ORIGINAL ARTICLE

CD40 ligand and P-selectin in heterozygous Beta-thalassemia

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Abstract

Objective: To investigate platelet functions and measure soluble CD40 ligand, soluble P-selectin, beta-thromboglobulin and platelet factor 4 levels in the blood of heterozygous beta thalassemia patients.

Methods: The cross-sectional case-control study was conducted at Bezmialem Vakif University, Istanbul, Turkey, between September 2013 and April 2014, and comprised heterozygous beta thalassemia patients who were compared with 41 gender-, age- and body mass index-matched controls for platelet function markers. The two groups were also compared for co-morbidities, smoking, and regular medications.

Results: Of the 78(78.78) subjects, 50(64%) were women and 28(36%) men with an overall mean age of 39.4±12.7 years (range: 18-79 years). The mean body mass index was 26.3±4.2. The heterozygous beta thalassemia group included 37(47%) subjects [24(65%) females; 13(35%) males] while the control group had 41(53%) [26(63%) females; 15(37%) males]. Soluble CD40 ligand and soluble P-selectin were lower in the heterozygous beta thalassemia group (p=0.009; p=0.010). Beta-thromboglobulin and platelet factor 4 levels were comparable between the groups (p=0.497; p=0.507.)

Conclusion: Some platelet functions may be reduced in heterozygous beta thalassemia patients, which may be related to their lower incidence of cerebral and cardiac ischaemic events.

Keywords: P-Selectin, CD40 ligand, Beta thromboglobulin, Platelet factor 4, Heterozygous beta thalassemia. (JPMA 66: 699; 2016)

Introduction

Heterozygous beta thalassemia (HBT) is a benign blood disorder caused by a hereditary reduction in beta globin synthesis, which often leads to mild anaemia and is characterised by hypochromic microcytic erythrocyte indexes.[1] HBT is prevalent in many regions of the world, including Mediterranean countries, the north coast of Africa, the Middle East, Central Asia, Southeast Asia, the Far East and South America.[1] A lower incidence of cerebral and ischemic events has been reported in individuals with HBT compared to the control groups, which was supported by a meta-analysis.[2] However, it is not known how this happens. haemorrhagic events like epistaxis, easily bruising and menometrorrhagia have been reported in some HBT patients, and in vitro platelet aggregation tests reveal that platelets from these patients have a reduced aggregation response.[3] These studies suggest that the platelets of individuals with HBT might be functionally impaired and less likely to mediate cerebral and cardiac ischaemic events.

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The current study was planned to assess the levels in the blood of soluble P-selectin (sPS), soluble CD40 ligand (sCD40L), beta thromboglobulin (BT) and platelet factor 4 (PF4) in patients with HBT.

Patients and Methods

The prospective cross-sectional case-control study was conducted at Bezmialem Vakif University, Istanbul, Turkey, between September 2013 and April 2014, and comprised two groups of age-, gender- and body mass index (BMI)-matched adults with or without HBT. The controls were patients who presented to the same institution between April 1 and 20, 2014. All the participants gave informed consent and the study was approved by the institutional review board.

Those with conditions that are either known to affect or likely to affect platelet functions were excluded.[4,5] Exclusion criteria comprised acute and chronic infections, nonsteroidal anti-inflammatory drugs (NSAID) or aspirine use less than 10 days prior to recruitment, anti-aggregant and anti-coagulant use, oral contraceptive use, lipid-lowering agent use, uncontrolled hypertension, ischaemic heart disease, heart failure, peripheral artery disease, cerebrovascular disease, venous thrombosis, solid tumours, haematologic malignancy, pregnancy, lactation, rheumatic collagen tissue disorders, Familial

Mediterranean fever, type 1 diabetes, any of vitamin B12, folic acid, or iron deficiency-associated anaemia, > stage 2 chronic kidney disease, chronic hepatitis, portal hypertension, thrombocytopenia, thrombocytosis, hypothyroidism, hyperthyroidism, adrenal dysfunction, pituitary dysfunction, colitis ulcerosa, Crohn's disease, Behçet's disease, sarcoidosis, chronic obstructive pulmonary disorder (COPD), asthma, and any haemolytic anaemia other than HBT.

The subjects were considered to have HBT if their mean corpuscular volume (MCV) was lower than 80 fL, mean corpuscular haemoglobin (MCH) was lower than 27 µg and HbA2 $\geq$ 3.5%. The control group comprised non-anaemic individuals (haemoglobin $\geq$13 g/dL for males and haemoglobin $\geq$12 g/dL for females) with normal MCV and MCH.

Medical, family and drug history was taken and a systemic physical examination was carried out for each person enrolled. Co-morbidities were determined by blood and urine analysis and by imaging techniques when necessary.

Blood samples were obtained early in the morning after overnight fasting. Routine blood analyses were performed the same day. A complete blood count (CBC) analysis was performed within one hour using a Sysmex XT 1800i apparatus (ROCHE-2011, Kobe, Japan). The biochemical assays were performed on a COBAS 8000 apparatus (ROCHE-2007, Tokyo, Japan) using kits specific for the COBAS-C system. The levels of thyroid hormones were examined using a Siemens Advia Centaur apparatus (S?emens-2006, Dublin, Ireland) by a chemiluminescent method using Advia Centaur kits (Advia-2013-Tarrytown, USA). Haemoglobin electrophoresis was carried out on a Shimadzu 20-A (Shimadzu-2013, Kyoto, Japan) using the high prominence liquid chromatography (HPLC) method. Citrate, theophylline, adenosine, dipyridamole (CTAD) tubes containing special anticoagulant mixture were used to obtain platelet depleted plasma. Venous blood was taken, and first portion of 2ml was discharged and subsequent 4.5ml of venous blood was transferred to the CTAD tube. The tube, after being instantly placed in the ice/water bath for at least 15 minutes, was later centrifuged at 2.500g for 20 minutes at 2-8°C within an hour. As soon as centrifugation was completed one-third volume of the plasma supernatant in the middle region of the liquid portion was transferred to another CTDA tube, which was again centrifuged at 2500g for 20 minutes at 2-8°C. Then one-third of the supernatant that was in the middle region of the CTDA tube was transferred to plastic polypropilene tube. Plasma samples were stored at -20°C for approximately one month until the time of analysis. The blood samples collected in plastic tubes were left to stand at room temperature for 20 minutes to allow clot retraction, and centrifuged for 15 minutes at 2500g for obtaining serum. Then the serums were transferred into polypropylene plastic tubes. The serum samples were frozen at -80ºC and stored for approximately one month until the time of analysis. The levels of sCD40L and sPS were analysed in serum samples, and the levels of PF4 and BT were analysed in plasma samples. Kits used to measure platelet function markers included: Human soluble CD40L Platinum enzyme-linked immunosorbent assay (ELISA) (Bender Med Systems, cat no: BMS265, lot no: 87059020, Vienna, Austria), Human sPS Platinum ELISA (Bender Med Systems; cat no: BMS219/4; lot no: 93950016 Vienna, Austria), HBT Assera Chrom Stago ELISA (cat no: 00950, lot no: 111398, Seine, France), and Human PF4 Assera Chrom Stago ELISA (cat no: 00951, lot no: 111112, Seine, France). The results were visualised using a Multiskan FC® Microplate Reader (Thermo Scientific, USA) at 450 nm. The intra-assay coefficient of variation (CV) for BT was 5.3% and the inter-assay CV was 5.5%. The intra-assay CV for PF4 was 3.6%, and the inter-assay CV was 6.3%. The intra-assay CV for sPS was 7.8%, and the inter-assay CV was 5.4%. The intra-assay CV for sCD40 L was 5.5%, and the inter-assay CV was 7.0%.

Numerical variables were presented as means with standard deviations, and nominal variables as frequencies and percentages. The nominal independent variables were compared between the groups using the Chi-square test. A one-sample Kolmogorov-Smirnov test was performed to determine if the continuous (numerical) independent variables were normally distributed. Normally distributed independent continuous variables were compared using Student's t-test, whereas non-normally distributed ones were compared between the groups using the Mann-Whitney U test. A two-tailed p value of <0.05 was considered statistically significant.

Results

Of the 99 subjects screened, 78(78.78) were included. Of them, 50(64%) were females and 28(36%) males with an overall mean age of 39.4±12.7 years (range: 18-79 years). The mean BMI was 26.3±4.2).

The HBT group had 37(47%) subjects [24(65%) females; 13(35%) males] and the control group had 41(53%) subjects ([6(63%) females; 15(37%) males].

The serum levels of sCD40L and sPS were significantly

Table-1: Comparison of demographics and blood analysis results between the HBTM and control groups.

Variable	HBT: 37 subjects Mean±SD	Controls: 41 subjects Mean±SD	Normal range	P value	Type of distribution
Age, years	39.90±13.94	39.02±11.68		0.760	ND
BMI, kg/m^2	26.92 ±4.76	25.83±3.57	19-25	0.248	ND
RBC x 10^{12} /L	6.11±0.88	4.79±0.60	4.0-6.2	<0.001	ND
Hb, g/L	117.5±13.6	135.1±16.8	130-175	<0.001	ND
Htc, %	36.67±3.90	40.50±4.04	40-52	<0.001	ND
MCH, pg/cell	19.81±2.51	27.49±5.09	27-34	<0.001	NND
MCV, fL	61.34±3.79	84.78±6.53	80-100	<0.001	ND
RDW, %	16.90±1.47	13.28±1.28	11.5-14.5	<0.001	ND
WBC, x10^9/L	7.23±2.03	6.62±1.99	3.8-10.0	0.184	ND
Platelets, x10^9/L	288.15±73.26	264.49±73.02	150.0-400.0	0.152	ND
MPV, fL	10.32±0.91	10.07±0.63	7-11.5	0.156	ND
PDW, %	14.17±2.47	12.00±1.47	9-17	<0.001	ND
HbA2, %	5.15±1.32		2-3.5		ND
HbA, %	93.81±6.71		95.8-98.0		ND
HbF, %	0.72±1.51		0-0.9		ND
ESR, mm/h	12.73±12.27	15.87±11.69	<20	0.142	ND
Sensitive CRP, mg/dL	0.28±0.17	0.31± 0.17	0- 0.48	0.344	ND
Creatinine, mg/dL	0.68±0.16	0.67±0.18	<1.3	0.868	ND
Glucose, mg/dL	96.40±11.89	96.76±9.73	70-100	0.402	ND
HbA1C, %	5.25± 0.66	5.28±0.49	4.5-5.7	0.385	ND
LDL-cholesterol, mg/dL	109.65±35.14	111.20±30.12	<100	0.838	ND
HDL-cholesterol, mg/dL	50.97±20.08	49.03±16.63	>55	0.942	ND
Triglyceride, mg/dL	116.81 ±62.83	135.40±83.19	<150	0.435	ND
AST, IU/L	21.86±4.74	22.63±9.46	10-30	0.485	ND
ALT, IU/L	21.88±16.20	23.68±9.46	10-40	0.760	NND
Transferrin saturation rate,%	29.26±11.39	23.27±10.63	20-40	0.020	ND
TSH, mIU/L	1.81±0.93	1.49±0.86	0.63-4.82	0.124	ND
FT3, pmol/L	4.64±0.60	4.80±0.71	3.5-6.5	0.328	ND
FT4, pmol/L	14.37±1.53	14.86±1.93	10-18.7	0.229	ND
Albumin, g/L	46.00±2.50	45.30±2.60	35-55	0.222	ND
Total protein, g/L	72.80±3.60	72.80±3.90	60-80	0.963	ND
sCD40 ligand, IU/mL	35.24±5.15	50.45±26.89	Unknown	0.009	NND
sP-Selectin, IU/mL	250.10±80.24	291.95±57.18	Unknown	0.010	ND
Beta thromboglobulin, IU/mL	355.06±40.25	348.68±41.46	Unknown	0.497	NND
Platelet factor 4, IU/mL	159.63±22.68	155.90±25.79	Unknown	0.507	ND

HBT: Heterozygous Beta Thalassemia. BMI: Body Mass Index. RBC: Red Blood Cell. Hb: Haemoglobin. Htc: Hematocrit. MCH: Mean Corpuscular Hemoglobin. MCV: Mean Corpuscular Volume. RDW: Red Cell Distribution Width. WBC: White Blood Cell. MPV: Mean Platelet Volume. PDW: Platelet Distribution Width. HbA2: Hemoglobin Alpha2. HbA: Hemoglobin A. HbF: Hemoglobin F. ESR: Erythrocyte Sedimentation Rate. CRP: C-reactive Protein. HbAlc: Hemoglobin AlC. LDL: Low Density Lipoprotein. HDL: High Density Lipoprotein. AST: Aspartate Amino Transferase. ALT: Alanine Amino Transferase. FT3: Free Triiodothyronine. FT4: Free Thyroxine. SCD40: Soluble CD40. SP-Selectin: Soluble Platelet Selectin.
ND: Normal distribution - NND: Nonnormal distribution.

Table-2: Gender, smoking status, and co-morbidities of subjects included in the study.

Variable	BTM 37 subjects Number	Controls 41 subjects Number	P value
Gender, Female/male	24/13	26/15	0.918
Diabetes mellitus type 2	3	3	0.768
Essential hypertension	4	5	0.870
Smokers	10	11	0.813
Ex-smokers	2	3	0.906

BTM: Beta thalassemia major.

lower in the HBT group. The two groups were similar in BT and PF4 plasma levels. Besides, the platelet distribution width (PDW), red blood cell (RBC), and red cell distribution width (RDW) values were higher in the HBT group compared to the controls. The haemoglobin (Hb), haematocrit (Htc), MCV and MCH values were lower in the HBT group than in the control group. The MPV, platelet count and white blood cell (WBC) values were similar between the two groups. The frequency of patients either on anti-diabetic medicines or with type 2 diabetes

Table-3: Drugs used regularly.

Variable	HBT 37 subjects Number	Controls 41 subjects Number	P value
Sulfonylurea	1	1	0.520
Metformin	3	3	0.768
ACE inhibitor	1	2	0.617
Angiotensin receptor blocker	2	1	0.496
Amlodipine	1	1	0.520
Beta-blocker	1	2	0.617
Diuretic	1	1	0.520

ACE: Angiotensin-converting-enzyme.

mellitus, haemoglobin A1c (HbA1c) and fasting glucose values were also similar between the two groups, while the lipid profiles and sensitive C-reactive protein (CRP) values were also comparable (Table 1).

The two groups were comparable in terms of the proportion of smokers and ex-smokers, and they did not differ significantly in the frequency of either patients with hypertension or patients using antihypertensive drugs (Tables-2, 3).

Discussion

In 1963, Hilgartner et al. observed signs of skin and mucosal haemorrhage in some patients with BTM despite the fact that liver parenchymal damage did not occur and clotting factors (factor I, factor II, factor V, factor VII, and factor IX) and prothrombin time was normal.[3] In 1977, Gruppo et al. observed that platelets responded to adenosine diphosphate (ADP), epinephrine and collagen with reduced aggregation in patients with sickle cell anaemia during vaso-occlusive crisis and that PF3 availability was also high. This was interpreted as: "Platelets may give in vitro reduced aggregation response since they were previously activated in vivo." However, they detected that platelets responded to in vitro ADP, collagen and epinephrine with decreased aggregation in the period during which patients with sickle cell anaemia were completely asymptomatic as well, and they could not explain it.[6] In a 1978 investigation, Eldor et al., who took those studies into consideration, observed a reduced platelet aggregation response to collagen, ADP, adrenaline, ristocetin in patients with BTM and HBT compared to controls. The patients in this study had signs of skin and mucosal haemorrhage such as bruising easily, epistaxis and menometrorrhagia.[3] In this study, PF3 availability, bleeding time and clot retraction was normal in patients with BTM.[3] After the re-suspension of the platelets of patients with BTM in normal plasma, reduced aggregation response did not resolve. Eldor et al. interpreted these results of their study as platelet dysfunction due to platelet membrane disorder.[3]

Our patients with HBT had lower levels of sCD40L and sPS than controls. The CD40/CD40L receptor/ligand pair belongs to the tumour necrosis factor superfamily and has dual prothrombotic and pro-inflammatory roles.[7] The CD40/CD40L molecules are expressed by lymphocytes, components of the vascular wall and activated platelets.[7] However, platelets are the primary source of the sCD40L found in the serum.[8] sCD40L, which may trigger the key mechanisms involved in atherothrombosis, has a prognostic role not only in individuals with advanced atherosclerosis but also in the general population.[7] Increased sCD40L levels have been demonstrated not only in patients with clinically evident atherosclerotic disease but also in patients who have not experienced the complications of atherosclerosis but carry the risk factors for atherosclerosis, such as either type 2 diabetes mellitus, hypercholesterolemia, hypertension or obesity.[4]

P-Selectin is an adhesion molecule found in the Weibel-Palade bodies of endothelial cells and the alpha granules of platelets.[4] Although P-Selectin is present on the extracellular surfaces of both activated platelets and activated endothelium, most of the sPS detected in the blood is derived from platelets.[4] Activated platelets express P-Selectin on their membranes, and from there, it dissociates and is disseminated by the blood.[4] Because P-Selectin is involved in the interaction between the blood cells and the endothelium, and it has a role in the adhesion of platelets to the endothelium, it may be used as an indicator of adverse cardiovascular events.[9] In animal models, while active atherosclerotic plaques express increased levels P-Selectin, fibrotic, inactive plaques do not express P-Selectin.[9] Animals lacking P-Selectin have a reduced tendency to develop atherosclerotic plaques compared to wild-type controls.[9] The levels of sPS increase under certain conditions, including hypertension, coronary artery disease and atrial fibrillation and the levels of sPS were found to be associated with the clinical outcome.[9]

Lower levels of sPS and sCD40L may be related to the lower incidences of cerebrovascular and cardiovascular ischaemic events in HBT patients.[2] In our study, although the controls and patients with BTM had the same cardiovascular risk factors (age, gender, BMI, lipid profile, sensitive CRP, proportion of diabetics, hypertensive subjects and history of smoking), patients with HBT had lower serum levels of sPS and sCD40L.

This might be originated from reduced platelet functions in HBT.

BT and PF4 are platelet-specific chemokines and are stored in the platelet alpha granules and released into the blood once the platelets are activated.[4] Based on initial observations, these two molecules seemed to represent promising markers of platelet activation; however, very few studies have demonstrated a clinical benefit associated with these markers in acute coronary syndrome and these studies have had inconclusive results.[4] We found comparable levels of BT and PF4 in the HBT and control patients.

Reduced platelet functions in HBT patients may not be reflected uniformly in all classes of platelet function markers. In patients with BTM and HBT, reduced platelet aggregation response to epinephrine, collagen, ADP and ristocetin could be explained by a defect or an impairment in glycoprotein structure of receptors on platelet membranes.[3,10,11] Impairment of platelet function might be congenital or acquired in beta thalassemia. Acquired platelet function defect may result from oxidative stress. Increased oxidative stress desialylates platelet glycoproteins, disrupting its function and structure.[12] In patients with BTM, serum glycoprotein levels are lower and desialylated serum glycoprotein quantities are higher compared with the control group.[13] Oxidative stress increased in both BTM and HBT.[13,14] which may disrupt the structure of the membrane glycoproteins on platelets and cause reduced platelet functions. While sCD40L and sPS are platelet function markers from membrane glycoproteins group, BT and PF4 are alpha granule chemokines.[4]

Compared with general population, while the frequency of venous and arterial thrombotic event increased in BTM, the frequency of arterial thrombotic events decreased in HBT, despite both had the same platelet dysfunction.[2,3,15] Moreover, the platelet dysfunction in BTM was more severe than HBT.[16] Despite the reduced platelet function in BTM, severe haemolysis increases the tendency to coagulation. Damaged erythrocyte membranes may lead to increased thrombin generation, increased platelet activation, and endothelial inflammation, and reduction of protein C and protein S may occur because of damaged liver.[2,15,17] The factors preventing coagulation (reduced platelet functions) in BTM might be overcome by the factors that increase the tendency to coagulation (heavy haemolysis), resulting in increased thrombotic events. Haemolysis associated with ineffective erythropoiesis in HBT is much milder.[18] Coagulation activation was reported in HBT as well.[19] As the factor causing coagulation activation in HBT is a quite mild haemolysis, it may be overcome by congenital or acquired platelet dysfunctions, and thrombotic events could occur less frequently compared to general population.[2,3,18]

It has been proposed that HBT patients may experience less frequent cerebrovascular and cardiac ischemic events because of lower cholesterol levels, less frequent and more moderate blood pressure increases and lower blood viscosity.[2] We believe that the lower incidence of ischaemic events in HBT may be multifactorial.

The relatively small population was a limitation of our study.

To date, very limited research has been conducted on the platelet functions in HBT. New studies to examine different platelet function markers by different methods taking into consideration the results of this study are needed.

Conclusion

The lower levels of sCD40L and sPS observed in HBT patients may indicate impaired platelet function. This finding may be related to the lower incidences of cerebral and cardiac ischemic events in HBT patients compared to the general population.

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Conflict of Interest: All author state that they have no conflict of interest.

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