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Laboratory Investigation of Platelet Function in Patients with Thalassaemia

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Key Words
Light transmission aggregometry · Platelet function analyser PFA-100 · Thalassaemia major · Platelet function

Abstract
The aim of this study was to investigate platelet function in patients with thalassaemia and to detect any relation to chelation treatment (deferasirox or deferiprone/deferiprone plus desferioxamine). Thirty-three transfusion-dependent patients with thalassaemia were included. The investigation consisted of aggregation testing of platelet-rich plasma by light transmission aggregometry (LTA) with the use of 5 agonists as well as the global test of haemostasis by means of the PFA-100 platelet function analyser. In 66.67% of the patients, there was reduced LTA to at least one agonist and in 18.18% there was reduced LTA to two or more agonists. The PFA-100 test was prolonged in 60.6% of the cases. An abnormal LTA and a prolonged PFA-100 time were recorded in 33.3% of the patients and 27.4% had a normal aggregation and PFA-100 test. No correlation between chelation regimen and either LTA or PFA-100 test was found. The abnormal LTA can be explained either by the release of ADP from the haemolysed red blood cells, which leads to defective platelet aggregation, or by the presence of two platelet populations. An in vitro effect without an in vivo impact could be an alternative explanation. In patients with thalassaemia, the reduced LTA and the prolonged PFA-100 closure time could be an in vitro effect and has a close correlation to the bleeding phenotype of each patient.

Introduction

Platelet function disorders constitute a rare cause of symptomatic bleeding. Muco-cutaneous bleeding of varying severity is the result of several genetic defects of platelet function [1]. Haemostatic disorders are reported in patients with thalassaemia [2, 3] as easy bruising or epistaxis, while it is generally accepted that thalassaemia is a hypercoagulable state [4]. Patients with β-thalassaemia are reported to have activated platelets and enhanced platelet aggregation [5] based on the increased expression of the in vivo platelet activation of P-selectin and CD63. Several studies on platelet function investigation in β-thalassaemia major report an in vitro defect of platelet function, when tested by light transmission aggregometry (LTA) [6–10]. We found only one report in the literature concerning the evaluation by PFA-100 platelet function analyser in 14 patients with thalassaemia [11]. To our knowledge, there

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is no other report examining the combined use of both the LTA and PFA-100 tests in such patients.

The aim of this study was to evaluate the in vitro platelet function of patients with thalassaemia and to detect any relation to the chelation treatment.

Patients and Methods

A total of 33 patients with thalassaemia major (19 males and 14 females), with a mean age of 20.82 ± 9.80 years (range 7–45 years) were included. All patients were transfused in order to maintain a pre-transfusion haemoglobin value of >9 g/dl (9.85 ± 0.83). They were on chelation treatment with either deferasirox (n = 17) or deferiprone/deferiprone plus desferioxamine (n = 16). Seven patients had had a liver biopsy and 5 had undergone splenectomy, though no haemostatic cover was used and no bleeding was reported. Only one had experienced bleeding of a peptic ulcer. Four patients with chronic viral hepatitis were included. Seven had slightly elevated transaminases due to medications. Renal function was normal in all patients. A complete medical history was taken, and the following analyses were performed: complete blood count, peripheral blood smear, prothrombin time (PT), activated partial thromboplastin time (aPTT) and hepatic and renal function markers. Patients receiving prophylactic aspirin were not included in the study as such patients demonstrate an iatrogenic cyclooxygenase (COX)-like defect due to this medication.

The control group consisted of 33 healthy blood donors of a similar age and sex distribution to the patient group.

The platelet examination consisted of the platelet count number and the blood smear report for white cell inclusions and platelet size. Diagnostic laboratory investigation of the platelet function consisted of the gold standard method of LTA (Bio/Data Corporation, Horsham, Pa., USA) in platelet-rich plasma (PRP) by the use of 5 agonists (arachidonic acid, adenosine-ADP, adrenaline, collagen and ristocetin) and the performance of the global test of haemostasis by the PFA-100 platelet function analyser (Dade Behring Inc., Deerfield, Ill., USA). The principle of PFA-100 is platelet plug formation in vitro under shear stress, with either collagen and epinephrine or collagen and ADP as agonists.

For the aggregation test, 20 ml of blood in 3.8% tri-sodium citrate was collected in polystyrene tubes (before transfusion). It was centrifuged at 200 *g* for 12 min at room temperature to obtain PRP and then at 1,000 *g* for 10 min to obtain platelet-poor plasma. After centrifugation, the supernatant PRP was carefully transferred to a 10-ml polypropylene tube, avoiding contamination with either red cells or the buffy coat, and it was then kept undisturbed for 30 min at room temperature to recover from refractoriness before testing. Platelet and red cell counts were measured on PRP samples and when platelet count was >600 × 10^9/l, a further dilution with PPP from the patient was made, in order to adjust the platelet count to a 'normal' level as the aggregation response could be modified (diminished or enhanced) in high PRP counts.

According to the protocol, the traditional baseline panel of agonists for LTA was used: ADP 2 μM (3 and 10 μM if full aggregation was not achieved), adrenaline 2 μM (3 and 10 μM if full aggregation was not achieved), collagen 1 μg/ml, ristocetin 1.25 mg/ml and arachidonic acid 1 mM. All the concentrations listed above are the final concentrations added to the PRP. Each patient's LTA test was compared to a normal control-sample LTA test with the same batch of agonists (normal values: >50–60% aggregation compared to control samples).

Platelet aggregation testing was completed within 4 h of preparation.

In addition to PFA-100, the INNOVANCE PFA P2Y (Dade Behring, Inc.) was also tested. It consists of ADP, prostaglandin E1 (PGE1) and ionic calcium.

For both the PFA-100 and PFA P2Y, the measurements were performed in whole blood, collected in 3.8% tri-sodium citrate.

It is generally accepted that the LTA test is time-consuming and labor-intensive, while the PFA-100 test is considered to be a rapid screening test that measures both platelet adhesion and aggregation.

Statistical Evaluation

The data statistical analysis was carried out by Statistica® (StatSoft, version 8) and Microsoft Excel. All results are expressed as mean ± standard deviation (SD). Statistical significance was estimated by the Student t test or the non-parametric Mann Whitney U test for the difference between groups depending on the normal or non-normal distribution of the values. The Spearman rank (r) correlation was used for the estimation of relationships. The χ^2 test was used for the analysis according to the iron chelation therapy. Differences were considered statistically significant when p values were ≤0.05.

Table 1. LTA differences between patients with thalassaemia and the control group

LTA	Patients	Controls	p value
ADP	62.6±17.9	73.2±5.9	0.00
Adrenaline	42.1±24.3	72.3±5.9	0.00
Collagen	69.4±13.9	77.2±8.1	0.01
Arachidonic acid	66.3±8.3	68.9±7.2	0.14
Ristocetin	75.4±11.5	78.6±6.9	0.23

Results

A mild prolongation in PT and aPTT was observed in 2 patients. Platelet counts in PRP were <600 × 10^9/l in the patient group. The peripheral blood smears that were examined presented platelet populations of varying sizes and no evidence of white cell inclusions.

A statistically significant difference in LTA (3/5 agonists) was found between the patients and the controls (table 1). In the patient group, there was reduced LTA to adrenaline in 57.57% (19/33), to ADP in 18.18% (6/33) and to collagen in 9.09% (3/33). In addition, 18.18% (6/33) displayed reduced LTA to two or more of the used

agonists. The PFA-100 test was prolonged (>165 s) in 60.6% of the cases. Platelet plug formation was prolonged under shear stress with collagen and epinephrine. In total, 33.3% of the patients had both a defect in platelet aggregation and a prolonged PFA-100 test, while 27.4% had an abnormal LTA and a normal PFA-100 time, 12.1% had normal aggregation and an abnormal PFA-100 test and 27.4% had both normal aggregation and PFA-100 time. PFA P2Y closure time was found to be within 'normal' limits in all patients except in 5 cases that exceeded the cut-off (>106 s). In these cases, there was a normal aggregation test with ADP and 4 of them had normal PFA-100 closure times (collagen/ADP). It is interesting that among the 28 patients with 'normal' PFA P2Y, there were 7 with abnormal aggregation to ADP.

Twelve patients had undergone surgery, either splenectomy or liver biopsy, without surgical or postoperative bleeding complications. Two of them had normal platelet aggregation and PFA-100. Nine of them had both abnormal aggregation and PFA-100 and only 1 had abnormal aggregation only to adrenaline. Just one patient had experienced a bleeding episode due to a peptic ulcer. This patient presented normal PFA-100 and normal aggregation to all agents except adrenaline.

No statistical correlation was found between bilirubin or LDH measurements and LTA. An interesting negative correlation ($r = -0.39$, $p = 0.02$) was observed between PFA-100 (collagen/ADP closure time) and total bilirubin; it was more intense ($r = -0.45$, $p = 0.01$) than the correlation between PFA-100 and unconjugated bilirubin. No statistically significant difference was found concerning PFA-100 or LTA measurements between the patients on different chelation regimens (tables 2, 3). No correlation was found between LTA and PFA-100 or PFA-P2Y measurements. No statistically significant difference was found concerning age, haemoglobin, hematocrit, PT, aPTT, PRP and platelet counts between the patients on different chelation regimens.

Table 2. No statistical significant difference between PFA-100 and iron chelation regimen

	DFX	L1 or L1 + DFO	Total	p value
Abnormal PFA-100	9	11	20 (60.6%)	
Normal PFA-100	8	5	13 (39.4%)	
Total	17	16	33	0.35

DFO = Desferioxamine; DFX = deferasirox; L1 = deferiprone.

Table 3. No statistical significant difference between LTA and iron chelation regimen was detected

LTA	DFX	L1 or L1 + DFO	p value
ADP	59.5±22.2	61.0±11.6	0.60
Adrenaline	43.4±26.1	40.8±22.9	0.80
Collagen	64.5±14.7	74.6±11.1	0.06
Arachidonic acid	68.4±8.0	64.0±8.2	0.08
Ristocetin	75.8±13.2	75.1±9.7	0.54

DFO = Desferioxamine; DFX = deferasirox; L1 = deferiprone.

Discussion

Accurate measurement of platelet function and identification of a congenital platelet disorder constitute a complex procedure [12, 13] which includes multiple assays and often repeated testing. The major problem encountered with this testing is the difficulty of simulating haemostasis in vitro as platelets are sensitive to manipulations.

Moreover, there is a lack of consensus about the diagnostic tests concerning the platelet function disorders [13]. Each haemotology centre uses a diagnostic algorithm ranging from simple screening tests to more sophisticated assays; this needs to be evaluated from time to time. An ideal test for the platelet function does not exist. We performed classic platelet aggregometry with the LTA test and a panel of 5 agonists, a time-consuming method which provides a great deal of information. We also used the PFA P2Y (in the literature for the first time in such patients) and the PFA-100, a simple, rapid screening tool and a potential alternative to the in vivo bleeding time test [14].

The results of this study are indicative of an in vitro defect of platelet function and aggregation in 22 of 33 patients with β-thalassaemia major. The aetiology of platelet defect appears to be unclear since there are few clinical trials that study platelet aggregability. In 19 cases, the aggregation abnormality was restricted, particularly to adrenaline in all three concentrations. It is possible that ADP released from haemolysed red blood cells has an impact on platelet aggregation, although we found no statistically significant correlation of the basic markers of haemolysis (bilirubin/LDH) and LTA. In view of the low incidence of bleeding manifestations in the patients with abnormal platelet aggregation, the al-

ternative explanation of an in vitro abnormality is more probable. Due to the reduced size and volume of red blood cells in β-thalassaemia major, it is difficult to produce PRP to be able to satisfactorily monitor the platelet aggregation. Prolonged centrifugation is needed and this leads to PRP platelet counts that are lower than those in whole blood. It is also possible that prolonged centrifugation selectively separates the young and active platelets, which are larger, from the older ones. The remaining older platelets are less active and show a somewhat reduced aggregability. Another factor that may influence the platelet aggregation is the platelet refractoriness induced by the variety of procedures undertaken, e.g. centrifugation.

In conclusion, the high incidence of abnormal platelet aggregation in patients with thalassaemia major may be due to artifacts induced by the in vitro technical manipulations. The low incidence of bleeding episodes supports this suggestion, although the existence of one of the already rare platelet function defects should give rise to bleeding of varying intensity [1]. Another issue is that the contents of the platelet-dense granules were not investigated by measuring total ATP and ADP with a luciferin/luciferase technique [15]. Thus, platelet function defect in these patients must be neither overestimated nor ignored, but, on the contrary, carefully co-examined with the clinical phenotype, bearing in mind the problems that attenuate the interpretation of the laboratory tests.

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