COMPARATIVE ANALYSIS OF RBC MEMBRANE FATTY ACIDS, PROTEINS AND GLYCOPHORIN IN PATIENTS WITH HETEROZYGOUS BETA THALASSEMIA AND IRON DEFICIENCY ANEMIA

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ABSTRACT

Membrane lipid and protein composition was compared in erythrocytes from iron deficiency anemia (IDA) and heterozygous beta thalassemia patients. The study was planned to correlate the influence of iron deficiency with the intrinsic defect of the heterozygous condition on the membrane structural integrity as well as to investigate whether there are differences in membrane changes between the two conditions. Results indicate high levels of saturated fatty acids and low unsaturated fatty acids in both disorders although arachidonic acid and the unsaturation index were lower in heterozygous thalassemia than IDA. Nevertheless, neither of the conditions provoked any alterations in membrane protein or glycoporphin suggesting alterations in the lipid moiety only. Present findings indicate that irrespective to the etiology, both, iron deficiency and the heterozygous condition show a common pattern of lipid derangement, which may in turn result in increased membrane rigidity and decreased cellular deformability.

KEY WORDS

Iron deficiency anemia, heterozygous beta thalassemia, fatty acids, proteins, glycoporphin.

INTRODUCTION

The Beta thalassemias and Iron deficiency anemia (IDA) are characterized by modified features of erythrocytes, including decreased cellular deformability, in part due to anomalies in the structure and function of erythrocyte membrane, microcytosis and hypochromia (1, 2). Increased susceptibility of these microcytic red blood cells to in vitro oxidative stress (1, 3) has also been demonstrated. Indeed, alterations in proteins and lipids may often be more important in oxidative stress situations in vivo, since they provide the viscoelastic properties necessary for membrane deformability (4, 5). While the length and degree of unsaturation of phospholipid fatty acids particularly influence the lipid bilayer fluidity (6), the proteins and glycoporphin play a crucial role in maintaining erythrocyte integrity and antegenecity (7). Although studies

have described modifications in the erythrocyte membrane structure and function in beta thalassemia major and intermedia (7-10), few and rather conflicting data was available on the Indian population with IDA (10-15) and heterozygous beta thalassemia (16, 17). We therefore investigated the erythrocyte membrane fatty acid composition and the protein and glycoporphin profiles and observed for changes that may contribute to erythrocyte pathology in heterozygous beta thalassemia and IDA. The purpose of the study was also to identify whether the iron deficiency or the intrinsic defect of the heterozygous condition is more critical in causing cell damage.

MATERIALS AND METHODS

Blood samples were collected from patients attending the hematology Out Patient Department (OPD) of Bai Yamunabai Laxman Nair Charitable Hospital, Mumbai.

Based on the Clinical and laboratory data, 35 patients with IDA (22-40 years) and 25 heterozygous beta thalassemia carriers (19-43 years) were included in the study with their consent. Blood samples from IDA patients were collected

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before they were put on iron and vitamin supplementation.

The heterozygous beta thalassemia carriers who volunteered for the study were the parents of the beta thalassemia homozygous children attending the OPD. The patients were not suffering from any other disease and were not under any drug therapy during the period of study. The control group comprised of age and sex matched 30 adult volunteers. Ghosts were prepared from packed red blood cells according to the method of Kuross et al (18). Two ml of washed cells were hemolysed in 5mM Sodium Phosphate buffer (pH 8.0) containing 0.5 mM/L EDTA and centrifuged at 15,000rpm at 4°C for 40 min repeatedly until colourless membranes appeared. The final volume of the erythrocyte membrane suspension was made to 3ml with 5mM phosphate buffer. Lipids were extracted from 2ml of the erythrocyte membrane suspension following a modification in the procedure of Folch et al (19), using a chloroform – methanol mixture containing 15mg of butylated hydroxy toluene (BHT) in the proportion 2:1 (v/v). The fatty acid composition was analysed by Gas Chromatography (20). Total membrane protein was estimated from the erythrocyte membrane suspension by using the method of Lowry et al (21). Membrane Proteins and glycophorins were electrophoresed in 10% acrylamide gels containing 2% Sodium Dodecyl sulphate (SDS-PAGE) (22, 23) and the SDS-PAGE bands were identified using molecular weight markers (kit no. MW-SDS-200, Sigma chemicals, St. Louis, USA) (Fig1). Gels were stained for glycophorins (carbohydrates) in periodic acid Schiff's reagent (PAS) (23) (Fig 2). All the chemicals used were of analytical grade. The nomenclature of the polypeptide bands was according to Fairbanks et al (23). Gels were scanned on a LKB laser

FIGURE 1. SDS-PAGE of erythrocyte membrane proteins from a thalassemia heterozygous patient (lane a), IDA patient (lane b) and control (lane c). The numbers in the left-hand margin identify the protein bands.

densitometer at 570nm for proteins and at 560nm for glycophorins. Statistical analysis was carried out employing paired and unpaired t- test as applicable.

Table 1
Hematologic Parameters of heterozygous beta thalassemia, IDA and control subjects.

	Controls (n=30)	Heterozygous Beta Thalassemia (n=25)	IDA (n=35)
Hemoglobin (gm%)	13.50±1.08	11.50±2.00 **	8.22±2.10 ****
PCV (%)	42.30±4.49	35.46±4.70 ***	29.75±4.30 ****
RBC (x10 ¹² /L)	5.45±1.00	5.10±0.65 £	4.10±1.10 **
MCV (ft)	84.10±5.30	63.53±5.31 ***	61.68±6.54 ***
MCH (pico-gram)	32.56±5.93	21.50±3.39 ***	23.30±3.84 ***
MCHC (%)	34.30±3.05	31.80±3.50 **	29.10±2.00 **
WBC (x10 ⁹ /L)	7.90±2.50	8.50±1.85 £	7.12±2.10 £
Hb F (%)	-	1.30±0.55	-
HbA ₂ (%)	1.91±0.60	4.30±1.80 ****	2.10±0.70 £

** P<.02, *** P<.01, **** P<.001 £-Non Significant. Values are expressed as mean ±SD

PCV-Packed cell volume; RBC-Red blood cells; MCV-Mean corpuscular volume;

MCH-Mean corpuscular hemoglobin; MCHC-Mean corpuscular hemoglobin concentration;

WBC-White blood cells.

Table 2
Comparative analysis of fatty acids in control subjects and patients with heterozygous beta thalassemia and IDA in terms of percentage.

	Controls (n=30)	Heterozygous Beta Thalassemia (n=25)	IDA (n=35)
C16:0	17.57±3.36	21.10±4.55 ***	20.50±3.75 **
C18:0	17.44±4.46	20.00±4.27 *	17.88±2.84 £
C20:0	3.78±2.11	6.58±3.50 £	5.29±2.38 £
C22:0	2.70±1.51	4.19±2.31 £	4.40±1.48 £
C16:1	3.81±2.27	4.80±3.50 £	4.04±0.45 £
C18:1	15.33±3.16	12.00±3.42 £	14.95±2.41 £
C18:2	11.91±2.06	11.08±2.62 £	10.19±2.66 £
C20:4	16.51±3.82	9.23±3.88 ****	12.99±4.50 **
Unsaturation Index	104.24±10.42	62.01±17.67 ****	80.82±16.72 ***

* P<.05, ** P<.02, *** P<.01, **** P<.001 £-Non Significant. Values are expressed as mean ±SD

C16:0- Palmitic acid, C18:0- stearic acid, C20:0- Arachidic acid, C22:0- Behnic acid

C16:1- Palmitoleic acid, C18:1- Oleic acid, C18:2- Linoleic acid, C20:4- Arachidonic acid.

RESULTS AND DISCUSSION

The mean hemoglobin level as well as the total RBC count was significantly lower in IDA than beta thalassemia heterozygous ($P < 0.001$) (Table 1). Both conditions were associated with higher levels of erythrocyte membrane saturated fatty acid, palmitic acid as compared to controls (thalassemia heterozygous: $P < .01$ and IDA: $P < .02$) with no significant difference between the two experimental groups. The stearic acid content was slightly higher in thalassemia heterozygous than Controls and IDA ($P < .05$) (Table 2). The unsaturated fatty acid, arachidonic acid was significantly lower in both conditions as compared to controls (thalassemia heterozygous: $P < .001$ & IDA: $P < .02$). Between the two experimental groups arachidonic acid was significantly lower

in heterozygous thalassemia than IDA ($P < .01$). Moreover the unsaturation index was decreased in both the conditions than controls (thalassemia heterozygous: $P < .001$ & IDA: $P < .01$) and was significantly lower in the heterozygous condition than IDA ($P < .001$). The rest of the fatty acid fractions remained unaltered (Table 2). However, the sodium dodecyl sulphate electrophoresis of erythrocyte membrane proteins and glycoporphins was normal in both conditions. (Tables 3 & 4). Workers have demonstrated conflicting results in IDA (10-15) and beta thalassemia heterozygous (16, 17) on erythrocyte lipid profile and membrane proteins. Volkov et al (11) and Mkhilarian et al (12) have reported significant changes in the erythrocyte lipid profile in IDA, similar to the present study, while Acharya et al (13) and Ramchandran et al (14) have reported no significant difference between normal and anemic

Table 3
SDS – Polyacrylamide electrophoretic profiles of membrane proteins from erythrocytes of heterozygous beta thalassemia, IDA and controls, in terms of percentage.

	Controls (n=30)	Heterozygous Beta Thalassemia (n=25)	IDA (n=35)
Bands 1 & 2 (Spectrin)	34.83±3.20	32.81±4.60 £	33.39±3.00 £
Band 3 (Anion transporting Protein)	30.86±2.56	30.91±3.04 £	29.93±3.12 £
Band 4	16.01±2.10	17.33±2.90 £	16.44±2.40 £
Band 5 (Actin)	5.72±1.10	5.84±1.08 £	6.47±1.20 £
Band 6 (GAPD)	6.50±1.43	6.91±1.95 £	6.79±1.20 £
7 – Globin Area	5.69±1.72	7.00±2.50 £	7.01±2.30 £

£-Non Significant. Values are expressed as mean ±SD GAPD – glyceraldehyde phosphate dehydrogenase.

Table 4
The sialoglycoproteins (Glycophorins) of the erythrocyte membrane from heterozygous beta thalassemia, IDA and controls, in terms of percentage.

	Controls (n=30)	Heterozygous Beta Thalassemia (n=25)	IDA (n=35)
PAS 1	50.53±4.40	48.96±4.50 [£]	49.05±5.04 [£]
PAS 2	31.16±4.00	32.50±2.53 [£]	32.00±3.90 [£]
PAS 3	18.31±3.60	18.54±2.90 [£]	18.95±3.60 [£]

£- Non Significant Values are expressed as mean ±SD

PAS - Periodic Acid-Schiff positive bands.

PAS 1 - Glycophorin A, PAS 2 - Glycophorin C, PAS 3 - Glycophorin B.

membrane lipids when subjected to peroxidation. Kalofoutis et al (16) have however reported alterations in all major phospholipid fatty acids in beta thalassemia minor including C16:0, 18:0, 18:1, 18:2, 22:0, 24:0 and 24:1, while our results indicate fatty acid fluctuations only in C16:0, 20:4 and 24:4, while the rest of the fractions remained unaltered.

Rouyer-Fessard et al (17) have reported no significant changes in the SDS-PAGE profile of membrane proteins in beta thalassemia heterozygous, similar to the present observation, while contradictory to our findings, LI et al (15) have reported alterations in band 3 and band 4.1 proteins, albeit in rats with IDA. In general, in the present study, the fatty acid profile followed a similar pattern of derangement in both conditions, while neither of the conditions provoked damage to membrane proteins or glycophorin.

The severity of lipid or protein damage directly relates to the concentration of oxidants in the cells and hence on the efficiency of the lipid repair mechanisms and the proteolytic process to clear damaged substrates (5, 24, 25). It has also been proposed that oxidative stress significantly increases the intracellular degradation of cellular proteins by proteases (5, 24). Moreover, under conditions of oxidative stress the incorporation of free fatty acids into RBC can either be stimulated or inhibited (6, 26). Tiurin et al (26) have reported that the induction of lipid peroxidation resulted in a significant decrease in the content of Phosphatidyl-ethanolamine polyenoic fatty acids and an increase in incorporation of palmitic acid into Phosphatidyl-ethanolamine. Moreover, Arduini et al (27) have studied the membrane phospholipid and protein organization in human erythrocytes exposed to phenyl hydrazine, an oxidant and pointed out that the oxidant, having a peak value at 0.1mM, did not induce any modification of the SDS-PAGE electrophoresis membrane protein pattern whereas induced the translocation of phosphatidyl serine to the membrane outer leaflet, causing lipid imbalance in intact

erythrocytes. Previous study from our laboratory has also shown altered RBC membrane phospholipid composition and peroxidative damage to membrane lipids in IDA and thalassemia (10). Increased repair of oxidatively damaged lipids on account of peroxidative environment in both the pathologic erythrocytes may cause disturbances in fatty acid

FIGURE 2. SDS-PAGE of erythrocyte membrane glycophorin from a thalassemia heterozygous patient (lane a), IDA patient (lane b) and control (lane c). The numbers in the left-hand margin identify the glycophorin bands. MD-Marker dye.

composition resulting into high membrane saturated fatty acids and low unsaturated fatty acid content. An unchanged SDS-PAGE pattern of proteins and glycophorin indicates two possibilities. (1) Either the concentration of the oxidant in these pathologic erythrocytes is inadequate to produce major changes in these membrane constituents or (2) the rate of proteolytic degradation of oxidized proteins balances the rate of protein oxidation. Oxidation of fatty acyl groups might locally disrupt membrane structure (6) and quantitative changes in cell membrane lipids may decrease cellular deformability and may be considered as an important molecular mechanism of Ca²⁺ transport disorder and subsequent metabolic homeostatic disturbances (6,12).

In conclusion, the effect of iron deficiency and the intrinsic defect of erythrocytes demonstrated in heterozygous beta thalassemia showed a similar pattern of lipid derangement. Arachidonic acid content and the unsaturation index were more severely affected in thalassemia heterozygous than IDA, which may contribute towards increased membrane rigidity and decreased cellular deformability in both conditions supporting the findings of Vaya et al (1) and Yip et al (2) in heterozygous beta thalassemia and IDA, respectively. Furthermore, the peroxidative environment of the red cells was not intense enough to alter the SDS-PAGE protein or glycophorin pattern of both experimental groups and / or probably the oxidatively modified proteins were removed by intracellular proteolytic degradation.

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