DIFFERENCES IN ERYTHROCYTE MEMBRANE PROTEINS AND GLYCOPROTEINS IN SICKLE CELL DISEASE

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Summary: Erythrocyte membrane proteins obtained from individuals with sickle cell anemia show an SDS polyacrylamide gel pattern that differs in five regions from the normal pattern. These membranes when compared with membranes from normal individuals also show a marked decrease in sialic acid content which correlates with a marked reduction of the periodic acid-Schiff staining of the three major glycoprotein components. The observed membrane protein and glycoprotein changes are a characteristic of all the red cells in sickle cell anemia and do not correlate with the proportion of irreversibly sickled cells.

When erythrocytes in sickle cell anemia become deoxygenated they assume bizarre shapes caused by the crystallization of the internal hemoglobin. Upon reoxygenation the hemoglobin redissolves and most of the cells assume a normal shape, but many remain irreversibly sickled and rigid (1). Apparently a permanent alteration remains in the membrane. There have been conflicting reports that cell fragmentation during the sickle-unsickle cycle eventually renders the cell irreversibly sickled (2,3). Another approach to the problem of the irreversibly sickled cell (ISC) is to look for changes in the molecular composition of the membranes of such cells. Durocher and Conrad (4) reported the protein patterns from normal (AA) and sickle cell (SS) erythrocyte membranes are similar. However, we have looked for and have found alterations among the membrane protein and glycoprotein patterns of SS erythrocytes.

METHODS

Cells and membranes were prepared as in reference 5, except that 0.01M Tris, 0.15M NaCl, pH 7.4 was used for washing cells, and 0.01M Tris pH 7.4 was used for preparing membranes. Protein determination was performed by the Lowry procedure (6). SDS-polyacrylamide gel electrophoresis of 25µg dissolved membranes was performed as in reference 5, except 4.5% acrylamide, 0.17% bisacrylamide; 0.4% SDS and 80mM dithiothreitol in sample buffer; 0.2% SDS in electrophoresis buffer. Quantitation of staining intensity (Coomassie blue) of bands was with a Gilford spectrophotometer Model 6040/2410-S attached to an Autolab digital integrator Model 6300. For glyco-

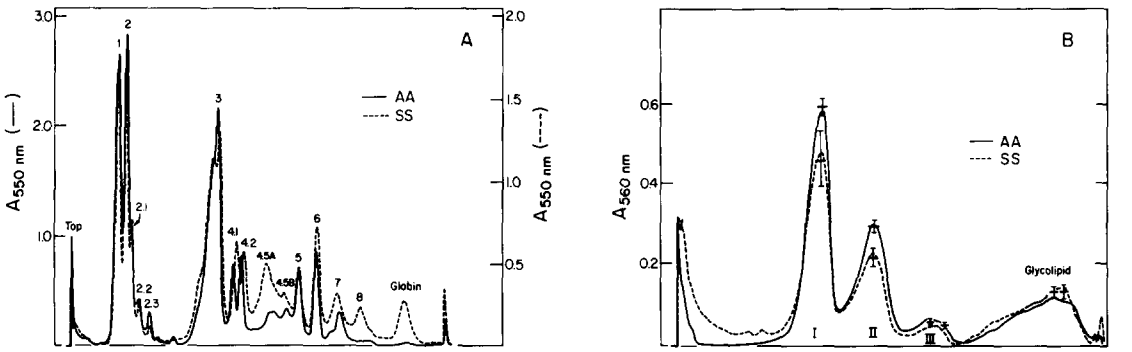

Figure 1: Proteins and glycoproteins of normal and sickle cell anemia erythrocyte membranes. (A) Protein gels loaded with 25 µg total protein, stained with Coomassie blue; bands numbered as in (7). This SS sample had 16% irreversibly sickled cells. The full scale absorbance of the gels is different since the gels were run and destained on separate occasions. (B) Glycoprotein gels. All gels were loaded with equal amounts of membrane protein (total protein minus hemoglobin), handled together under the same conditions and scanned at the same full scale absorbance. Bars indicate mean and range of five normal and seven sickle cell samples.

Table I

Analysis of Proteins in Normal and Sickle Cell Membranes

Band	AA[a]	SS[b]	Significance[c] of Difference	Direction of Difference (AA→SS)
Pre-1	0.8 ±0.28	0.7 ±0.42	<0.01	-
1	14.7 ±0.67	13.6 ±1.41	<0.01	-
2	12.4 ±0.55	11.3 ±1.11		
2.1	3.9 ±0.70	3.7 ±0.49		
2.2	2.0 ±0.20	1.8 ±0.20	<0.01	-
2.3	1.3 ±0.20	1.0 ±0.17	<0.01	-
3	29.1 ±0.90	27.1 ±1.15	<0.01	-
4.1	4.5 ±0.17	4.7 ±0.32		
4.2	4.6 ±0.37	4.3 ±0.26		
4.5A	6.8 ±0.57	8.5 ±1.17	<0.01	+
4.5B	2.9 ±0.36	3.0 ±0.39		
5	5.0 ±0.24	5.1 ±0.46		
6	5.3 ±0.66	6.3 ±1.07	<0.01	+
7	3.4 ±0.45	4.3 ±0.81	<0.01	+
8	1.6 ±0.39	2.6 ±0.81	<0.01	+
Globin	0.6 ±0.22	2.7 ±0.97	<0.01	+

(a) Mean ±S.D., 15 donors, 20 samples; expressed as % of total protein less globin.
(b) Mean ±S.D., 10 donors, 15 samples; expressed as % of total protein less globin.
(c) From *t* distribution.

protein quantitation gels were loaded with 150µg protein, not including globin, stained with PAS reagent (5), quantitated by weighing the peak areas cut from photocopies of gel scans.

In vitro "sickling" is an overnight incubation with gentle shaking of whole blood at 37°C under a humidified atmosphere of 5% CO_2 in nitrogen. Penicillin and streptomycin were present at 100 U/ml and 100 µg/ml respectively.

RESULTS

Our gel patterns of erythrocyte membrane proteins from donors with sickle cell anemia show several changes compared with patterns obtained from normal individuals, black or white, who have only hemoglobin A (Fig. 1A,Table 1). While all the changes are fairly small, several are significant. Band 3 decreases and splits into 2 or 3 overlapping components; bands 4.5A, 6 and 7 increase and band 8 appears. As expected, globin is present in SS membrane preparations (8), probably as an occluded precipitate. The magnitude of these changes varies from patient to patient, whereas normal samples remain constant. While all the decreases are among the high molecular weight proteins, the increases begin in the lower molecular weight classes (Table 1), suggesting one or several degradative events. These changes are increased further (data not shown) when aliquots of the same SS blood sample are subjected to an in vitro "sickling" procedure. These changes in relative amounts of membrane proteins are among those seen when AA cells are lysed in the presence of calcium (9). Sickle cells are known to contain abnormally high levels of calcium (10), especially under conditions of energy depletion (11). Such conditions favor the generation of irreversibly sickled cells (3). It is possible that the changes which we observe are calcium induced effects.

The data shown in Table 1 are also compatible with the hypothesis that the membranes from persons with sickle cell disease are "sticky" in the sense that they might adsorb more plasma proteins or intracellular proteins in the molecular weight range of bands 4.5 to 8. Such stickiness might be due to the lower sialic acid content of sickle cell membranes which we

PAS = periodic acid-Schiff's reagent.

Table II

Analysis of Glycoproteins in Normal and Sickle Cell Membranes

	AA[a]	SS[b]	% Change AA → SS	Significance of Difference
Glycoprotein[c]	592 ±21	460 ±26	-22.3	$p<<0.01$
Sialic Acid[d]	31 ± 1.5	25 ± 2.7	-19.0	$p < 0.01$
Neutral Carbohydrate[e]	55 ± 5.5	55 ± 6.9		
N-acetyl glucosamine[f]	92 ± 8	88 ±12		
N-acetyl galactosamine[f]	69 ± 5	64 ± 7		

(a) Mean ±S.D., 6 donors.
(b) Mean ±S.D., 7 donors. Counts of ISCs in oxygenated smears varied from 16 to 61%.
(c) Arbitrary units, determined as in Methods.
(d) µg/mg membrane protein, determined by the method of Warren (13) after hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 hour.
(e) µg/mg membrane protein, determined by the method of Dubois et al. (14).
(f) n moles/mg membrane protein determined on the amino acid analyser (Durrum D 500) after hydrolysis in 4 N HCl at 100°C for 6 hours.

report below. Rehfeld et al. (12) describe increased binding of human albumin to sickle cells. If this were the case, then the relative amounts of polypeptides 1 to 4.2 would appear to be smaller than normal, as is in fact observed. However, in four experiments which indicate against the hypothesis that our increase in band 4.5 is merely due to albumin binding, washed red cells from persons with sickle cell anemia were artificially sickled under 5% CO_2 in N_2 with 20mM NaF in Tris-saline 1mM Ca^{++} buffer in the absence of serum or plasma. In every case there was an increase of bands 4.5, 6, 7, 8 and globin. Thus the quantitative changes in polypeptide composition described in our Table I for sickle cell membranes are increased by an *in vitro* sickling incubation in the absence of serum proteins. Although we feel certain of the occurrence of the protein changes in the gel

patterns from sickle cell membranes, we cannot yet be sure of the cause of these changes.

The quantitative examination of the three major human glycoproteins - PAS I, II and III - showed significantly lower staining intensities of all three glycoproteins in seven SS samples (Fig. 1B, Table II). Interestingly, the PAS reactivities of all three glycoproteins are lower by the same amount in a given sample; the relative mobilities of the glycoproteins in the gel are unchanged.

Erythrocyte membranes contain three classes of carbohydrates (15): sialic acid, hexosamines and neutral carbohydrate. Assays for each of these classes were performed to determine the nature of the reduction of PAS staining ability. In the SS samples of Fig. 1B there is a lower content of sialic acid (Table II). Sialic acid content and the staining intensity of all three PAS-positive glycoproteins in all the SS and AA samples are highly correlated (regression line almost through origin, $r = 0.89$, $p<0.01$). Table II shows no change in total neutral carbohydrate and no statistically significant changes in N-acetylglucosamine and N-acetylgalactosamine content.

We conclude that the portion of the PAS reactive material which is decreased in SS membranes is sialic acid, especially in light of the observation that gels of membrane preparations from neuraminidase treated erythrocytes show decreased PAS staining in proportion to the amount of sialic acid liberated (5, 16). Counts of the proportion of irreversibly sickled cells in the SS samples were made. These correlate negatively, but weakly, with sialic acid content, glycoprotein content and total neutral carbohydrate. The plot of sialic acid content and percent irreversibly sickled cells suggests that two thirds of the sialic acid residues are not susceptible to removal under conditions of irreversible sickling.

The AA samples of Table II were from white donors. To determine whether particular blood group frequencies among black individuals might be responsible for showing an unusual sialic acid distribution, samples

from four white AA and five black AA unrelated donors were compared. Hemoglobin type was determined on pH 8.9 acrylamide gel electrophoresis as described previously (17). We found no differences in the levels of PAS-positive glycoprotein or sialic acid, nor were any differences seen in the gel protein pattern. The observed lower PAS positive glycoprotein and sialic acid content of SS cells must therefore be connected with the disease process.

In order to look for a connection between the level of ISCs and the loss of sialic acid, we employed the ultracentrifugation technique of Bertles (18) to fractionate an SS blood sample, obtaining fractions containing ISC levels varying from 5 to 45% of the red cell population. Sialic acid content and intensity of PAS stain varied only slightly and were not correlated with the percentage of ISCs in the fraction. Furthermore, the Coomassie blue protein staining pattern showed no changes among the fractions, except for a higher percentage of globin (16% vs. 5%) in the bottom fraction which also contained the most ISCs (45%). We feel that the sialic acid decrease in sickle cell anemia is a characteristic of the red cell population as a whole and not of the ISCs in particular.

Since it is reasonable to assume that at least some of the sialic acid is in a terminal position on the membrane glycoproteins which are known to be on the external surface of the red cell (19), it is possible that some factor in SS serum removes such terminal residues. To test this idea, washed AA cells in heparinized SS plasma and washed SS cells in AA plasma were incubated overnight at 37°C. The homologous incubations were also performed. The membranes were isolated and the staining intensity of the glycoprotein bands was determined as in Table II. The staining intensity of the glycoproteins of the AA cells after incubation in either heterologous or homologous plasma decreased only 3 percent. On the other hand the PAS staining of the SS cell glycoproteins which before incubation was 84% of the AA control, fell to 71 and 65% of the AA control after incubation in homologous and heterologous plasma, respectively. We conclude that the loss of sial-

ic acid from SS membranes is an intrinsic property of the cells and not caused by a plasma factor.

DISCUSSION

The mechanism of loss of sialic acid from SS cell membranes is unclear, as is the impact which loss of sialic acid may have on the subsequent behavior and fate of the SS erythrocyte. It has been suggested that a lower sialic acid content of older erythrocytes could provide a means of recognizing and destroying these cells (16, 20). Since the partial loss of sialic acid from its membrane is characteristic of all SS erythrocytes, irreversibly sickled or not, we must look further for the membrane defect which causes ISCs to retain their sickle shape.

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