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COMPARATIVE ANALYSIS OF RBC MEMBRANE LIPIDS IN THALASSEMIA AND IRON DEFICIENCY ANEMIA IN RELATION TO HYPOCHROMIA AND OXIDANT INJURY

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ABSTRACT

The effect of an intrinsic defect in the red cell and pronounced hypochromia on oxidative damage to RBC membrane lipids was compared in beta-thalassemia and iron deficiency anemia (IDA), which have a varied etiology but equivalent low hemoglobin content. The study was planned to correlate the etiology of the disorders to the severity of lipid imbalance and RBC hemolysis in membranes of both the conditions. Results indicated a fall of lysophosphatidylcholine(LPC), phosphatidylethanolamine(PE) and the unsaturated to saturated fatty acid ratio in both conditions, while phosphatidylcholine(PC) increased only in thalassemia. However, irrespective of the disease, sphingomyelin(SM), total cholesterol and phospholipid levels elevated and the hydrogen peroxide stress test indicated increased susceptibility of both pathologic RBCs to peroxidation. Present findings indicate that IDA and thalassemia allow for considerable amounts of oxidative damage to membrane lipids, irrespective of their etiologies, and thus point hypochromia as an important contributor for inducing lipid imbalance and RBC hemolysis.

KEYWORDS: Hypochromia, intrinsic defect, oxidation, RBC membrane, lipid imbalance.

INTRODUCTION

An intracellular defect in the red blood cell (RBC) can accentuate oxygen radical formation and risk damage to cellular components (1,2). On the other hand, there is some evidence that hypochromia may facilitate oxidation of the membrane by reducing the buffering protection of hemoglobin (3,4). The mechanisms facilitating oxidative damage to red cells in thalassemia are multifactorial and result from the presence of excess unpaired globin chains and high intracellular content of nonhemoglobin iron, in addition to hypochromia (2,3,5). However, in iron deficiency anemia (IDA), hypochromia is the lone factor that may enhance oxidant- induced threat to the RBC membrane (6). The accumulation of activated oxygen can potentially cause hemolysis (3,5,7). Nevertheless it is not clear whether the presence of an "intrinsic" defect in the RBC or "hypochromia" is the more important factor as far as lipid damage and hemolysis is concerned.

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In order to correlate the etiology of the disorders to the severity of lipoperoxidation, lipid imbalance and RBC hemolysis, we conducted a comparative investigation between beta thalassemia homozygous and IDA membrane lipid profiles.

MATERIALS AND METHODS

In the present study, 25-transfusion dependent, non-splenectomized (non-spx) beta thalassemia homozygous children (5-12 years) and 35 patients afflicted with IDA (22-40 years) attending the hematology out patient department of B.Y.L.Nair Charitable hospital were included with their consent. Age and sex matched 20 normal children and 30 adult volunteers formed the control groups for thalassemia and IDA respectively. In thalassemia patients, blood was collected prior to blood transfusion, in an effort to minimize the influence of donor blood cells.

Six ml of venous blood were drawn into heparinized tubes and packed red cells were isolated by centrifugation at 3000 rpm. The level of malondialdehyde (MDA), a lipid peroxidation product was determined in terms of thiobarbituric acid reactive substances (TBARS) by the method of Stock et al. (8), and total hemoglobin was measured using Drabkin's reagent (9).

Two ml of washed cells were hemolysed in 5 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM/L EDTA and centrifuged at 15000 rpm at 4° C for 40 min. repeatedly until colourless membranes appeared (10). Lipids were extracted, employing a modified method of Folch and Lees (11), from 2ml of RBC membrane suspension using chloroform-methanol mixture containing 15mg of butylated hydroxy toluene (BHT), in the proportion 2:1 by volume. Total membrane cholesterol was estimated by the CHOD-PAP method (kit no. 1.14366.001 cholesterol enzymatic, E.Merck, Darmstadt, Germany) (12) and phospholipids by the method of Stewart (13). Individual phospholipid fractions were separated by high performance thin layer chromatography (HPTLC) by a modified method of Johannes Muthing (14). (HPTLC plates: 10X20cm, pre-coated with 0.2mm Silica gel 60 on aluminum, No.94069, Aldrich, USA). Lipid preparations equivalent to 50 micrograms of total phospholipids were spotted on TLC plates employing an automatic sample applier (Linomat IV, CAMAG, MUTTENZ, Switzerland). Respective phospholipid standards were obtained from Centre for Biochemical Technology, Delhi, India (batch No.4D-3). The developing solvent used was chloroform/ methanol/water (65/25/4, by volume). Phospholipids were quantified by scanning the plates with a CD 60 densitometer (CAMAG, Switzerland), operating in absorption-reflection mode at a wavelength of 210nm employing the deuterium lamp. Fatty acid analysis was carried out by gas chromatography (GC) according to the method of Morrison et al. (15). The unsaturated/saturated fatty acid ratio was calculated thereon.

RESULTS AND DISCUSSION

As indicated in Table 1, the patients included in the present study suffered from severe anemia. The mean hemoglobin level of beta thalassemia patients was 5.52g/dl, while in IDA it was 8.52g/dl. Our results indicate that both, thalassemic and IDA membranes were associated with significantly elevated levels (Table 1) of TBARS ($P < .001$), cholesterol (thalassemia -$P < .001$; IDA- $P < .01$) and total phospholipids ($P < .001$). A complete imbalance in the entire phospholipid profile (Table 2) was observed, thus suggesting that both pathologic RBCs were more susceptible to auto-oxidation than normal cells. Lysophosphatidylcholine (LPC) levels dropped massively ($P<.001$) in thalassemia (66.30%) and IDA (77.32%), and phosphatidylethanolamine (PE) decreased significantly in both conditions by 33.40% ($P<.001$) in thalassemia and 12.72% ($P<.01$) in IDA. However, phosphatidylcholine (PC) elevated significantly only in thalassemia (27.38%; $P<.001$), while, sphingomyelin (SM) was elevated almost to the same extent in both IDA (17.96%) and thalassemia (15.56%). A significant fall in the unsaturated to saturated (U/S) fatty acid ratio was also observed in both conditions. (Table 2)

Contradictory results indicating an imbalance in the lipid profile in thalassemia homozygous (16-18) and IDA (19-25) have been reported by workers. Ramchandran and Iyer (22) have reported no significant difference between normal and anemic membrane lipids when subjected to peroxidation. Acharya et al (23) have also not evidenced an increased susceptibility of RBC to lipid peroxidation in iron deficiency. As against these findings, increased RBC lipid peroxidation has been observed by Vives Corrons et al (24) and Kumerova et al (25) in IDA, similar to the present observations. Maggioni et al (16) have indicated a decrease in total RBC cholesterol and phospholipids in thalassemia, while Rice-Evans et al (18) have observed an increase of these lipids. Rachmilewitz et al (17) have reported a two fold increase in lipid phosphorus in spx. thalassemia patients, and have indicated that non-spx patients have total lipid phosphorus closer to the control level. However, our results indicate elevated levels of cholesterol and phospholipids and considerable amount of lipid imbalance in both conditions, irrespective of their etiologies.

Table 1. Levels of hemoglobin, cholesterol, phospholipids and TBARS in thalassemia and IDA against the respective controls.

	Hb(g/dl)	Membrane Cholesterol (mg/ml of Packed Cells)	Membrane Phospholipids (mg/ml of Packed Cells)	TBARS (nmoles/gHb)
Normal adults (n=30)	13.50©1.08	1.40©0.21	2.99©0.39	54.20©22.00
Normal Children (n=20)	12.20©1.62	1.29©0.16	3.04©0.26	79.70©22.06
Thalassemia homozygous (n=25)	5.52©1.48 **	1.75©0.02 **	4.70©0.56 **	185.00©47.10 **
IDA (n=35)	8.52©1.95 **	1.61©0.30 *	3.30©0.61 **	100.00©23.30 **

*P < .01, ** P < .001, Values are mean©S.D.

Table 2. Individual Phospholipid fractions in terms of percentage of total phospholipids and the U/S ratio in thalassemia and IDA against respective controls.

	LPC	SM	PC	PE	U/S ratio
Normal adults (n=20)	3.97± 2.00	26.58± 4.95	36.59± 4.00	32.86± 3.73	1.04± 0.19
Normal Children (n=20)	3.71± 2.00	27.05± 4.60	35.17± 4.00	34.07± 2.06	1.07± 0.27
Thalassemia homozygous (n=22)	1.25± *** 0.92	31.26± * 5.80	44.80± *** 5.68	22.69±*** 6.00	0.58± *** 0.26
IDA (n=35)	0.90± *** 0.79	31.36± ** 5.70	39.06±NS 7.00	28.68±** 5.90	0.78± *** 0.28

*P < .02, **P < .01, *** P < .001, NS Non-Significant, Values are mean©S.D.
LPC - Lysophosphatidylcholine, SM - Sphingomyelin, PC - Phosphatidylcholine
PE - Phosphatidylethanolamine, U/S ratio - unsaturated to saturated fatty acid ratio.

Chiu et al (26) have pointed that phospholipids rich in PUFA are particularly susceptible to peroxidation. The decrease of PE (the phospholipid rich in PUFA) and of U/S fatty acid ratio observed in the present study are thus an index of oxidation of erythrocyte lipids in both conditions. Since the RBC lacks the ability for denovo synthesis of phospholipids, it depends for the repair processes of its oxidized lipids on the various lipid renewal pathways (26). These are, transport of lipids between plasma and RBC and across the lipid bilayer, breakdown of phospholipids by phospholipase into lysophospholipids and acylation of lysophospholipids to form phospholipids (26). Chiu et al (26) have pointed that oxidant stress can lead to

increased repair, but oxidant damage can lead to insufficient repair, impaired repair or even disrepair of phospholipids in the RBC membrane. Kuypers et al (27) have hypothesized that the presence of oxidative stress to the RBC membrane could lead to alterations in membrane lipid organization. They studied the transbilayer movement of spin labeled phospholipids in model ß thalassemic cells and observed a faster rate of PC movement in these cells. The retention of LPC as against the complete loss of it from control RBC suggested an altered phospholipid molecular species turnover, possibly as a result of an increased repair of oxidatively damaged phospholipids (27). Further, Giardini et al (28) have reported significantly low 'plasma' levels of phospholipids, PC and SM in thalassemia. In our laboratory also plasma phospholipids in all lipoprotein fractions were found to be decreased not just in thalassemia but even in IDA where unlike the homozygous condition the liver is unaffected by iron overload. This supports the concept of increased uptake of lipids by the membrane from the plasma as a repair process in both conditions. Therefore, we suggest that increased level of lipids and low LPC levels in both membranes could be on account of the attempt on the part of the RBC to restore its oxidatively damaged lipids by both, increased uptake from the plasma, and acylation in the RBC membrane.

Such pathologic cells, characterized by an altered lipid profile, if subjected to higher oxidative stress would have a greater predisposition to hemolysis, as indicated by increased levels of TBARS following an exogenous oxidant stress. Lipid peroxidation is known to cause polymerization of membrane components, thus decreasing cell deformability (29), while a fall in PUFA and an elevation of cholesterol and SM can increase the rigidity of the phospholipid bilayer (30). Hence, low levels of PE and PUFA, accompanied by an increase in membrane cholesterol and SM in thalassemia and IDA in the present study may contribute towards membrane rigidity and decreased cellular deformability, thus enhancing RBC hemolysis in both conditions.

In conclusion, despite the mechanisms facilitating ROS generation in thalassemia being multifactorial, resulting from the presence of unmatched alpha globin chains, high intracellular concentration of nonhemoglobin iron and hypochromia (3), against just hypochromia in IDA, our findings indicate considerable lipid imbalance in both conditions, irrespective of their etiology. Although, the level of significance was almost equivalent in both conditions, in terms of percentage, lipid peroxidation and damage was more pronounced in thalassemia than IDA. Nevertheless, we point hypochromia as an important contributor for inducing membrane lipid oxidation and RBC hemolysis.

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