Hyperactivation and Hyperfunction Platelets in Beta-thalassemia

ภาวะการกระตุ้นเกร็ดเลือดอย่างสูง และการทำงานมากกว่าปกติในโรคเบต้าธาลัสซีเมีย

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ABSTRACT

Thalassemia is a common hereditary anemia in Thailand. Thromboembolic complications occur frequently in beta-thalassemia patients. The role of platelets in the pathogenesis of thrombotic events in beta-thalassemia was determined by analysis of platelet activation in 15 beta-thalassemia/HbE patients and 8 healthy control individuals. The results showed significant increase in level of baseline platelets activation of beta-thalassemia/HbE patient compared with normal individuals (6.67±3.38 and 0.46±0.15, respectively, $p < 0.05$). The beta-thalassemia/HbE platelets are significantly hyperactivation in response to low dose of agonist, 5 and 10 µg/ml collagen and 0.1 and 0.5µM adenosine diphosphate (ADP) when compare to normal individuals group ($p < 0.05$). The hyperactivation and hyperfunction platelets are likely to play pathogenetic role in the thrombotic risk in beta-thalassemia/HbE.

บทคัดย่อ

ธาลัสซีเมียเป็นโรคทางพันธุกรรมที่พบได้บ่อยในประเทศไทย อุบัติการณ์ของภาวะลิ่มเลือดอุดตันในหลอดเลือดพบได้บ่อยในผู้ป่วยเบต้าธาลัสซีเมีย บทบาทของเกร็ดเลือดต่อพยาธิกำเนิดของภาวะลิ่มเลือดอุดตันในผู้ป่วย โดยทำการศึกษาการกระตุ้นเกร็ดเลือดของผู้ป่วยเบต้าธาลัสซีเมีย ฮีโมโกลบินอี 15 ราย และคนปกติที่มีสุขภาพแข็งแรง 8 ราย จากการศึกษาที่ระดับพื้นฐาน พบว่า เกร็ดเลือดจากผู้ป่วยอยู่ในภาวะถูกกระตุ้นสูงกว่าคนปกติอย่างมีนัยสำคัญ (6.67±3.38 และ 0.46±0.15 ตามลำดับ, $p < 0.05$) เกร็ดเลือดจากผู้ป่วยที่อยู่ในภาวะถูกกระตุ้นสูงอยู่แล้ว พบว่า มีการตอบสนองต่อสารกระตุ้น ได้แก่ คลอลาเจน ที่ระดับความเข้มข้น 5 และ 10 µg/ml เอดีพี ที่ระดับความเข้มข้น 0.1 และ 0.5µM เมื่อเปรียบเทียบกับกลุ่มคนปกติอย่างมีนัยสำคัญ ภาวะที่การกระตุ้นเกร็ดเลือดอย่างสูง และการทำงานมากกว่าปกติของเกร็ดเลือดนั้น อาจเป็นสาเหตุหนึ่งที่ทำให้ผู้ป่วยมีความเสี่ยงต่อภาวะลิ่มเลือดอุดตันในหลอดเลือดได้

Key Words: Thalassemia, Platelet activation

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Introduction

Thalassemia is a common hereditary anemia in Thailand that defects in globin chain synthesis. Beta-thalassemia is caused by deletion or point mutation at beta-globin gene that leads to absence or reduced beta-globin chain production. The excess unbound alpha-globin chains and their degradation products precipitate in red blood cell (RBC) precursors, causing defective maturation and ineffective erythropoiesis.The complication of patients depends on disease severity ranging from a mild form of thalassemia intermedia to severe transfusion dependent (Rund and Rachmilewitz, 2005).

Frequent occurrence of thromboembolic complicationsin beta-thalassemia, particularly in patients who have undergone splenectomy or who have severe blood transfusion was reported (Greenberg et al., 2001). Patients have shown high levels of thrombin, platelets activation, coagulation factors,soluble tissue factor and other cellular elements.While abnormal activation of fibrinolysis, and decreased levels of anticoagulant proteins were observed. These finding leds to the hypercoagulable state in patients (Eldor et al., 1991; Eldor and Rachmilewitz, 2002).

Platelet function anomalies including increased circulating aggregates have been observed in 71% of splenectomized and 35% of non-splenectomizedbeta-thalassemia/hemoglobin (Hb) E patients(Winichagoon et al., 1981).

Uncontrolled platelet activation under different pathophysiological condition is associated with the many pathogenesis including thromboembolic phenomena in beta-thalassemia patient (Pattanapanyasat et al., 2007; Solum, 1999).

Objectives of the study

In this study, we evaluated the role of platelets on thromboembolic events in beta-thalassemia/HbE patients. Baseline platelets activation of patients and control subjects were examined by using flow cytometry. Then, effects of agonists including adenosine diphosphate (ADP), collagen and thrombin to platelet activation were investigated. This study aimed to demonstrate platelet activation in beta-thalassemia/HbE patients.

Materials and method

Blood sample collection

Three milliliters of venous blood samples were collected into 3.2% trisodium citrate (Becton Dickinson Biosciences [BDB], San Jose, CA, USA) from 23 volunteers; 15 beta-thalassaemia/HbE patients and 8 normal subjects (normal Hb typing and DNA analysis). The diagnosis of thalassemia for all subjects was made by standard hematological techniques,Hb analysis and DNA analysis. Hematological data of the normal subjects and beta-thalassaemia/HbE patients is showed in Table 1.Inclusion criteria, all subjecthad received no blood transfusion, no aspirin, no Non-steroidal anti-inflammatory drugs (NSAID drug), and no antioxidant drug treatment (e.g. vitamin E, curcummin) at least 8 weeks prior the study, no evidence ofconcurrent infection, and none had been hospitalized. There was no history of vaso-occlusive episode or atherosclerosis vascular disease in thesepatients prior to the initiation of the study or during the sampling period. The study was approved by the Institutional Review Boards of Mahidol University.

Reagents and mAbs

mAbs were purchased from BDB:mAb to platelet glycoprotein IIb/IIIa (anti-CD41a, clone HIP8),P-selectin (anti-CD62P, clone AC1-2). FITC and PE conjugated mouse IgG were used as isotype control. Adenosine diphosphate (ADP), thrombin and collagen were purchased from Sigma (Steinheim, Germany). All mAbs and agonists were utilized at the protocol recommended by the manufacturer**.**

Table 1 Characteristics of healthy control subjects and beta-thalassemia/HbE patients.

	Control	NS	S
Number	10	9	6
Hb(g/dl)	14.5±1.3	7.2±1.7	5.9±1.8
Hct(%)	45±2.5	27±5.1	20±3.0
WBC ($x10^3$/µl)	8.5±1.8	7.4±3.2	10.2±5.1
Platelet ($x10^3$/µl)	270±42.6	265±50.6	809±118.7*

*Data are expressed as the mean ± SD. NS, nonsplenectomy beta-thalassemia/HbE patient; S, splenectomy beta-thalassemia/HbE patient; Hb, hemoglobin; Hct, hematocrit; WBC, white blood cell.

Platelet analysis

Platelet activation was determined immediately within 2-3 hours after venous puncture.Ten microliters of 3.2% trisodium citrate whole blood samples were diluted with 1×PBS buffer at 1:10 (vol/vol). Five microliters of diluted whole blood were incubated with absence or presence of agonists at 100 µl total volume. Briefly, diluted whole blood samples were treated with either collagen at final concentration 0.1, 1, 5, 10 and 100 µg/ml or ADP at final concentration 0.1, 0.5, 1, 10, 20 and 30 µM or thrombin at final concentration 0.001, 0.01, 0.1, 0.5 and 1 U/ml. After that, Treated whole blood samples were incubated at RT for 15, 45 and 75 min, in the dark. Samples were then stained with FITC conjugated anti-CD41a and PE conjugated anti-CD62P. The percentages of activated platelets ($CD41a^+/CD62P^+$) were analyzed by using flow cytometry.

Statistical analysis

Descriptive statistics (mean ± SD) were performed by using the SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Comparisons of statistical difference between parameters were performed by Student's t test. The threshold for statistical significance for all comparisons was chosen as $p< 0.05$.

Results and discussion

Chronic platelet activation in peripheral blood from beta-thalassemia/HbE patients

Flow cytometric analysis of baseline activated platelets in whole blood was determined as logarithmic amplification of FSC-H and SSC-H signal (Fig 1). Classical identification of RBCs and platelets according by size and granularity had shown in R1 and R2 region, respectively (Fig 1A). Platelet population in R2 region was determined as $CD41a^+$platelets (R3 region) (Fig 1B). The percentages of activated platelets were identified as $CD41a^+/CD62P^+$platelets (R4 region) (Fig 1C). Isotype control was used as negative signal (Fig 1D).

Baseline platelets activation of patients and normal controls were examined. The percentage of $CD41a^+/CD62P^+$platelets (mean±SD) in patients was significant higher than control subjects (6.67±3.38 and 0.46±0.15, respectively,$p< 0.05$) (Fig 2).

The increased in platelets activation in beta-thalassemia/HbE patients may resulted from higher levels of phosphatidylserine (PS), the tenase and prothrombinase complex binding site, exposures on outer RBC membrane in the patients. Hemichromes include heme disintegrates from excess alpha-globin are resulting in reactive oxygen species (ROS) production via Fenton reaction. ROS causes RBC membrane damage, loss of plasma membrane asymmetry and PS exposure on cell surface. Moreover

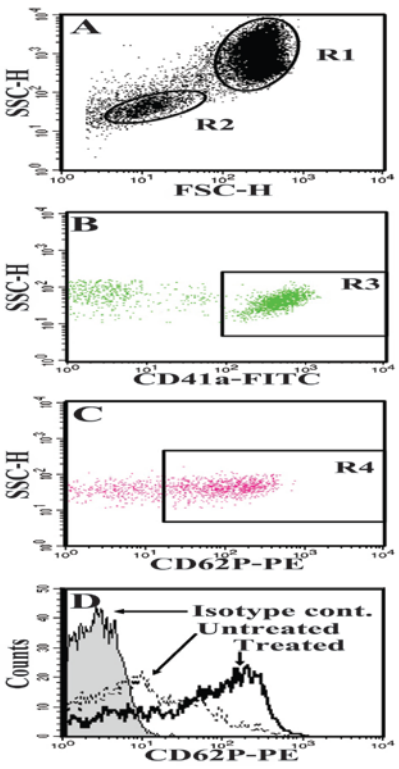

Fig1 Flow cytometric analysis of activated platelets. (A) Dot plot analysis of RBCs (R1 region) and platelets (R2 region) (B) Platelets ($CD41a^{+}$) were identified in region R3. (C) Percentage of $CD41a^{+}CD62P^{+}$ as markers of activated platelet (R4 region). (D) Histogram analysis of $CD62P^{+}$ platelets of isotype staining control and plateletuntreated and treated.

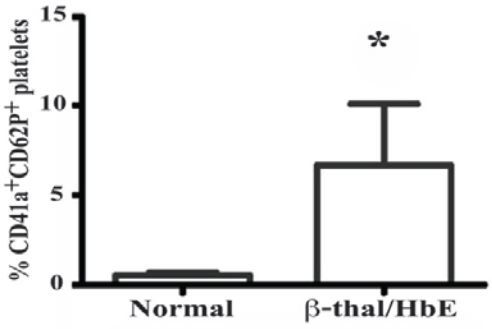

Fig2 Activated platelet analysis. Whole blood samples obtained from beta-thalassemia/HbE patients and normal individuals were measured the percentage of CD41a+CP62P+ platelets at baseline. *Significant difference at $p< 0.05$ when compared to normal subjects.

RBC vesicles and microparticles (MPs) were generated from the damaged RBC. The level of PS-baring MPs was increased in the patents compared with normal individuals. These PS-bearing MPs have procoagulant properties as measured by assessing the platelet factor-3-like activity (Pattanapanyasat et al., 2007).In addition, another fundamental mechanism of platelet activation is oxidative stress. This caused by iron overload resulted from multiple transfusions and increased intestinal iron absorption and lower of intracellular glutathione caused continuous oxidative stress leading to expression of platelet hyperactivity (Eldor and Rachmilewitz, 2002; Rund and Rachmilewitz, 2005).

Effect of agonists on platelet activation

Several agonists including collagen, ADP and thrombin effect on platelet activation via transmembrane receptors (Eldor et al., 1991). Time course effect of ex vivo platelet activation by 3 different agonists as recommended concentration; 10 µg/ml collagen, 10 µM ADP and 0.5 U/ml thrombin (Aslam et al., 2013) at 15, 45 and 75 min were examined (Fig 3). The results showed that percentage of activated platelets were increased when stimulated with thrombin > ADP > collagen at the same incubation period. Stimulating condition promotes platelet activation act as time-dependent manner. Therefore, we further performed activated platelet analysis at 15 min incubation.

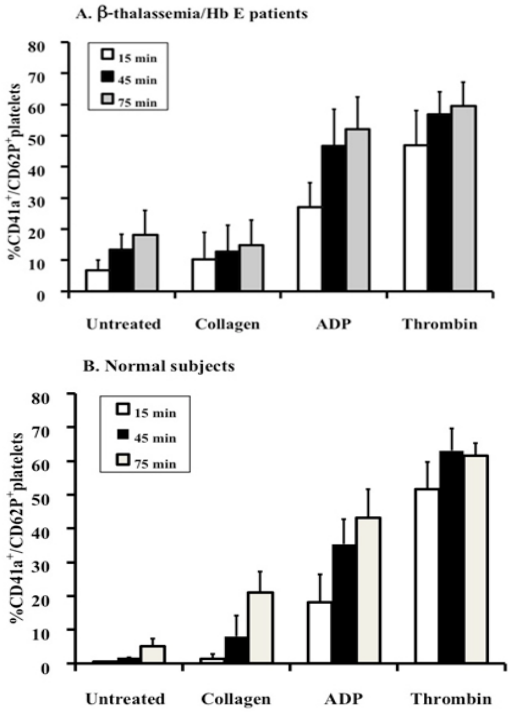

Fig3 Agonists activated platelets at different incubation time. Whole blood samples obtained from beta-thalassemia/HbE patients (A) and normal individuals (B) were treated with either 10 µg/ml collagen, 10 µM ADP or 0.5 U/ml thrombin at 15, 45 and 75 min. Untreated samples were analyzed as baseline control. After incubation, the percentage of $CD41a^{+}CP62P^{+}$platelets was determined by using flow cytometry.

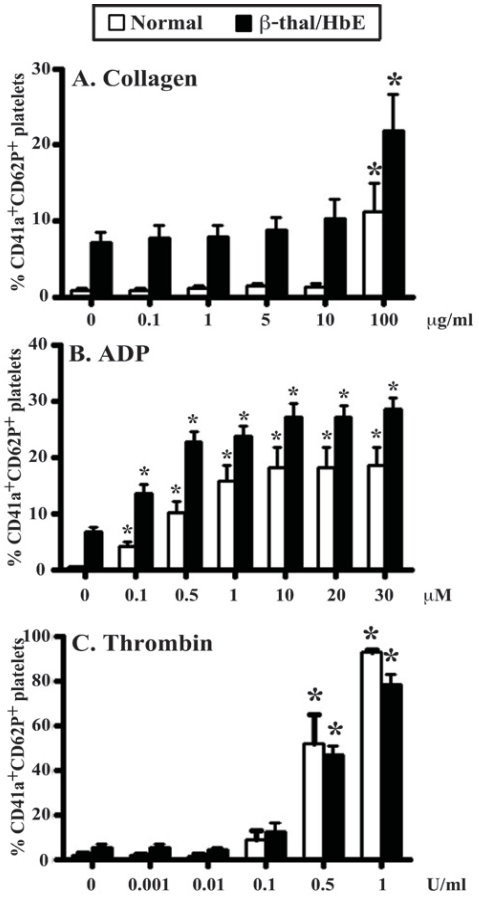

Fig4 Agonists activated platelets at different concentration. Whole blood samples were treated with collagen, ADP or thrombin at 15 min. Untreated samples were analyzed as baseline control. After incubation, the percentage of $CD41a^{+}CP62P^{+}$platelets was determined by using flow cytometry. *Significant difference at $p< 0.05$ when compared to baseline.

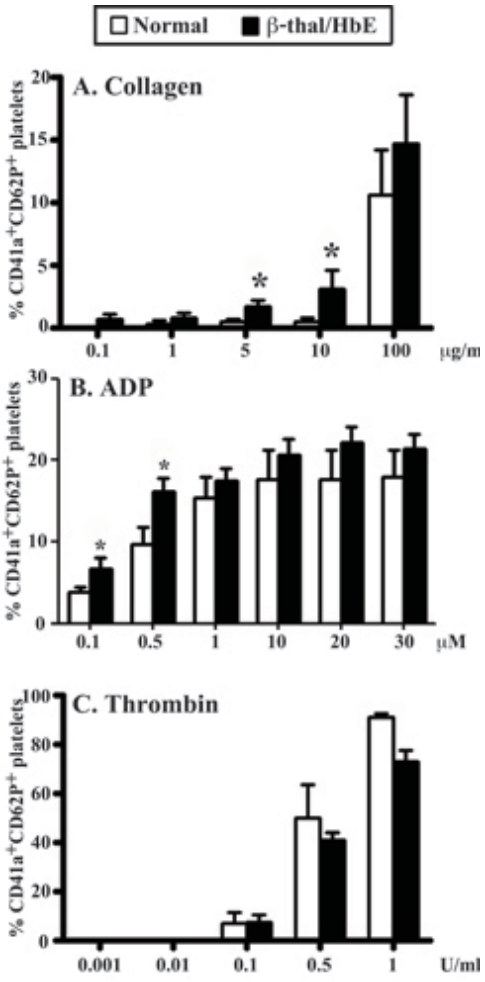

Fig5 The percent difference of treated and untreated platelet activation in the individual subjects. The percent changes of platelet activation were normalized with baseline (Δ treated – untreated). *Significant difference at p< 0.05 when compared to normal subjects.

Consequently, the dose effect of agonists on platelets activation was determined. Whole blood samples from patients and normal subjects were treated with the 3 agonists and measure platelet activation at 15 min of incubation (Fig 4). The results showed that the 3 agonists induced activation of platelets obtained from both normal individuals and beta-thalassemia/HbE patients in dose dependent manner. There was significant different of platelet activation at 100 µg/ml collagen, 0.1-30 µM ADP and 0.5-1 U/ml thrombin treated samples when compared to untreated samples in the individual groups (p< 0.05) (Fig 4).

As the baseline platelet activation of the patients was higher than normal those of normal individuals, the platelets activation potential was determined by analysis the percentage of CD41a+ CD62P+platelets at treated conditions refuted those at untreated condition (Δtreated – untreated) (Fig5). The statistical analysis of difference in platelet activation at baseline compare with agonists activation of patients showed significant difference compared to those of normal individuals when treated with 5µg/ml collagen (1.63±1.96 and 0.51±0.50, respectively);10 µg/ml collagen (3.09±4.88 and 0.46±0.69, respectively); 0.1 µM ADP (6.77±5.07 and 4.23±1.49, respectively) and 0.5 µM ADP(16.12±6.32 and 10.17±4.58, respectively) (p< 0.05). However, the percentage of CD41a+CD62P+platelets was no significant different between groups when treated whole blood with 0.001-1 U/ml thrombin. The results suggested that beta-thalassemia/HbE platelets are easier to activate as the cells can be activated with lower amount of agonist, ADP and collagen. Several etiologic factors may play a role in the platelet activation in patients such as increased coagulation factors, PS-bearing cells and MPs and decreased protein C and protein S (Eldor et al., 1991).Collagen, ADP and thrombin effect on platelet activation via different transmembrane receptors; GPVI for collagen, P2Y1 and P2Y12 for ADP, and PAR1 for thrombin. For this reason may be cause thrombin activation pattern different from another agonist. The hyperactivated platelets stage

might contribute to the more sensitive to agonist activation in beta-thalassemia/HbE patients.

Conclusions

Beta-thalassemia/HbE patients had higher levels of baseline activated platelets compare to normal. In addition, they were easier to be stimulated by agonists than those from normal subjects. Chronic platelet activation and pathological complication could be cause of hypersensitive of platelets in patients. Platelets play important roles on hemostasis. The hyperfunction of platelets could be high risk to thromboembolic events in patients.

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