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Activation of endothelial cell, coagulation and fibrinolysis in children with beta- and alpha-thalassemia

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Introduction : *Thromboembolic complications due to hypercoagulable states are common in thalassemic patients, mostly adults. The change in the endothelial cell from resting to procoagulant state could be associated with expression of adhesion molecules and selectins. Subsequently, endothelial cell injury can alter hemostasis by promoting fibrin-clot formation.*

Objective : *To determine plasma levels of endothelial, coagulation and fibrinolysis markers (soluble intercellular adhesion molecules (sICAM-1), vascular endothelial growth factor (VEGF), soluble endothelial selectin (sE-Selectin), factor VIII activity (FVIII: C) and D-dimer) in children with thalassemia.*

Design/Methods : *This is a cross sectional study. Plasma samples from 120 children with thalassemia (30 with non-splenectomized alpha-thalassemia (NSA), 65 with non-splenectomized beta-thalassemia major (NSB) and 25 with splenectomized beta-thalassemia major (SB) aged 1 to 16 years old were assayed for sICAM-1, VEGF, sE-Selectin, FVIII:C and D-dimer, whereas samples from thirty-five healthy children served as controls.*

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Results : *Plasma levels of sICAM-1 were significantly elevated in patients with NSA, NSB and SB compared to controls (324.7 ± 83.9; p ≤ 0.001, 277.5 ± 99.6; p ≤ 0.001and 422.3 ± 128.9; p ≤ 0.001 vs. 150.9 ± 41.9 ng/ml, respectively). Plasma E-selectin levels were significantly increased in NSA and SB versus controls (60.4 ± 32.8; p = 0.008, 63.9 ± 34.2; p = 0.003 vs. 39.5 ± 11.3 ng/ml, respectively). Plasma VEGF levels were elevated; however, the statistical difference was not significant. Plasma D-dimer levels were significantly increased in SB versus controls (365.5 ± 200 vs. 200.6 ± 54.1 ng/ml; p = 0.02, respectively). On the contrary, FVIII: C levels were significantly decreased in NSA and NSB versus controls (57.8 ± 13.5; p ≤ 0.001, 67.4 ± 23.9; p ≤ 0.001 vs. 96.7 ± 27.7 ng/ml, respectively). Comparing NSB to SB patients, SB patients had significantly higher plasma concentrations of sICAM-1, E-selectin and D-dimer than NSB patients (p ≤ 0.001, p = 0.003 and p = 0.005, respectively).*

Conclusion : *There is evidence of endothelial cell activation and alteration of coagulation and fibrinolysis in children with thalassemia which increases the risk of thromboembolic complications.*

Keywords : *Thalassemia, Endothelial cells, Thrombosis.*

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ภูมิหลัง : *ภาวะลิ่มเลือดอุดตันจากการแข็งตัวของเลือดที่ผิดปกติ พบได้บ่อยในผู้ใหญ่โรคธาลัสซีเมีย สาเหตุสำคัญเชื่อว่ามาจากการเปลี่ยนแปลงของเซลล์ผนังหลอดเลือดในผู้ป่วยโรคนี้ และนำไปสู่การกระตุ้นปัจจัย การแข็งตัวของเลือด จนเกิดเป็นก้อนไฟบรินอุดตันในผนังหลอดเลือด*

เป้าหมาย : *เพื่อศึกษาระดับของค่าชี้วัดของการกระตุ้นเซลล์ผนังหลอดเลือด ปัจจัยแข็งตัวของเลือดและปัจจัย ต่อต้านการแข็งตัวของเลือด ได้แก่ soluble adhesion molecular (sICAM-1), vascular endothelial growth factor (VEGF), selection family (SE-selectin), แฟคเตอร์แปด และ D-dimer ในเด็กโรคธาลัสซีเมีย*

วิธีการศึกษา : *การศึกษาแบบโคฮอร์ตไปข้างหน้า โดยการเจาะเลือดเด็กโรคธาลัสซีเมีย อายุ 1-16 ปี จำนวน 120 คน (30 คน ชนิดแอลฟาธาลัสซีเมียที่ไม่ได้รับการตัดม้าม, 65 คน ชนิดเบต้าธาลัสซีเมียที่ไม่ได้รับการตัดม้าม และ 25 คน ชนิดเบต้าธาลัสซีเมียที่ได้รับการตัดม้าม) เพื่อนำไปศึกษาระดับของตัวชี้วัดการกระตุ้นเซลล์ผนังหลอดเลือด ปัจจัยแข็งตัวของเลือด และปัจจัยต่อต้านการแข็งตัวของเลือด เปรียบเทียบกับเด็กปกติที่อายุใกล้เคียงกัน จำนวน 35 คน*

ผลการศึกษา : *ในผู้ป่วยโรคธาลัสซีเมียทั้ง 3 ชนิด ระดับของ sICAM-1 จะสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มเด็กปกติ ระดับของ E-selectin ในผู้ป่วยชนิดแอลฟาธาลัสซีเมียที่ไม่ได้รับการตัดม้าม และชนิดเบต้าธาลัสซีเมียที่ได้รับการตัดม้าม จะสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มเด็กปกติ แต่ระดับของ VEGF จะสูงขึ้นในกลุ่มเด็กโรคธาลัสซีเมีย แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มเด็กปกติ ผู้ป่วยชนิดเบต้าธาลัสซีเมียที่ตัดม้ามเท่านั้นพบมีระดับ D-dimer สูงขึ้นอย่างมีนัยสำคัญทางสถิติเพื่อเปรียบเทียบกับกลุ่มเด็กปกติ นอกจากนั้น ในกลุ่มชนิดแอลฟาธาลัสซีเมียและเบต้าธาลัสซีเมียที่ไม่ได้รับการตัดม้ามพบระดับของแฟคเตอร์แปดลดต่ำลงอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มปกติ เมื่อเปรียบ เทียบกลุ่มชนิดเบต้าธาลัสซีเมียที่รับการตัดม้ามและกลุ่มที่ไม่ได้รับการตัดม้าม พบว่ากลุ่มเบต้าธาลัสซีเมียที่ได้รับการตัดม้ามจะมีระดับของ sICAM-1, E-selectin และ D-dimer สูงกว่ากลุ่มที่ไม่ได้รับการตัดม้ามอย่างมีนัยสำคัญทางสถิติ*

สรุป : *การศึกษานี้ พบหลักฐานของการกระตุ้นเซลล์ผนังหลอดเลือด ปัจจัยการแข็งตัวของเลือดและปัจจัยต่อต้านการแข็งตัวของเลือดในผู้ป่วยเด็กโรคธาลัสซีเมีย และเป็นเพียงส่วนหนึ่งของสาเหตุจากหลายปัจจัย ที่อาจนำไปสู่การเกิดภาวะลิ่มเลือดอุดตันในหลอดเลือดในอนาคต*

คำสำคัญ : *โรคธาลัซซีเมีย, เซลล์เอนโดธีเลี่ยม, ภาวะลิ่มเลือดอุดตัน.*

Thalassemia is the most common disease of congenital hemolytic anemia which results in reduced synthesis of the alpha- or beta-globin chain. The clinical severity of this disease is varying spectrums from mild to severe subtypes which are known as thalassemia intermedia and thalassemia major, respectively. With the current improvement of the standard care of thalassemic patients, life expectancy of the patients is now almost double; however, the thromboembolic complications are still relatively common in splenectomized thalassemic patients especially among thalassemia intermedia.[1] These events are reported in both arterial and venous thromboemboli.[1-4] Previous autopsy findings confirmed peripheral arterial and venous thrombosis with an increase of pulmonary emboli in splenectomized patients with beta-thalassemia/Hb E.[5] The pathophysiology of this phenomenon is thought to be hypercoagulable state.[1, 6] Vascular endothelial cells play a major role in every mechanism that contributes to inflammation-induced activation of coagulation. Under the stimulatory influence of various factors, the endothelial cells can increase the expression of endothelial surface adhesion molecules and lead to the rolling, activation, and firm adhesion of leukocytes to the endothelium. [7, 8] Furthermore, the endothelial cell injury interferes directly with the initiation and regulation of fibrin formation.[9,10] Previous studies showed evidence of endothelial cell activation, acquired changes in coagulation factors and fibrinolysis in thalassemic patients, mostly adults. [11, 12] Thus, we designed and conducted a cross sectional study to determine the extent of activation of endothelial cells, coagulation and fibrinolysis and to correlate these findings with clinical severity in children with thalassemia.

Patients and Methods

Study setting

Study subjects were recruited from thalassemic patients who visited the Pediatric Hematology Clinic of King Chulalongkorn Memorial Hospital, Bangkok, Thailand from December 2007 to March 2008. The study protocol has been reviewed and approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Thailand. Written informed consents were obtained from the parents or guardians of the patients.

Subjects

One hundred and twenty thalassemic patients (67 males, 53 females) aged 1-16 years were recruited and further divided into three groups, namely: non-splenectomized alpha-thalassemia (NSA), non-splenectomized beta-thalassemia major (NSB) and splenectomized beta-thalassemia major (SB). Thalassemia major patients received regular blood transfusions every 3 to 4 weeks to maintain pretransfusional hemoglobin levels more than 9 grams/deciliter. Thirty-five age-match healthy children served as the control group. The normal healthy children consisted of those who had recovered from acute idiopathic thrombocytopenic purpura (ITP) at least one year earlier, or who had come in for a routine medical visit at the Pediatric Hematology Outpatient Clinic. None of them had a coagulation or thrombotic disorder or was taking any medication.

Blood sampling

Blood (3 ml) was obtained with a clean venipuncture technique into 3.2% buffered sodium citrate and EDTA vacutainers (Beckton Dickinson, NJ,

USA). Blood samples were obtained from each patient at least three weeks after their last blood transfusion and also collected from 35 healthy children. The samples were centrifuged at 4 °C at 2,000 g for 30 min, aliquoted and stored frozen at -70 °C until analysis.

Assay methods

Endothelial cell activation

Soluble adhesion molecules (sICAM-1), vascular endothelial growth factor (VEGF), selectin family (sE-Selectin) levels were determined by specific immunoassay, using ELISA commercially kits (R&D Systems, Minnesota, USA) according to the manufacturer's instructions.

Coagulation and Fibrinolysis

Factor VIII (FVIII:C) levels were measured by one-stage clot based assay, Instrumentation Laboratory, Lexington, MA, intra-assay CV 3.0 %. D-dimer was assayed based upon an ELFA technique by using VIDAS® D-dimer Exclusion, Biomerieux, Lyon, France.

Statistical analysis

The data were analyzed using SPSS (version 12) software. Statistical significance was assumed at $P< 0.05$. Continuous data were expressed as the mean +/- SD. Nominal data were presented as percentage (%). As for comparison of more than 2 groups, an analysis of variance (ANOVA) tests with additional Bonferroni adjustment was used for multiple comparisons.

Results

Patient characteristics

Recruited in this study were 30 patients into the NSA group (25 Hb H, 5 H/CS disease), 65 in NSB (50 beta-thalssemia/Hb E, 15 homozygous beta-thalassemia), and 25 in SB (15 beta-thalssemia/Hb E, 10 homozygous beta-thalassemia). Basic characteristics of the patients in the study are shown in Table 1. There was no significant difference between the age and sex of the three studied groups compared to the controls. However, their hemoglobin and ferritin levels were significantly higher and lower respectively in the NSA when they were compared to both the NSB and SB groups. None of these patients developed thromboembolic phenomenon before and during this study.

Endothelial cell activation assays

The thalassemic patients (both alpha- and beta-thalassemia) had significantly higher plasma SICAM-1 and E-selectin levels than the controls. Plasma VEGF levels were elevated in our patients; however, the statistic was not significant. Compared to the NSB group, the SB group had significantly elevated plasma levels of SICAM-1 and E-selectin as shown in Table 2.

Alteration of Coagulation and Fibrinolysis

The NSA and NSB groups had significantly lower of plasma levels of FVIII: C than controls. FVIII: C was also decreased in the SB group but the statistics was not significant. In terms of alteration of fibrinolysis, only the SB group had significantly higher plasma levels of D-dimer than the controls (Table 2).

Table 1. Basic characteristics of study patients.

Parameters	Non splenectomized α - thalassemia (n = 30)	Non splenectomized β - thalassemia (n = 65)	Splenectomized β - thalassemia (n = 25)	Control (n = 35)
Age (years)	9.1 ± 4.2	10.1 ± 3.8	11.1 ± 3	9.6 ± 4.6
Sex (M:F)	1.1:1	1.1:1	2:1	1.2:1
Hemoglobin (g/dl)	9.1 ± 1.5 **$^{\#\#ถ}$	7.9 ± 1.1 **	7.4 ± 0.9 **	13.8 ± 1.4
Ferritin (ng/ml)	590.2 ± 397.2$^{\#\#ถ}$	2380.5 ± 1211.4 **	2725 ± 1431.5 **	99.5 ± 38.3

Values were expressed as means ± SD.

*P ≤ 0.05 and ** P ≤ 0.001 compared with normal control

$^{\#}$P ≤ 0.05 and $^{\#\#}$ P ≤ 0.001 compared with non- splenectomized Beta-thalassemia major

^{ถ}P ≤ 0.05 and ^{ถถ} P ≤ 0.001 compared with splenectomized Beta-thalassemia major

Table 2. Activation of endothelial cells, coagulation and fibrinolysis in thalassemic patients.

Parameters	Non splenectomized α - thalassemia (n = 30)	Non splenectomized β - thalassemia (n = 65)	Splenectomized β - thalassemia (n = 25)	Control (n = 35)
SICAM-1 (ng/ml)	324.7 ± 83.9 **	277.5 ± 99.6 **	422.3 ± 128.9 **$^{\#\#}$	150.9 ± 41.9
E-Selectin (ng/ml)	60.4 ± 32.8 *	45.4 ± 19.2	63.9 ± 34.2 **$^{\#}$	39.5 ± 11.3
VEGF (pg/ml)	168.5 ± 115.9	151.9 ± 124.6	214.8 ± 211.8	111.0 ± 25.1
D-dimer(ng/ml)	163.6 ± 87.2	202.7 ± 162.5	362.5 ± 200 *	200.6 ± 54.1
FVIII Level (%)	57.8 ± 13.5 **	67.4 ± 23.9 **	92.6 ± 34.7$^{\#\#}$	96.7 ± 27.7

* P ≤ 0.05 and ** P ≤ 0.001 compared with normal control

#P ≤ 0.05 and ## P ≤ 0.001 compared with non-splenectomized Beta-thalassemia major

Values were expressed as means ± SD

Discussion

SICAM-1 and E-selectin levels were significantly increased in our patients suggesting that thalassemic patients, both alpha- and beta-thalassemia, have activated endothelial cells and this concurs with previous studies, mostly in adults. [11, 13] Interestingly, the highest concentrations of these molecules appeared in the SB group, which was correlated well with the clinical severity of thalassemia and may be involved in vascular occlusion. Activation of vascular endothelial cells promote mainly the inflammatory response and also affects the coagulation response. FVIII is a glycoprotein procofactor synthesized and released into the blood

stream by the endothelium. Our studies showed the decreased of FVIII: C in both alpha- and beta-thalassemia compared to the controls. We speculated that FVIII: C was consumed by the process of activation of the endothelial cells more than decreased production. D-dimer, a marker of the formation and lysis of cross-linked fibrin, was significantly elevated in the SB groups compared to the controls. Thus, there is evidence of activation of coagulation and fibrinolysis only in children with splenectomized beta-thalassemia major which is suggestive of thrombotic complications.

In conclusion, the present study confirms that the activation of endothelial cells, coagulation and fibrinolysis in children with thalassemia especially in splenectomized beta-thalassemia major, however, it does not show any clinical evidence of thromboembolic phenomenon. Further studies to follow up these patients closely until they reach the adulthood are suggested.

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