells in carcasses demonstrated that sialic acid decreased during the early postmortem period when an increase in osmotic fragility also occurs. This result supports the view that old red cells, distinguished by the difference in osmotic fragility, show a significant decrease in sialic acid content, and that glycoproteins may undergo significant chemical modifications with aging.[23]

Lenard[24] and Sarris *et al.*[25] compared completely disaggregated polypeptide chains from red cell membranes of different mammals by PAGE in 1% SDS. They concluded that the proteins in red cell membrane from different species show many similarities, and the very large degree of homology between distantly related mammalian species suggests that each of the conserved proteins plays

a necessary role in the structure, function and viability of intact red cells. There are, however, conflicting reports concerning the comparative analysis of mammalian erythrocyte membrane proteins.[26-28] In this experiment, rat red cell membrane was found to have protein and glycoprotein subunit patterns identifiable with human ones. The discocyte-echinocyte transformation and the decrease in deformability associated with red cell ATP depletion or amphipathic drugs have been attributed to changes in the physical properties of spectrin and actin.[29] Palek *et al.* reported that the high-molecular weight spectrin-rich complexes results from rearrangement of spectrin and other polypeptides in membranes of ATP depleted red cells.[30] In this experiment, however, there were few or no changes in the distribution pattern of proteins obtained by both Coomassie brilliant blue and PAS staining up to 18 hours after death, so it was thought membrane proteins did not change markedly during this early postmortem period.

PC, PS, PE and SM are quantitatively the major membrane phospholipids. Free cholesterol also is a lipid constituent of red cell membranes. In this study it was found that the lipid constituents of red cell membrane changed within 3 hours of death, and that fragments were observed. Red cell membrane fragmentation is among the hemolytic mechanisms under consideration.[31] Fragmentation may be defined as the loss from the cell of a piece of membrane which may or may not contain hemoglobin. Rous *et al.* suggested that fragmentation is the major mechanism of normal destruction of red cells and an important mechanism for destruction of pathologic red cells.[32,33] In a variety of hemolytic disorders associated with fragmentary loss of membrane from the cell, a parallel decrease in all membrane lipids can be found, in fact, such a finding is a good biochemical definition of fragmentation[31] though the details of fragmentation remain obscure. A parallel lipid loss was not observed in this experiment, however. The mechanism of fragmentation in the postmortem period may be different than that described up to this time, and further studies into this problem seem to be necessary.

Red cell membrane lipids are in a dynamic state of exchange with plasma lipids,[34-36] and free cholesterol is in especially rapid equilibrium between the plasma and red cells.[31,37,38] Cooper *et al.* described that during incubation in normal serum possessing an active cholesterol-esterifying agent, red cells lose cholesterol and thereby become more spheroidal, less resistant to osmotic lysis.[7] Available evidence indicates that cholesterol is an important determinant of red cell shape and osmotic fragility.[37,39,40] PC and especially free cholesterol were reduced during the early postmortem period, whereas PE decreased only a little and PS and SM not at all. These results suggested that a significant decrease in free cholesterol in red cell membrane might be responsible for the changes in osmotic fragility that are of great value in estimating the postmortem period.

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