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THE ERYTHROCYTE MEMBRANES IN β-THALASSEMIA

LOWER SIALIC ACID LEVELS IN GLYCOPHORIN

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Summary

The sialic acids content of glycophorin of thalassemic erythrocyte membranes is about 25% lower than in glycophorin of normal erythrocyte membranes. Glycophorin extracted from old thalassemic erythrocytes separated by density centrifugation, has about half the sialic acids content found in glycophorin extracted from young thalassemic erythrocytes. Possible sialidase activity was sought in the plasma and erythrocyte membranes of thalassemic erythrocytes. No increased sialidase activity was detected in the plasma of the patients as compared to that of normal donors. Thus, other sites for sialidase activity, or other possibilities have to be explored to account for the increased sialic acid hydrolysis of glycophorin of the thalassemic erythrocytes.

Introduction

Thalassemia is a congenital hemolytic anemia caused by absent or decreased levels of messenger RNA directing the synthesis of globin chains of hemoglobin [1]. Recent studies indicated that in addition to this basic abnormality, alterations also occur in the erythrocyte membrane. These include membrane cation

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Abbreviations: SDS, sodium dodecyl sulfate; Fuc, fucose; GlNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

permeability [2—4], changes in the lipids [5] and protein [6] composition, as well as a 25% decrease in the content of sialic acids [7,8]. Using cytochemical methods in conjunction with transmission electron microscopy it was found that sialic acids residues on the surface of the thalassemic erythrocytes are distributed in an uneven manner and are less abundant than those present on the surface of normal erythrocytes [8]. As the majority of the sialic acids of the erythrocytes are components of the sialoglycoproteins (glycophorins) one would assume that the decrease in the sialic acids content results either from a quantitative decrease of glycophorin in the membrane or from a decrease in the amount of sialic acids on the glycophorin molecules. These possibilities were studied and are reported here. In addition, we studied the sialidase activities of the plasma and the erythrocyte membrane as a possible mechanism to account for the lower sialic acids levels in the thalassemic erythrocytes.

Materials and Methods

Blood sources. Blood was drawn [6] from β^{o} or β^{+}-thalassemia major and β-thalassemia intermedia patients (who are infrequently transfused) and also from healthy donors. Details on the clinical and laboratory findings of the patients have been previously reported [9].

Erythrocyte isolation and separation into different populations according to age. Erythrocytes were separated into different populations according to their age by density centrifugation following the method of Murphy [10]. The 10% top and bottom fractions were obtained and the degree of separation was verified by estimation of mean corpuscular hemoglobin concentration, reticulocyte count, glutamate oxaloacetate transaminase and in several instances by measuring ^{59}Fe radioactivity of newly formed erythrocytes following ferrokinetic analysis [11]. The radioactivity was analyzed for erythrocyte suspensions (at 20% hematocrit) before and after separation of the cells population. The results were expressed as the percent of the total radioactivity.

Isolation of erythrocyte membranes and glycophorin. The erythrocytes were washed and lysed and the membranes were washed and their glycophorin isolated by the lithium diiodosalycilate-phenol extraction procedure [12]. When membranes were prepared for sialidase assay special care was taken to eliminate the white blood cells by using the α-cellulose/microcrystalline cellulose column method of Beutler [13]. The cells were washed, lysed, the membranes were collected and washed and an aliquot was separated on a 25—50% sucrose gradient and the membrane band collected and washed free of the sucrose [14].

Chemical analysis. Protein was assessed either according to Lowry et al. [15] or by measuring absorption at 280 nm. Carbohydrates were analysed by gas-liquid chromatography using the trimethylsilyl derivatives of the monosaccharides according to Clamp [16]. Sialic acid was determined by the thiobarbituric assay procedure according to Aminoff [17]. Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described previously [6].

Enzymic assay. Sialidase was determined according to Schauer et al. [18] by the release of tritium-labeled sialic acid derivatives from labeled substrates

(16 h at 37°C). The system was slightly modified as follows: (a) Plasma was separated from 5 ml whole blood anticoagulated with 250 units heparin. The cells were centrifuged at 850 × *g* for 15 min at 4°C and the supernatant was separated and centrifuged at 30 000 × *g* for 60 min at 4°C. The plasma (supernatant) was separated and if not assayed directly was stored in small aliquots at −20°C. (b) The control was not a boiled sample, since it was found difficult to handle, but a reaction mixture to which about 1000 times excess non-labeled substrate was added. (c) The hydrolysed sialic acids were separated by dialysis tubing and not by the ultra-thimbles [18]. The substrates α_1 acid glycoprotein and glycophorin were labeled according to Schauer et al. [18].

The source of exogenous sialidase was the enzyme from *Vibrio cholerae*, obtained from Behring Werke, F.R.G., and a homogenate from human lymphocytes which was prepared as follows: 20 ml blood were collected with heparin (1000 U) as anticoagulant. The lymphocytes were separated on Ficoll Paque (Pharmacia Fine Chemicals) gradient. They were washed in sterile phosphate-buffered saline and resuspended in 3.4 ml of plasma isolated from the same donor. The cells were sonicated at 4°C for two 30-s 140-W pulses, with 1 min interval, using a Heat Systems Ultrasonic Inc. Model 350.

Results

Glycophorin content of thalassemic erythrocyte membranes

Glycophorin comprised 9.1 ± 1.8% and 9.8 ± 0.9% of the total proteins of thalassemic and normal erythrocyte membranes, respectively. Thus, it seems that the amount of glycophorin is similar in both types of membranes. This is within an experimental error which is slightly higher for the thalassemic membranes, as only small amounts of membranes can be obtained (up to 30 ml blood from a single patient) and analyses were carried out on individual patient blood samples.

The SDS-polyacrylamide electrophoretic profile of glycophorin of the thalassemic erythrocyte membranes resembled that of normal membranes. On the other hand, the carbohydrate composition of the thalassemic preparations indicated a lower mole ratio particularly in regard to that of sialic acid (Table I).

The lower ratio of sialic acid to galactose may have reflected abnormal membrane synthesis during cell maturation in the bone marrow or modification of the cell membrane after the erythrocytes had been released into the peripheral circulation. In order to differentiate between these possibilities, the erythrocytes from β-thalassemia intermedia patients, at least 6 months after blood transfusion and therefore free from donors erythrocytes, were separated by their density into different populations according to age, followed by analysis of glycophorin. As seen in Table II, the separation was successful. The more dense cell population had higher mean corpuscular hemoglobin values, was almost devoid of reticulocytes, had glutamate oxaloacetate transaminase activity about three times lower than the top erythrocyte fraction. In addition, the amount of radioactive iron in newly formed erythrocytes, which resulted from the ferrokinetic studies, was much lower in the more dense cell population as compared to the top less-dense fraction.

The carbohydrate composition of glycophorin extracted from the youngest

TABLE I

CARBOHYDRATE CONTENT OF GLYCOPHORIN OF THALASSEMIC ERYTHROCYTE MEMBRANES

The results are the mean of the analyses obtained for the number of subjects in parentheses.

Erythocytes	Carbohydrate (mol/mol galactose)						
	Fuc	Man	Gal	Glc	GlcNAc	GalNAc	Sialic acid
β-Thalassemia major (4)	0.1 ± 0.01	0.2 ± 0.02	1	0.5 ± 0.1	0.4 ± 0.01	1.0 ± 0.2	1.4 ± 0.1
β-Thalassemia intermedia (8)	0.1 ± 0.01	0.2 ± 0.01	1	0.2 ± 0.02	0.4 ± 0.05	0.9 ± 0.1	1.2 ± 0.2
Control subjects (4)	0.1 ± 0.01	0.2 ± 0.01	1	0.3 ± 0.09	0.5 ± 0.05	1.0 ± 0.1	1.9 ± 0.2

and oldest populations of the thalassemic erythrocyte were compared. The major difference was found in the mole ratio of sialic acids to galactose (Table III). A value of about 2 was found for glycophorin extracted from young thalassemic erythrocytes whereas the sialic acids to galactose mole ratio was only 1 for the glycophorin extracted from old thalassemic erythrocytes. It seems therefore that sialic acids are hydrolyzed off the glycophorin of the erythrocytes while the cells are in the peripheral circulation.

TABLE II

HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS OF YOUNG AND OLD ERYTHROCYTES OF β-THALASSEMIA INTERMEDIA PATIENTS

MCHC, mean corpuscular hemoglobin concentration; GOT, glutamate-oxaloacetate transaminase. The numbers in parentheses indicate the range obtained in the study of eight samples. Average percent cpm ^{59}Fe data are from samples of four patients.

Erythrocytes	MCHC (%)	Reticulocytes (%)	GOT (−ΔE/HB g%)	Average % cpm ^{59}Fe/20% hematocrit
Top (10%)	22.3 (21—28)	15.0 (15—85)	17.0 (17—24)	79.5
Bottom (10%)	27.1 (27—34)	0 (0—1)	5.7 (5—17)	13.0
Not separated	24.6 (22—30)	3.5 (1—25)	9.0 (9—15)	

TABLE III

CARBOHYDRATE CONTENT OF GLYCOPHORIN OF YOUNG AND OLD ERYTHROCYTES OF β-THALASSEMIA INTERMEDIA

The results are the mean ± S.D. of four analyses.

Erythrocytes	Carbohydrate (mol/mol galactose)					
	Man	Gal	Glc	GlcNAc	GalNAc	Sialic acids
Young (10% top)	0.2 ± 0.02	1	0.7 ± 0.1	0.5 ± 0.2	1.0 ± 0.1	2.0 ± 0.1
Old (10% bottom)	0.2 ± 0.03	1	1.1 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.1

TABLE IV

SIALIDASE ACTIVITY IN PLASMA OF β-THALASSEMIC PATIENTS

The substrate included about 120 000 cpm/reaction mixture (0.4 or 0.6 nmol sialic acids in glycophorin or α_1-acid glycoprotein, respectively). Sialic acid hydrolysis with competition is in the presence of non-labeled α_1-acid glycoprotein (2 mg).

Sialidase		Substrate	Sialic acid hydrolysis (cpm ± S.D.)	
Plasma	*V. cholerae*			With competition
β-Thalassemia	—	Glycophorin *	721 ± 96	664 ± 57
Control	—	Glycophorin	916 ± 153	880 ± 153
β-Thalassemia	—	α_1-Acid glycoprotein	314 ± 65	318 ± 46
Control	—	α_1-Acid glycoprotein	299 ± 54	319 ± 75
Control	$1.5 \cdot 10^{-3}$ U	α_1-Acid glycoprotein	17 259	3732
—	$1.5 \cdot 10^{-3}$ U	α_1-Acid glycoprotein	24 714	8857

* The major sialogylcoprotein of the erythrocyte membrane.

Sialidase activity in plasma and erythrocyte membranes

One explanation for the increased hydrolysis of the sialic acids from the thalassemic erythrocyte membranes could be due to increased sialidase activity, which was identified in plasma [18] and in erythrocyte membranes of normal adults. The results for the sialidase activity in the plasma are summarized in Table IV. There is only a minimal hydrolysis of the substrates (about 2 pmol sialic acid/min) when either thalassemic or normal plasma were used as the source for sialidase activity. The question whether this low hydrolysis represents enzymic activity is difficult to answer since the values are very low. Using glycophorin as the substrate there is somewhat lower hydrolysis in the presence of about 1000 times excess of non-labeled substrate but the difference is still statistically not significant (Table IV). The capacity of the system to detect sialidase activity is indicated when very small amounts of exogenous enzyme were added (Table IV). With this control it is also shown that an excess of non-labeled substrate decreased considerably the hydrolysis of the labeled sialic acid derivative. In order to confirm that the system can detect also sialidase activity from human origin, lymphocytes were sonicated and used as an enzyme source. Low but significant sialidase activity was detected with this enzymic preparation.

The possibility that sialidase activity resides in the erythrocyte membrane was also tested. In these experiments special care was taken to separate erythrocytes from the white blood cells and other blood elements. This could be achieved only for the normal erythrocytes, while those from the thalassemic patients included also normoblasts [14]. As membranes from these could contaminate the erythrocyte membrane preparation, the study was therefore performed only on erythrocyte membrane obtained from normal donors. The results showed no significant sialidase activity in the erythrocyte membranes obtained from normal donors.

Discussion

Sialic acids seem to have an important role in the physiology of the erythrocytes [19]. Aging of circulating normal erythrocytes is associated with a reduc-

tion of 15—30% sialic acids which may be an important determinant of erythrocytes survival in the eventual recognition and sequestration of aged cells by the reticuloendothelial system [20—23]. Recent studies indicate that other carbohydrate moieties may also vary during the aging of erythrocytes [24,25]. Because of this complexity, the study of isolated erythrocyte membrane components may be desired.

Lower sialic acids content in erythrocyte membranes was recently documented in several hemolytic anemias. These include sickle cell anemia [26], paroxsymal nocturnal hemoglobinuria [27] and thalassemia [7,8]. Thalassemic erythrocytes have a much shorter life span [28] which may be related to an increased role of sequestration due to the loss of surface sialic acids. The findings of the current study demonstrate that alterations in the sialic acids occur on the glycophorin of the thalassemic erythrocyte membranes. It was noteworthy that glycophorin extracted from erythrocytes of β-thalassemia intermedia patients had lower sialic acid values (Table I). This may possibly be explained by the fact that since these patients have less severe anemia, they do not require blood transfusions and therefore, the extracted glycophorin originated from genuine patients' erythrocytes. On the other hand, in samples from patients with β-thalassemia major who require frequent blood transfusions, the extracted glycophorin included some of the transfused normal erythrocytes glycophorin. Since most of the erythrocyte sialic acid is located on the glycophorin molecules, the alteration of the membrane sialic acid can be attributed to the changes recorded on glycophorin. Due to the limitations in the amounts of erythrocytes that may be obtained from the patients, the amounts of glycophorin obtained were relatively small and thus the changes were recorded as mole ratio to galactose, which is commonly the carbohydrate residue penultimate to the sialic acid in oligosaccharides which are attached to glycoproteins. The present study, however, cannot reveal changes that occur if the entire oligosaccharide has been eliminated or altered, a process which was reported to occur in aging erythrocytes [24,25].

The present findings that the glycophorin obtained from the young thalassemic erythrocytes is fully sialylated as the mole ratio of sialic acids equals that of galactose and *N*-acetylgalactosamine (Table III). The latter are the residues to which sialic acids are usually attached. On the other hand, the glycophorin of old thalassemic erythrocytes was sialylated to about 50% of its capacity (Table III). These findings indicate that the alterations on the glycophorin of the thalassemic erythrocytes do occur after the cell has emerged from the bone marrow to the peripheral cirulation. We cannot deduce that all alterations in the sialic acids of thalassemic membranes occur while the cells are in the circulation. Other sialic acid-bearing molecules of the erythrocyte membrane should also be investigated. Studies on phagocytosis of thalassemic erythrocytes suggest that alterations of thalassemic erythrocyte suggest that alterations of thalassemic erythrocyte membranes are evident on the young erythrocytes, rendering them more susceptible to phagocytosis as compared to normal erythrocytes. However, the young thalassemic erythrocytes were phagocytized less than old thalassemic erythrocytes [29].

How is the sialic acid hydrolysed? Enzymic hydrolysis is the most reasonable explanation and the decrease in the sialic acid in the thalassemic erythrocytes

may result from higher sialidase activity. The plasma could be a medium for the enzyme, albeit a very nonspecific milieu, but good enough to reach all erythrocytes. If the plasma is the site of the sialidase its activity must be very low in order to allow for the 120 days life span of the normal erythrocytes, as reported by Schauer et al. [18] using a very sensitive method. In our hands, using almost the same method, the activities were at the lower limits of detection and not increased in the patients with thalassemia.

Another site for the location of the sialidase could be the erythrocyte membrane itself [30]. However, using pure erythrocyte membranes derived from normal erythrocytes we could not confirm the previous observation which has been carried out with a much less sensitive method [30] and could probably have been a result of contamination of the erythrocyte membranes with white blood cells or platelets [13]. This was found to be the case in studies of other glycosidases activities which were reported to be present in the erythrocyte membrane [14]. Based on the assumption that the mechanism for removal of sialic acids is the same for both normal and thalassemic erythrocytes, though that it occurs at a higher rate in the latter, the absence of sialidase in the membrane of normal erythrocytes may suggest its absence also from thalassemic pure erythrocyte membranes. The final proof awaits a successful separation of pure thalassemic erythrocyte population. Other sites for sialidase activities in cells which the erythrocytes do encounter may be looked for (e.g. macrophages or endothelial cells). If endothelial cells have sialidase on their outer surface it may occur that sialidase that hydrolyzes sialic acid on the red blood cell is not elevated, but due to the less fragile and more rigid membranes of the thalassemic red blood cells [1,7,28], the cells may migrate slowly in the capillaries allowing more time for a regular amount of sialidase to hydrolyze their substrate.

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