

Erythrocyte Phosphatidylserine Exposure in β -Thalassemia

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Abstract

Introduction: Phospholipid asymmetry is well maintained in erythrocyte (RBC) membranes with phosphatidylserine (PS) exclusively present in the inner leaflet. Eryptosis, the suicidal death of erythrocytes, is characterized by cell shrinkage, membrane blebbing and cell membrane phospholipids scrambling with PS exposure at the cell surface. Erythrocytes exposing PS are recognized, bound, engulfed, and degraded by macrophages. Eryptosis thus fosters clearance of affected erythrocytes from circulating blood which may aggravate anemia in pathological conditions. Thalassemia patients are more sensitive to the eryptosis depletion and osmotic shock which may affect RBC membrane Phospholipid asymmetry.

Aim: We aimed in this work to determine the erythrocyte PS exposure in splenectomized and non splenectomized β -Thalassemia (β -TM) patients and correlate it with the clinical presentation and laboratory data.

Methods: RBCs were stained for annexin V (AV) to detect phosphatidylserine (PS) exposure in 46 β -TM patients (27 splenectomized and 19 non splenectomized) compared to 17 healthy subjects as a control group. Results: We reported significant increase in erythrocyte PS exposure in β -TM patients compared to control group ($p=0.0001$). Erythrocyte PS exposure was significantly higher in splenectomized β -TM patients as compared with non splenectomized β -TM patients ($p=0.001$). No correlation was found between erythrocyte PS exposure and clinical or hematological data of β -TM patients but there was positive correlation between erythrocyte PS exposure and ferritin level in β -TM patients.

Conclusion: These findings suggest that β -TM patients have higher level of erythrocyte PS exposure and splenectomy was shown to aggravate erythrocyte PS exposure without aggravation of anemia.

Keywords: Flow cytometry; Thalassemia; RBCs; Phosphatidylserine; Annexin V

Introduction

The thalassemias are a group of inherited hematologic disorders caused by defects in the synthesis of one or more of the hemoglobin chains. Alpha Thalassemia is caused by reduced or absent synthesis of alpha globin chains, and beta Thalassemia is caused by reduced or absent synthesis of beta globin chains. Imbalances of globin chains cause hemolysis and impair erythropoiesis [1].

Historically, β -Thalassemia has been divided into three clinical syndromes; β -Thalassemia minor (heterozygous), a mild microcytic, hypochromic hemolytic anemia; β -Thalassemia major (homozygous), a severe transfusion-dependent anemia; and β -Thalassemia intermedia, with symptoms of severity between the other two types. A fourth syndrome is now recognized, which has been designated as silent carrier status, for patients with genetic changes in one of the two β genes that results in no hematologic abnormalities [2].

Individuals suffering from Thalassemia major usually present at less than one year of age with severe anaemia. These are at risks of complications such as hepatosplenomegaly, bone deformities and growth delay [3]. Current management of Thalassemia consists of blood transfusion, iron chelation, splenectomy and bone marrow transplantation [4]. Splenectomy is performed if patients develop hypersplenism or an increased requirement for blood transfusion.

Thalassemia is characterized by morphological and functional RBC anomalies that lead to shortening of the RBC life span. Most patients suffer from chronic hemolytic anemia because of untimely RBC destruction in the bone marrow and spleen. Several studies have proposed mechanisms that lead to the premature removal of abnormal RBCs. These include extraordinary ineffective erythropoiesis in the

marrow [5] and altered deformability due to the rigidity of the RBC membrane, which impair passage of RBCs through sinusoidal walls of reticuloendothelial organs, and which finally triggers the removal of these cells from circulation [6-8].

The RBC membrane undergoes vesiculation under a variety of conditions, including increased cytoplasmic calcium concentration, reduced ATP content, and disruption to the membrane lipid-protein organization [9], all of which are features characteristic of thalassemic RBCs. Severely affected thalassemic RBCs are known to correlate with increased accumulation of unmatched globin chains in the cytoskeleton; skeleton associated globin results in altered membrane function by producing oxidative damage to adjacent cytoskeletal proteins [7,10], which eventually induce loss of RBC membrane in the form of vesicles with phosphatidylserine (PS) exposure. It has been shown that vesicles and membrane-derived Micro Particles (MPs) from RBC strongly bind Annexin V (AV), a protein known for its interaction with negatively charged phospholipids such as phosphatidylserine (PS) [11-15]. Phosphatidylserine (PS) is one of the key membrane phospholipids that is normally located along the inner side of the membrane bilayer.

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The aim of our research was to determine the erythrocyte PS exposure in splenectomized and non splenectomized β -Thalassemia major (β -TM) patients and their relationship to the degree of severity in β -TM patients.

Subjects and Methods

Blood samples were collected from 46 β -TM patients (23 males and 23 females) with age ranged from (2 to 19 years) and 17 healthy volunteers with matched age (3.6-19 years) and sex (9 males and 8 females). The patients group included 27 splenectomized β -TM patients and 19 non splenectomized β -TM patients. They were selected in the period from January 2011 to December 2011 from Hematology Clinics of Children Hospital, Mansoura University. All patients were transfusion dependant. Exclusion criteria included evidence of concurrent infection, hospitalization or receiving blood transfusion for at least one month prior to the start of the study or during the sampling period. The study was approved by Institutional Review Board of Mansoura University.

Five ml of venous blood were obtained after attainment of informed consents. Three ml were preserved in K2EDTA for both flow cytometric analyses of erythrocyte PS exposure using the EPICS XL flow cytometer (Coulter Electronic, FL, USA) and hematologic parameters including complete blood count (CBC) using Cell- Dyn 3500.

(Abbott, USA), reticulocyte count and fetal hemoglobin percentage (HbF%) done by hemoglobin electrophoresis using Hydrasys (Sebia, France). Serum was obtained after centrifugation of the remaining blood sample and used for the determination of serum ferritin level using Elisa reader (Adaltis, Italy).

RBCs binding assay

A total of 200 μ L of each whole blood sample was fixed in 200 μ L of 1% paraformaldehyde in phosphate buffer saline (PBS) for at least 1 h or, if necessary, stored in fixative at 4°C until staining and flow cytometric analysis was performed. A total of 5 μ L of fixed blood sample was then incubated with 10 μ L of phycoerythrin (PE) conjugated anti-glycophorin A (GA), and 1 μ L of fluorescein isothiocyanate (FITC) conjugated annexin V (AV) for 30 min at room temperature in dark. All samples were collected at room temperature (18-29°C) and processed within 2 h.

Flow cytometric analysis

The Beckman coulter XL flow cytometry was used for analysis and data was collected in the list mode. The instrument was calibrated according standard protocols to achieve day-to-day reproducibility. The red cell population was defined by PE-glycophorin-A positivity. Fluorescence intensities were expressed in logarithmic mode. Data from at least 50,000 events were acquired and analysed. The control sample incubated without PE-glycophorin A and FITC-AV was used to set the region for positive fluorescence such that the fraction of cells with positive (auto-) fluorescence was lower than 0.2% of total. The population of cells labeled with FITC-AV and PE-anti-glycophorin-A above background was determined from the fraction of cells in this region in excess of that obtained with the (unlabeled) control. This approach was of particular importance because a number of thalassemic RBCs exhibited an increased autofluorescence as compared with normal control cells. RBC PS exposure were distinguished by double positivity for both FITC-AV and PE-anti-glycophorin-A which presented in the upper right quadrant, whereas intact RBCs were presented in the upper left quadrant of the biparametric histogram (Figure 1).

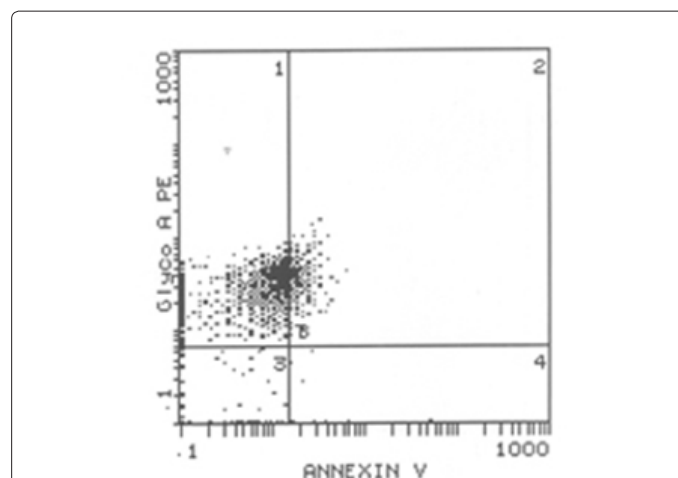


Figure 1: Representative flow cytometric dot plot of RBCs phosphatidylserine (PS) exposure in a β -TM patient stained with FITC-conjugated annexin V(AV) and PE- conjugated anti-glycophorin A. percentages of positive events are indicated in the upper right quadrant.

Statistical analysis

Data entry and analyses were performed using SPSS statistical package version 10. Quantitative data were presented as mean, standard deviation and range. Student t-test used to compare means of two groups. The one-way ANOVA procedure produces a one-way analysis of variance for a quantitative dependent variable by a single factor (independent) variable. Mann Whitney-U test and Kruskal-Wallis H are non parametric tests equivalent of the t-test and ANOVA test respectively. Correlation between variables was done using Pearson correlation for parametric data and spearman rank correlation for non parametric. For all above mentioned statistical tests, done, the threshold of significance was $P < 0.05$.

Results

There were significant decrease in RBCs count, Hb level, HCT %, MCV, MCH, RDW %, WBCs and platelets in β -TM patients compared to control group ($p < 0.05$). There was no significant difference in MCHC in β -TM patients compared to control group (Table 1).

Significant decrease was found in RBCs count, Hb level, Hct%, MCV and MCH in splenectomized β -TM patients compared to control group ($p < 0.05$). Regarding RDW%, WBCs count and platelets, there was a significant increase in splenectomized β -TM patients group compared to control group. There was no significant difference in MCHC in splenectomized β -TM patients compared to control group (Table 1). Significant decrease also was found in RBCs count, Hb level, HCT%, MCV and MCH in non splenectomized β -TM patients compared to control group ($p < 0.05$). RDW% showed a significant increase in non splenectomized β -TM patients group compared to control group. There was no significant difference in MCHC in non splenectomized β -TM patients compared to control group.

On the other hand, there was no significant difference in HbF% ($P = 0.094$) between splenectomized β -TM patients group (median-27.4)%, range (12.3-91.9)% and non splenectomized β -TM patients group (median-54.6)%, range (10.7-96.2)%.

A highly significant elevation was shown concerning ferritin level and RBC PS exposure % in β -TM compared to control group (Table 2). On the other hand, there was no significant difference in ferritin level in

| Parameter | Control N=17 | β -TM N=46 | β -TM/S N=27 | β -TM/NS N=19 Mean \pm SD | P | P ₁ | P ₂ | P ₃ |
|--------------------------------|--------------------|---------------------|---------------------|--------------------------------------|--------|----------------|----------------|----------------|
| | Mean \pm SD | Mean \pm SD | Mean \pm SD | | | | | |
| RBCs ($\times 10^6$ /ml) | 4.65 \pm 0.35 | 3.27 \pm 0.99 | 3.06 \pm 0.94 | 3.56 \pm 1.04 | 0.0001 | 0.0001 | 0.0001 | 0.103 |
| Hb (gm/dl) | 12.26 \pm 0.57 | 7.39 \pm 1.89 | 7.05 \pm 1.78 | 7.89 \pm 1.98 | 0.0001 | 0.0001 | 0.0001 | 0.142 |
| Hct (%) | 36.51 \pm 1.76 | 23.94 \pm 7.35 | 23.30 \pm 7.41 | 24.86 \pm 7.35 | 0.0001 | 0.0001 | 0.0001 | 0.484 |
| MCV (fl) | 78.74 \pm 3.55 | 74.53 \pm 7.21 | 75.54 \pm 5.71 | 73.09 \pm 8.91 | 0.003 | 0.045 | 0.018 | 0.261 |
| MCH (pg) | 26.51 \pm 2.33 | 23.91 \pm 3.38 | 24.20 \pm 3.37 | 23.50 \pm 3.45 | 0.004 | 0.01 | 0.005 | 0.497 |
| MCHC (g/dl) | 33.62 \pm 2.14 | 32.06 \pm 3.40 | 32.08 \pm 3.88 | 32.04 \pm 2.68 | 0.08 | 0.143 | 0.058 | 0.973 |
| RDW(%) | 14.59 \pm 1.54 | 23.76 \pm 7.74 | 24.95 \pm 8.09 | 22.07 \pm 7.08 | 0.0001 | 0.0001 | 0.0001 | 0.207 |
| WBCs($\times 10^3$ /ml) | 8.60 \pm 3.27 | 28.20 \pm 32.85 | 41.22 \pm 37.74 | 9.70 \pm 4.96 | 0.014 | 0.001 | 0.431 | 0.0001 |
| Platelets ($\times 10^3$ /ml) | 317.0 \pm 101.57 | 452.13 \pm 203.59 | 500.30 \pm 196.38 | 383.69 \pm 198.73 | 0.0001 | 0.0001 | 0.222 | 0.045 |

S, splenectomized; NS, non splenectomized.

P= β -TM patients versus control.

P1= β -TM/S versus control.

P2= β -TM/NS versus control.

P3= β -TM/S versus β -TM/NS.

Table 1: Comparison between healthy control, β -TM., β -TM (S) and β -TM (NS) patients regarding hematological data.

| | Control N=17 | | β -TM N=46 | | β -TM/S N=27 | | β -TM/NS N=19 | | P | P1 | P2 | P3 |
|-----------------------------------------|--------------|----------|------------------|----------|--------------------|----------|---------------------|----------|--------|--------|--------|-------|
| | Median | Range | Median | Range | Median | Range | Median | Range | | | | |
| Ferritin (ng/dl) | 33 | 10-79 | 747.5 | 192-3000 | 719 | 230-2500 | 776 | 192-3000 | 0.0001 | 0.0001 | 0.0001 | 0.422 |
| RBCs phosphatidylserine (PS) exposure % | 1.35 | 0.38-5.4 | 14.9 | 4-35 | 16.4 | 6.4-35.4 | 8.3 | 4.2-23 | 0.0001 | 0.0001 | 0.0001 | 0.01 |

Table 2: Comparison between healthy control, β -TM, β -TM (S) and β -TM (NS) patients regarding serum ferritin level and RBCs phosphatidylserine (PS) exposure %.

| RBC phosphatidylserine (PS) exposure % | r | RBCs ($\times 10^6$ /ml) | Hb (g/dl) | Hct (%) | MCV | MCH (pg) | MCHC (g/dl) | RDW (%) | WBCs ($\times 10^3$ /ml) | Platelets ($\times 10^3$ /ml) |
|----------------------------------------|---|---------------------------|-----------|---------|-------|----------|-------------|---------|---------------------------|--------------------------------|
| | | p | 0.222 | 0.240 | 0.366 | 0.405 | 0.888 | 0.519 | 0.182 | 0.076 |

Table 3: Correlation between RBC phosphatidylserine (PS) exposure percentage and hematological data in β -TM patients group.

splenectomized β -TM patients compared to non splenectomized β -TM patients but there was a significant elevation in RBC phosphatidylserine (PS) exposure in splenectomized β -TM patients compared to non splenectomized β -TM patients.

There was no significant correlation between RBC phosphatidylserine (PS) exposure % and hematological data in β -TM patients group ($P > 0.05$) (Table 3).

On the other hand, there was no significant correlation between RBC phosphatidylserine (PS) exposure % and HbF% in β -TM patients group but there is a positive correlation between RBC phosphatidylserine (PS) exposure and serum ferritin level (ng/dl) in β -TM patients group.

Discussion

Accumulation of unmatched globin chains at the cytoplasmic surface of the RBC membrane is an important feature of the pathophysiology of Thalassemia. These extra globin chains, and the associated iron, heme or hemichromes, induce oxidative damage to the membrane and, ultimately, contribute to premature cell destruction [9,16].

Normally, the erythrocyte membrane is asymmetric with respect to phosphatidylserine (PS). In β -Thalassemia patients, a subpopulation of erythrocytes was found in peripheral blood with higher level of PS exposed on the surface and is removed rapidly from the circulation [14,17]. Changes in this asymmetry are one of the hallmarks of eryptosis [18]. During in vivo ageing, the PS externalization triggers an "eat me" signal to the phagocytes [19,20].

Annexins are a family of proteins that bind to acidic phospholipids, particularly phosphatidylserine (PS) [21,22]. Annexin V was used as a marker for PS positivity, while anti glycophorin-A (CD235) is a sialoglycoprotein expressed on the surface of erythrocytes [23,24] and was employed as marker for intact red cells and red cell derived microvesicles [25].

The vesiculation process is probably more facilitated in β -thalassemic erythrocyte membranes, leading to faster shedding of glycophorin-containing microvesicles, leaving the highly PS-exposed erythrocytes accessible to the phagocytes or reticuloendothelial cells. This also explains the cause of survival of younger erythrocytes in normal individuals, where the cell surface glycophorins mask the exposed PS causing hindrance to phagocytic recognition [26]. In this study, we aimed to evaluate the PS externalization in 46 β -TM patients (27 splenectomized and 19 non splenectomized) and correlate it with the clinical presentation and laboratory data.

In the present study, there is a statistically significant increase in RDW, WBC count and platelet count in splenectomized Thalassemia cases as compared to controls ($P < 0.05$). Also, there is a statistically significant decrease in RBC count, Hb level, Hct%, MCV and MCH in splenectomized group compared to control group. Regarding MCHC, there is no statistically significant difference between splenectomized group and control group. Also, there is statistically significant increase in RDW % in non splenectomized as compared to the controls ($P < 0.05$). These results coincide with Hegazy et al. [27] who found a statistically significant increase in WBCs count, RDW % and platelet count in splenectomized Thalassemia cases as compared to controls

($P < 0.05$) [27]. Meanwhile, there was statistically significant increase in RDW and platelet count in non splenectomized Thalassemia cases as compared to controls ($P < 0.05$). Also, there was significant decrease in RBCs count, Hb level, MCV and MCH in non splenectomized group as compared to the control group. Regarding WBCs count and MCHC, there was no statistically significant difference between the 2 groups.

The statistical analysis of our results showed a highly significant elevation of serum ferritin levels in β -TM patients compared to healthy control ($P = 0.000$). This coincides with the finding of Ikram et al. [28], who described that the patients of β -TM have increase in serum ferritin levels [28]. In β -TM repeated blood transfusion, ineffective erythropoiesis and increased gastrointestinal iron absorption lead to iron overload in the body. The management of the iron overload in these patients requires the administration of iron chelators continuously and evaluation of serum ferritin levels at regular intervals [29].

In this study we found that percentage of erythrocyte PS exposure percentage were significantly elevated in β -TM patients group compared to control group ($P = 0.000$). These results coincide with Willekens et al. [26] who proved that 55 % of RBCs vesicles identified by glycophorin A were positive for annexin V and Lamchiaghase et al. [30] who stated that vesicles are part of the red blood cells membrane which can be found in a small number in normal apoptotic process and increased in β -Thalassemia [26,30].

In the present study there is elevation of erythrocyte PS exposure percentage in patients with β -TM/S compared to β -TM/NS ($p = 0.01$). This is in agreement with the finding of Pattanapanyasat et al. [15], who found higher percentage of RBCs vesicles in splenectomized β -Thalassemia patients than non splenectomized β -Thalassemia patients in which RBC vesicles identified by expression of both glycophorin A and annexin V [15]. These findings suggest that, although splenectomy improves Hb concentration and reduce the transfusion needs and total blood volume, it also leads to increase in percentage of circulating RBCs vesicles and erythrocyte PS exposure [31].

In the present study there is no significant correlation between erythrocyte PS exposure percentages, hematological data in β -TM patients. This is not in agreement with the finding of Pattanapanyasat et al. [15] who described inverse relationship between level of RBCs vesicles and Hb concentrations. However, those data are inconsistent with that of Hegazy et al. [27] who didn't find any correlation between RBC vesicles percentage and hematological data in β -Thalassemia patients [27].

In the present study we found no significant correlation between erythrocyte PS exposure percentage and HbF percentage in patients with β -TM which seems to contradict with previous report who found an inverse relationship between the levels of both RBCs microvesicles and PS positive cells with Hb F [25]. This contradictory may be related to the difference in methodology used for evaluation of Hb F percentage.

In conclusion, this study presented data that may be of important value to protect thalassemic patients from pathological complications by regular monitoring of erythrocyte PS exposure percentage and estimation of proper serum ferritin level.

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