The potential role of RBCs and activated platelets in the Thalassemic hypercoagulable state

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Abstract: Back ground: The presence of a high incidence of thromboembolic events has led to the identification of a hypercoagulable state in thalassemia. Several etiologic factors may play a role in the pathogenesis of the hypercoagulable state in thalassemia. **Objectives:** The aim of this study was to assess the existence of a chronic hypercoagulable state in thalassemic patients and *study* the potential role of thalassemic RBCs and activated platelets in the hypercoagulable state. **Patients and Methods:** Fifty- nine patients with thalassemia (30 with Thalassemia major (TM) and 29 with Thalassemia intermediate (TI) were used as the study group and 20 healthy volunteers were used as control group. Flowcytemerty was used to study the expression of anionic phospholipids (Annexin V) on the RBCs and CD62p (P-selectin) on the activated platelets. **Results:** Annexin V labelled RBC in TM and TI patients were significantly over expressed compared to control group ($p < 0.001$) with no significant difference between patients with and without spleenectomy. The expression of activation- dependent platelets neoantigen, P-selectin, was significantly higher ($p < 0.001$) in Thalassemic patients compared to the control. There was a strong association between the expression of Annexin V on the RBCs and P-selectin on the activated platelets. **Conclusion:** There is a strong association between chronic hypercoagulable state and levels of both RBCs expressing Annexin V and platelets expressing P-selectin in Thalassemia patients. Also there is a strong association between levels of RBCs expressing Annexin V and levels of platelets expressing P-selectin. The strong association between the expression of these two cellular markers and the tendency of the hypercoagulable state observed in patients with Thalassemia may help to predict and to avoid the development of this state in those patients.
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1. Introduction

Thalassaemia are a hetrogeneous group of genetic disorders of haemoglobin synthesis, all of which result from a reduced rate of production of one or more of the globin chains of haemoglobin. They are divided into α- β- δ β-, or γδβ – thalassaemias, according to which globin chain is produced in reduced amounts (1). Clinically the thalassaemias are classified according to their severity into major, intermediate and minor forms. Thalassaemia major is a severe, transfusion – dependent disorder. Thalassaemias intermedia is characterized by anemia and splenomegaly, though not of such severity as to require regular transfusion. Thalassaemia minor is the symptomless carrier state (2).

Thalassemias represent the most common monogenetic disorder worldwide (3), there is a particularly high incidence of thalassemia (2.5%-25%) in the Mediterranean basin, the Middle East, the tropical and subtropical regions of Africa, the Asian subcontinent, and Southeast Asia, where milder forms of the disease are most commonly seen. Cases of thalassemia also occur sporadically in virtually every ethnic group and geographic location (4).

Several clinical and laboratory finding suggest the presence of chronic hypercoagulable state in patients of thalassaemia major(TM) and thalassaemia intermedia (TI) including transient ischemic attacks, pulmonary embolism and deep venous thrombosis. Diverse factors contributing to the hypercoagulable state in patients with Thalassemia have been identified. In most cases, a combination of these abnormalities leads to clinical thrombosis. (5).

Among cellular factors, platelet activation contributes, to a significant extent. Many evidence suggesting that patients with thalassemia have activated platelets. As in thalassemia, there is evidence of increased platelet aggregation (6). Moreover, flow cytometric studies have also confirmed the chronic platelet activation status

manifested by an increased proportion of platelets expressing CD62P (P-selectin) and CD63 (7-8), in addition to shortened platelet survival due to enhanced platelet consumption (especially in splenectomized patients) (9-10).

Alteration in RBCs due to oxidation of globin subunits in thalassemia erythroid cells, leads to the formation of red-cell "senescence" antigens such as phosphatidylserine and phosphatidyletholamine cause thalassemic red cells to be rigid and deformed. These changes cause RBCs to aggregate, resulting in premature cell removal (11). So, Thalassemic RBCs expressing these negatively charged phospholipids were used as a source of phospholipids, enhancing and eventually increase thrombin generation in a prothrombinase assay where normal RBC had no effect (12).

This was evident by the finding that annexin V, a protein with high affinity and specificity for anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs (13). The procoagulant effect of thalassemic RBCs was suggested to contribute to the hypercoagulable state in thalassemia by amplifying thrombin generation and initiating platelet activation in vivo as one aspect of hypercoagulable state (8). These abnormalities have been reduced to normal range after the patients have received a blood transfusion (12).

The finding of elevated levels of endothelial adhesion proteins (E-selectin [ELAM-1], intercellular adhesion molecule-1 [ICAM-1] and von Willebrand factor [VWF] and vascular cell adhesion molecule-1 [VCAM-1] in thalassemic patients suggested that endothelial injury or activation may be a feature of this genetic disease which also plays an important role in the recruitment of white blood cells and RBCs and promote thrombosis at vascular inflammation sites, vessel obstruction, tissue hypoxia and death (14). More recently, it was shown that microparticles of red blood cell origins were elevated in patients with TI vessel having a potential to aggravate thrombotic events (15).

DNA mutations do not appear to play an important role in the pathogenesis of thrombosis observed in thalassemia. The presence of factor V Leiden, prothrombin mutation, and methylene tetrahydrofolate reductase (MTHFR) mutations was not significantly correlated with the thrombotic risk in Thalassemic patients (16). Other pathogenetic mechanisms have been correlated with hypercoagulability in thalassemia and these include cardiac dysfunction, hormonal deficiencies and liver dysfunction (5). Clinical observations have suggested that splenectomy in Thalassemia can contribute to an increased susceptibility to thrombosis (17-18). The development of these complications has been ascribed to the presence of high platelet counts following splenectomy and/or to increased number of abnormal RBCs (6, 10, and 19). Also, in splenectomized TI patients, thrombin generation was significantly higher than in control subjects and patients who had not undergone splenectomy (17 and 19).

The presence of a persistent hypercoagulable state combined with the infrequent occurrence of significant thrombotic events suggests that thrombosis is largely a subclinical process in thalassemia and has been associated with autopsy findings of platelet and fibrin thrombi in the microvasculature in the lungs (20) and the brain (18). Management of this hypercoagulable state has two arms: prevention and treatment. Prevention consists of proper anticoagulation to high-risk patients with Thalassemia who are exposed to transient thrombotic risk factors (eg, surgery, immobilization, pregnancy); Treatment entails the adequate use anticoagulation according to the recommendation for hypercoagulable state (21).

Therefore, the present study is an attempt to provide evidence for the existence of a chronic hypercoagulable state in patients with β -thalassaemia major, thalassaemias intermedia due to the expression of anionic red cell phospholipids leading to platelet activation.

We employed flow cytometry to determine the procoagulant properties of Thalassemic RBCs (by the binding of FITC annexin V), correlating the results with the fraction of circulating platelets expressing activation-dependent neoantigens (P-Selectin) (CD62p). The elucidation of the cause of platelet hyperactivity will improve the approach to avoid the thromboembolic events occur in thalassaemic patients.

2. Patients and Methods:

The present study includes 59 patients with Thalassemia (30 with Thalassemia major, TM, and 29 with Thalassemia intermediate, TI) who were compared to age and gender matched 20 healthy volunteers as a control group. This study protocol was approved by medical ethics review board of the College of Medicine, Taif University under the number (No.1833-433-1) in accordance with the guidelines of the protection of human subject. Informed written consent for the participant involved in the study was taken.

Patients and control were subjected to:

Complete history: including age, sex, age of disease onset, height, weight, spleenomegaly & hepatomegaly, onset and number of blood units received, spleenectomy, history of complications and treatment with iron chelating agent.

In all patients the results of complete blood pictures, hemoglobin electrophoresis, hemoglobin A2, hemoglobin F and the clinical course were used to classify our patients as TM & TI. The blood samples required for the study were taken immediately before a blood transfusion (at least 4 weeks after the last transfusion) and samples were obtained by standard venous puncture using a light tourniquet, where the first 2mL of blood were discarded to avoid platelet activation. The blood was drawn into EDTA tubes and the test has been strictly done within 4-6 hours.

Annexin V binding to RBCs:

The sample was mixed well and 10ul of EDTA blood were added to 40ul of HEPES buffer, mixed well then a saturating concentrations of specific monoclonal antibodies (Anexin V & Glucophorin) were added then incubated at room temperature in dark for 20 min and then read on the FACSscan flow cytometer (Becton Dickinson) after resuspension by isoton without lysis of RBCs. Light scatter fluorescent data were obtained with a gain setting in the logarithmic mode, and the data were analysed with Lysis II software (Becton Dickinson). The results were expressed as percentage (%) of positive cells for the co-expression of Anexin V and glycophorin on the surface of RBCs (22).

Circulating activated platelets:

Blood immediately was centrifuged at 750g for 5 minutes at 22°C. The supernatant separated as Platelet Rich Plasma (PRP) and diluted 1:10 in HEPES buffer saline. 50-µL aliquot from each diluted PRP sample was added to tubes containing saturating concentrations of specific monoclonal antibodies (the platelets activation markers P-Selectin (CD62p) and the normal platelets markers CD41). Samples were incubated in the dark at room temperature for 20minutes. After immunolabelling, the samples were diluted 1:10 HEPES buffer and analysed on FACSscan flow cytometer (Becton Dickinson). Platelets are distinguished by the characteristic light scatter; results were expressed as percentage (%) of positive cells for the co-expression of CD41 and CD62p on the activated platelets (23).

Statistics:

A statistical analysis was performed using SPSS version 14. Nonparametric Kruskal-Wallis test was used to compare between studied markers in each infected group. ANOVA test was used to compare between variance of each marker levels among two different groups of TM & TI and the control (24).

3. Results

Clinical & laboratory data: this study includes 59 patients with Thalassemia (30 TM& 29TI) in addition to 20 healthy voluntaries who are age &sex matched as a control. Age of patients was 12.8±4.2 years for TM, 13.5 ± 5.3 for TI and 11.9± 3.1 for control. Male to female ratio was 1:1 (15male/15 female) in TM, 1.2:1 (16male/13 female) in TI and 1:1(10male/10 female) in the control group.

Patients with TM have an early onset of the disease at age 0.9±0.2 months (within the first year of life); while those with TI have a late onset of disease at age 5.3 ± 1.7 years ($p< 0.001$). Also, those patients with TM have growth retardation as their height and weight were (128.6±21.1 cm & 27.8 ± 8.5 kg respectively) compared to both patient with TI (148.3 ± 23.3 cm &42.9 ± 14.9 kg) and the control group (149.7±15.9 cm & 44.5± 14.6 kg) ($p < 0.001$).

Spleenomegaly was reported in all cases (100%) of TM patients and in (34%) of patients with TI, while spleenectomy was a done for 7.7% of thalassamic patients, (13/30) in TM and none of cases with TI.

The results shows that all patients with both TM and TI need to receive blood transfusion and most of patients with TM (97%) receive at least one unit of blood or packed RBCs every month, while patients with TI need to receive one unit of blood or packed RBCs every four to six months. We observe a significant early onset of blood transfusion on patients with TM, within the first year of life (0.98 ± 0.27 year) compared to those with TI who need to receive blood later on (5.79 ± 2.13 year), ($p <0.001$). Also, we observed a significant difference regarding the need to receive iron chelating agent as 25/30 (83%) of the patients receive Desferol (iron chelating agent) and none of the patients with TI receive this drug.

The results showed a significant reduction in the Hb level in patients with TM and TI compared to the control group, as the Hb was 6.1 ± 0.5 gm/dl for TM, 7.4± 0.3 for TI and 12.3± 0.6 for healthy controls ($p < 0.001$). But, ferritin level was markedly higher in patients with TM (2626 ± 1094ng/ml) compared to (636 ± 172 ng/ml) for TI and (220 ± 59 ng/ml) for control group, ($p <0.001$).

Expression of platelets activation markers:

The fraction of platelets from healthy individuals expressing CD62p (p selectin) was very small (Fig, 1). The mean value was 10.9 ± 7.9% positive platelets for CD62p. The expression of activation- dependent platelets neoantigen was significantly higher ($p < 0.001$) in Thalassemic patients compared to the control (Fig, 1). The fraction of CD62p positive platelets clustered around a mean 50.1 ± 20.1 %. There was over co expression of CD 41 and CD62p on the activated platelets in patients with spleenectomy (54.3 ± 19.8%) compared to (47.9 ± 19.9%) on patient without Spleenectomy but this difference was not statically significant ($p > 0.05$).

Annexin V binding to RBCs:

Measurement of Annexin V binding to RBCs showed, in control normal individuals a very small fraction of RBC which had bound annexin V (Fig, 2). The values were clustered around a mean of 9.9 ± 3.6%. Meanwhile the RBC from thalassaemic patients showed a significantly ($p < 0.001$) higher binding of annexin V compared to the control (Fig, 2). The mean value for annexin V labelled RBC in thalassaemic patients was 68.4 ± 8.0%. There was no significant over expression on RBCs of patients with spleenectomy (69 ± 8.3%) compared to (66.9 ± 8.6%) on RBCs on patients without spleenectomy ($p > 0.05$).

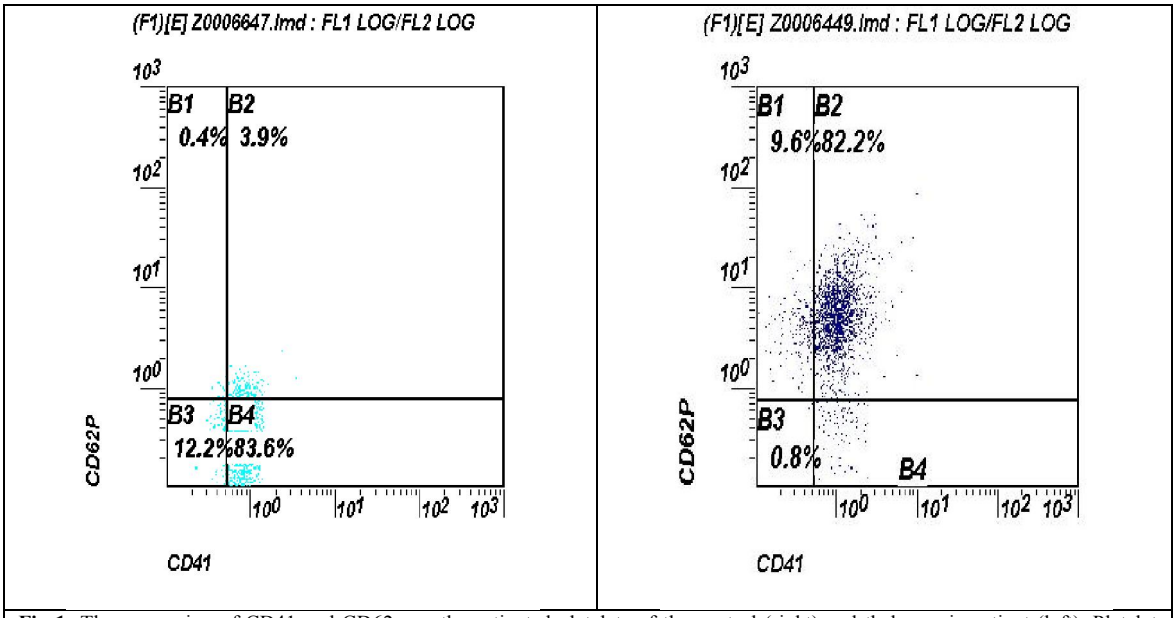

Fig 1: The expression of CD41 and CD62p on the activated platelets of the control (right) and thalassemic patient (left). Platelet Rich Plasma was diluted HEPES buffer saline then added to tubes containing saturating concentrations of specific monoclonal antibodies (the platelets activation markers P-Selectin (CD62p) and the normal platelets markers CD41). Samples were incubated in the dark at room temperature for 20minutes then analysed on FACSscan flow cytometer (Becton Dickinson). Platelets are distinguished by the characteristic light scatter; results were expressed as percentage (%) of positive cells where normal platelets express only CD41 (B4) and activated platelets co-express CD41 and CD62p (B2).

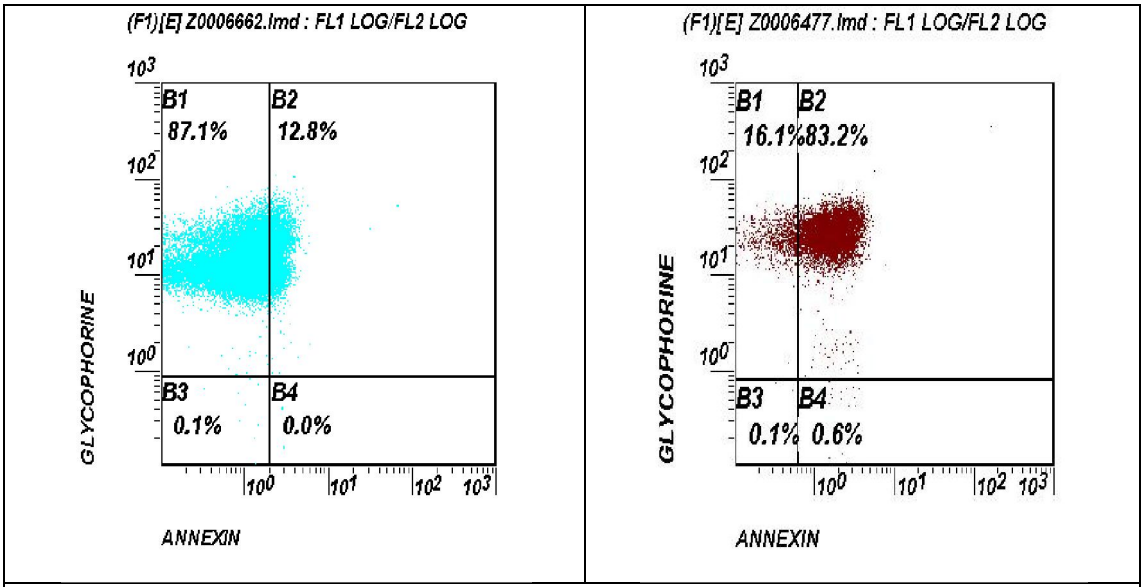

Fig 2: The expression of Annexin and Glycophorine on the RBCs of the control (right) and thalassemic patient (left). 10ul of EDTA blood were added to 40ul of HEPES buffer, mixed well then incubated at room temperature in dark for 20 min with saturating concentrations of specific monoclonal antibodies (Annexin V & Glycophorin) then and the read on the FACSscan flow cytometer (Becton Dickinson). The results were expressed as percentage (%) of positive cells where normal RBCs only express Glycophrin A (B1) and abnormal RBCs co-express Annexin V and Glycophorin A (B2).

4. Discussion

There is an increasing evidence of the presence of hypercoagulable state in patients with chronic hemolytic anemias (25-28) and those patients manifest thrombotic complications including venous thromboembolism (5), in situ pulmonary thrombosis (20) and stroke (29). Furthermore, the risk of thromboembolic complications appears to be higher following splenectomy (18 and 30).

The mechanism of coagulation activation in hemolytic anemia is likely multifactorial. Both SCD and Thalassemia are characterized by red blood cell (RBC) membrane abnormalities, with abnormal exposure of phosphatidylserine (25 and 31). External exposure of phosphatidylserine alters the adhesive properties of RBC (32-33) and appears to be involved in the hemostatic changes observed in hemolytic anemias (34 and 35).

In our study we found a significantly increased fraction of Annexin V labelled RBC in TM and TI patients compared to RBC from healthy individuals (fig, 2). These findings consistent with an abnormal membrane phospholipids asymmetry and exposure of phosphatidylserine (PS) in Thalassemic patients, which may increase thrombin generation and may enhance the hypercoagulable state in Thalassemic patients.

These findings are in line with previously reported data stated that the hypercoagulable state in TM &TI may result from procoagulant effect of abnormal RBCs of thalassemic patients, by amplifying thrombin generation and initiating platelet activation (5, 8 and 32-33). Furthermore it was found that annexin V antibody, a protein with high affinity and specificity for anionic phospholipids, could block the procoagulant effect of isolated thalassemic RBCs and that these abnormalities have been reduced to normal range after the patients have received a blood transfusion (13).

Similar results were reported by Capellini who found that Thalassemic RBCs expressing these negatively charged phospholipids may act as a source of phospholipids, enhancing and eventually increase thrombin generation in a prothrombinase assay where normal RBCs had no effect (12). Previously several reports stated the link between the persistent hypercoagulable state in thalassemic patients and the abnormal exposure of some phospholipids, especially Annexien V, on the surface of these RBCs (11, 15, 25 and 34-35).

In this study despite of the fact that none of TM or TI patients had an overt thrombotic event, a chronic hypercoagulable state was evident by the increased fraction of circulating platelets expressing activation dependent neoantigen, p-selectin (CD62p) (Fig, 1). In consistent with the current results, overt thromboembolic events (TEE) was demonstrated to occur only rarely in thalassaemia patients; however, laboratory tests have provided evidences for chronic hypercoagulable state to be exists early in thalassaemic childhood (7-9, 12 and 36).

On the other side higher, incidence of thrombotic events were observed in 4% of 683 patients with TM and in 9.6% of 52 patients with TI presented with TEE (37). The same group showed six years later lower incidence as only 1.1% of 720 patients from seven Italian centers with TM, had thrombosis. (38). In a large clinical study among 8860 thalassemia patients (6670 TM and 2190 TI), the cumulative prevalence of thromboemolic events was estimated at 1.65%, with thrombosis occurring 4.38 times more frequently in TI than TM (39).

In the current work the lower chance for developing overt thrombosis may be due to the young age as all patients were below 15 years old and spleenoectomy were done only to 7.7% of thalassemic patients. Indeed it was reported that the main risk factors for developing thrombosis were described as: age beyond 20 years, spleenectomy, family history of TEE and previous TEE (39).

In this work we found a strong association between the expression of the platelet activation markers, CD62p, and the Annexin V binding to thalassemic RBCs and the two cellular anomalies are highly correlated and the two cellular anomalies are linked together. In consistent with our results. Rulf et al., 1997 founded a strong correlation between the cellular anomalies and assumed that the abnormal RBCs might enhance thrombin generation in vivo and thus trigger platelet activation in thalassemia (8).

In parallel with the result of this study, it was hypothesized that a causal relationship and a significant association present between RBCs membrane anomaly and the degree of in vivo platelet activation (40). More recently, Monnucci et al., 2010 stated that the RBCs from thalassemic patients are an important player for the activation of platelets in patients with TM (41).

In conclusion, in our study we found a significant higher circulating number of activated platelets expressing CD62p (P-selectin) and increased fraction of Annexin V labelled RBCs in both TM and TI patients and a strong association between the expression of these two cellular markers. Although there are diverse factors contributing to the hypercoagulable state observed in patients with Thalassemia, the strong association between the expression of these two cellular markers and the tendency of the hypercoagulable state observed in patients with Thalassemia may help to predict and to avoid the development of this state in those patients.

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