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Altered erythrocyte surface electric charge in β- thalassemia: By Alcian Blue Dye binding

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Abstract

Aim: To evaluate erythrocyte surface electric charge in β- thalassemia by using Alcian Blue dye binding.

Objective: Estimate the binding of AB dye to Sialic acid on the surface of thalassemia and normal, healthy erythrocytes. Compare negative charge due to Sialic acid on the surface of erythrocyte of thalassemia with healthy control subjects.

Result: The study was performed on 60 thalassemia subjects (39 male, 21 female, mean age 8.3years) and 30 age and sex matched normal control subjects (17 male, 13 female, mean age 6.8years). Hematological parameters analyzed on beta thalassemia subjects' shows significant difference among control and Thalassemia group. Levels of HbA_2 showed a marginal difference ($p=0.041$). Cationic dye, Alcian Blue, in PBS (pH7.4) was used as a reagent to demonstrate binding of the dye to Sialic acid. Results of dye bound to thalassemic erythrocytes was significantly less ($p<0.05$) as compared to binding on erythrocytes of the control group.

Conclusion: We believe, our data support the perception that there is decreased negative charge on the erythrocyte membrane in thalassemia reflected by cationic Alcian Blue dye binding.

Keywords: Thalassemia, Erythrocyte, Alcian Blue Dye, RBC Membrane, Sialic acid.

Introduction

The clinical significance of Glycobiology has become the focus of today's research, as its role in the development, regulation and progression of the disease is slowly but, surely recognized. Sialic acid, a component of glycoprotein, plays a central role in the biochemical function of humans. Elevated levels of Sialic acid in metabolic diseases, infectious diseases, inflammatory diseases and carcinogenesis are well established. Altered sialoglycoprotein and Sialic acid on erythrocyte membrane has also been reported in various diseases like, Thyroid disorder, Diabetes, Jaundice, Nephrotic disease, glomerular disease, Alcoholism, cardiac disease, RA and various forms of malignant transformation. In addition, some physiological process may alter Sialic acid levels in plasma.[1]

Thalassemia, a genetic disease associated with abnormal hemoglobin, a defect in the beta globin chain cluster. The Beta thalassemia carrier rate in India is around 3-7% with higher frequency in certain ethnic groups.[2] Thalassemia is a form of transfusion dependent hemolytic type of anemia accompanied by iron overload and hemochromatosis as a side effect of repeated blood transfusion. Microcytic, hypochromic, anisocytic red cells with basophilic stippling and target cell appearance with increased osmotic fragility is a hematological hallmark of thalassemic erythrocytes.[3] Presence of unstable hemoglobin,[4] Iron overload,[5,6] Increased Reactive Oxygen Species along with reduced red cell Glutathione[7,8] and denatured membrane protein, loss of Sialic acid from glycol-conjugates of membrane[9] are some biochemical defects reported in thalassemia.

Morphological alterations and maldistribution of glycoprotein reported in thalassemic red cells are associated with damage of the red cell membrane skeleton.[10]

Recently, interest of Glycobiology has been shifted towards characterizing membrane flaw in hemoglobinopathies. Though such defect is not a primary cause, but it has some impact on survival of erythrocytes in these genetic diseases. The structure of Membrane micro domain or raft's contribution towards physical properties of live cell membranes continues to be clarified. Sialic acid a component of glycoprotein contributes almost all the negative charge present on the surface of erythrocyte membranes. Such high negative charge believed to suppress erythrocyte aggregation, but when removed results in altered rheological properties and causes aggregation of erythrocyte. It marks aging of circulating erythrocyte and causes early clearance from circulation. Thus Sialic acid has becomes a useful parameter to study the impact of genetic disease on modifications of cell surfaces micro domain.

Cationinc dye Alcian Blue (AB) is a routine histochemical stain used for staining sialylated glycocalyx of cell. AB dye specifically binds with sialylated glycocalyx of cells.[11] It has been used to compare Sialic acid and in turn map electric charge on erythrocyte in various diseases.[12-14] Gambaro G et al[15] and Baba Y et al[16] have reported altered erythrocyte charge in diabetes mellitus. Levin et al[17] reported reduced binding of cationic AB dye to erythrocyte in children with minimal change nephrotic syndrome. D Yavuz et al[18] found reduced binding of AB in hypertensive patients. Changes have also been

recognized in PAS pattern of beta thalassemia RBC membrane protein electrophoresis. In view of existing research, we undertook a study to evaluate the surface charge on thalassemia erythrocyte membrane by using Alcian Blue dye binding.

Material and Methods

The present study was performed at the Department of Biochemistry, of SDM College of Medical Sciences, Dharwad, Karnataka, as a part of ICMR-STS-2016 project. The Proposal was approved by the institutional ethics committee with a caution that the identity of the patient and results should be kept confidential.

The study was performed on 90 subjects of both genders between 5 and 21 years age group, living in and around Dharwad town in North Karnataka. The study population was categorized into two groups. Group A- consisted of sixty Thalassemia patients, diagnosed based on red cell indices (Hb < 10.0 gm/dl, RBC count > $5x10^6$ /cmm, RDWI < 17%, MCV <80fl, peripheral smear showing Microcytic, Hypochromic RBC with target cells and/or basophilic stippling as suggested by Shine and Lal[19] and showing HbA_2 > 3.5% by Hemoglobin electrophoresis.[20] Group B- comprised of thirty age and sex matched children visiting pediatric OPD of our hospital who are apparently healthy and without any history of blood abnormalities. Objective of our study was to estimate Sialic acid on the surface of erythrocyte in **t**halassemia and normal, healthy subjects by Alcian blue dye binding method and compare negative charge due to Sialic acid on the surface of erythrocyte of both above groups.

Written informed consent was obtained from each participant or family member included in the research study

Preparation of Alcian Blue (cationic dye) solution: As suggested by Winkel JGJ et al,[14] 5mg of Alcian Blue dye was completely dissolved in 100microliter of absolute ethanol and diluted to 10ml with Phosphate Buffered saline (PBS), pH7.4 containing 25mmol MgCl2. The final concentration of the AB dye was 500microgram/ml. Every time a fresh dye solution was prepared and extinction of dye solution was measured on the spectrophotometer (UV1700 Simadzu) at 650nm. Only the dye solution with extinction reading between 0.800 and 1.130 was accepted for further test.

Collection of Blood and preparation of washed red blood cells: After taking all aseptic precautions 2ml blood was collected from an anti-cubital vein in a tube containing 250microlit of 3.8% trisodium citrate solution. The blood sample was centrifuged at 3000 rpm for 10 min to obtain the packed erythrocyte fraction. Plasma was discarded and packed erythrocytes were washed thrice with Phosphate Buffered saline (PBS).

Cell count and Red Blood cell indices: Fifty microliter of packed and washed erythrocyte were transferred to 10.0 ml of PBS. It was divided into two portions of 5ml each. A first aliquot of 5ml suspension was subjected for blood indices and RBC count on Sysmax XN 1000 – Transasia blood counter in hematology laboratory of our hospital.

Cationic dye Alcian Blue binding to erythrocyte: To the second aliquot of 5ml erythrocyte suspension, 500microliter of cationic dye, Alcian Blue (AB) was added. The suspension was incubated for 30min at 37 ^{0}C. After incubation for 30 min, erythrocytes were removed by centrifugation and leftover concentration of AB dye in solution was estimated by measuring optical density reading at 650nm on UV1700 Shimadzu Spectrophotometer. The amount of AB dye bound to erythrocyte was calculated. The final result was expressed as amount of AB dye bound per 10^6 erythrocyte.

Hemoglobin electrophoresis: 250microliter of washed packed erythrocyte were used for preparation of hemolysate whch was subjected to alkaline hemoglobin electrophoresis on agarrose gel supplied by Helena Co (Alere) to estimate concentrations of Hemoglobin fractions. Electrophoretogram was quantitated on densitometer using Platinum Ver 3.0 Software supported by Helena Co.

The final result were expressed in terms of dye bound per 10^6 red blood cells. Results were compared in patients and control groups and presented as mean + SD. Data was analyzed for Students 't' test within 95% CI by SPSS statistical software. P value <0.05 was considered as significant.

Observations and Results

The study was performed on 60 thalassemia subjects (39 male, 21 female, mean age 8.3years) and 30 age and sex matched normal control subjects (17 male, 13 female, mean age 6.8years).

To support the selection of B thalassemia subjects hematological parameters were analyzed for whole blood collected from control and Thalassemia group. Hemoglobin A_2 levels were used as confirmatory parameter in diagnosis.

Table 1: Hematological parameter in Control and Thalassemia Group

	Control Group	Thalassemia Group	P value
Hemoglobin gm%	13.9 0.9 (11.8 – 15.7)	10.39 0.69 (5.3 – 17.6)	< 0.01
RBC count 106/cmm	4.58 0.36 (3.88 – 5.92)	5.56 0.4 (2.04 – 7.89)	< 0.001
MCV fl	86.2 4.7 (79.5 – 99.6)	60.11 3.49 (52.7 – 85.3)	< 0.01
MCH Pg	30.1 1.6 (25.4 – 35.8)	20.76 4.69 (16.5 27.2)	< 0.05
RCWD %	14.2 0.98 (9.9 – 34.4)	10.76 1.38 (9.3 – 31.6)	< 0.05
Hemoglobin A2 %	2.3 0.6 (1.8 – 3.3)	5.05 1.30 (3.6 – 8.5)	= 0.041

Result of control and Thalassemia groups for Hemoglobin, RBC count, MCV, MCH, RCWD and HbA2 from Table 1, when worked out for student t test showed a significant difference in hemoglobin ($p<0.01$), RBC count ($p<0.001$) MCV ($p<0.01$), MCH ($P<0.05$) and RCWD ($p<0.05$) between two groups. HbA2 showed a marginal difference ($p=0.041$) between both the groups.

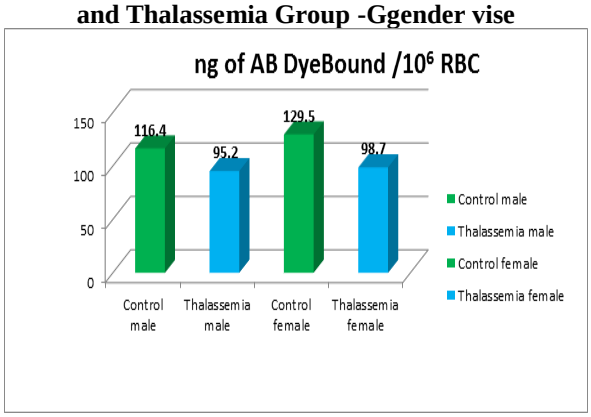
Cationic dye, Alcian Blue, in PBS (pH7.4) was used as a reagent to demonstrate binding of the dye to Sialic acid moieties on the surface of erythrocytes from thalassemia and control groups.

Table 2: Cationic Alcian Blue dye binding in Control and Thalassemia Group

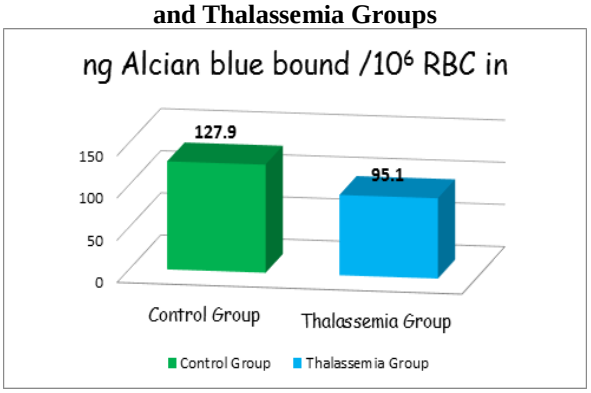
	Control Group Male n= 17(56.6%)	Control Group Female n= 13(43.3%)	Thalassemia Group Male n= 39(65%)	Thalassemia Group Female n= 21(35%)	P value
ng AB dye Unbound in sol /10^6RBC	405.2 ± 9.8	398.1 ± 6.2	301.2 ± 5.2	313.6 ± 8.3	
ng AB Dye Bound/10^6RBC	116.4 ± 1.8	129.5 ± 2.2	95.2 ± 0.9	98.7 ± 1.3	
ng AB dye bound to 10^6 RBC (Average)	127.9		95.1		<0.05

Binding of cationic dye Alcian Blue on erythrocyte in control and thalassemia group is shown in **Table 2**. In our study, Mean value of dye bound to male erythrocytes of control group was 116.4 ± 1.8 ng/10^6 RBC and female erythrocytes of control group was 129.5 ± 2.2 ng/10^6 RBC. Whereas the mean value of dye bound to male and female erythrocytes among thalassemia group was 95.2 ± 0.3 ng/10^6 RBC and 98.7 ± 1.3 ng/10^6RBC respectively. There was no difference observed in dye binding between male and female erythrocytes from the subjects of both the groups, but AB dye binding to thalassemic erythrocytes(95.1) was significantly less ($p<0.05$) as compared to binding on erythrocytes of the control (127.9)group.

Graph 1: ng of AB dye bound to 10^6 RBC in control and Thalassemia Group -Ggender vise



Graph 2: ng of AB dye bound to 10^6 RBC in control and Thalassemia Groups



Discussion

Glycobiology is the focus of today's research, as this post translational reaction is recognized playing role in the progression of the disease. There is a shift in research interest from metabolic diseases to congenital hemolytic disorders. Where changes in the erythrocyte membranes are not considered to be the primary defect, yet contribute to the pathophysiology of hemolytic process. Alteration in membrane structure of erythrocytes was reported in hemoglobinopathies such as HbS[21] G6PDH deficiency, hereditary spherocytosis[22] and paroxysmal nocturnal hemoglobinuria (PHN) a Genetic acquired hemolytic disease which show impaired sialylation of glycospingolipids and metabolic disorder of membrane Glycoconjugates.[23]

Sialic acids a component of Glycoconjugates, present on the surface of erythrocyte membrane contribute almost all negative charge,[24] thus binding of AB was indirectly used to assess the negative charge on these red cells. Our result indicates there is a significant difference in charge on erythrocytes between the groups. Thalassemic erythrocyte shows low AB dye binding, indicating less Sialic acid and in turn less negative charge on the membrane.

Our results are supported by existing results on erythrocyte membrane from thalassemics where, sialic acid (total, protein bound or lipid bound) has shown decrease level. Experimental data also suggest an increase of sialic acid in serum of thalassemic individuals. Parameters which alter rheological properties like decreased osmotic fragility, increased aggregation, microcytosis, low MCV of red cell also indirectly supports our findings of decreased charge on thalassemic red cell membrane.

Short life span of thalassemic erythrocyte could be because of loss of membrane Sialic acid, which mark them old and making them recognizable by RE cell receptor for early clearance[25] as desialylation, a negative consequence may appear to protect individual from non-functional red cells.

Erythrocytes in circulation constantly encounter elevated oxygen pressure. Ferrous ion acts as a strong catalyst to cause auto-oxidation stress. Even though defense enzymes of cell counteract this stress, iron overload and deficient antioxidant defense mechanism in thalassemia cause damage on membrane protein and lipid[26] such denatured rafts are shed off from the membrane as a part of cell protection process. Reduced Sialic acid to protein ratio has been reported in beta thalassemia.[27] Oxidative stress was also observed in certain genetic diseases like beta hemoglobinopathies (HbS, thalassemia) G6PDH deficiency, hereditary spherocytosis, PHN. Due to loss of Sialic acid erythrocytes become more sensitive to osmotic shock and thus leads to enhanced eryptosis, (suicidal death of erythrocytes).[22] Accumulation of alpha globin chain at cytoskeleton in beta thalassemia with loss of thiol group and denaturation of protein 3 & 4.1band designate oxidative damage on membrane.[28]

Kahane I et al[29] used cationised ferritin and Ruthenium red dye to visualize membrane based Sialyl residue, thalassemia erythrocyte membrane has exhibited less dense material as compared to normal. We are yet to know the exact molecular mechanism of observed reduction in Sialic acid. It could be defective biogenesis of membrane under genetic defect or early removal of damaged components from membrane.

Whatever may be the molecular mechanism involved in loss of Sialic acid carrying negative charge from thalassemic erythrocyte, we believe, in addition to a defective beta chain, exposure to oxidative stress could lead to loss of negatively charged Sialic acid augment removal of tinted erythrocyte from circulation thereby protecting the individual from non-functional red cells but in effect increasing the severity of disease.

Further research towards role of sialyltransferase and sialidase is needed. Also protecting Sialic acid on the erythrocyte membrane or masking of penultimate galactose epitope may provide defensive shield.

Conclusion

We believe that our data support the perception that there is decreased negative charge in the form of Sialic acid from the erythrocyte membrane in thalassemia which is reflected by cationic Alcian Blue dye binding.

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