Modification of Platelet Shape Change Parameter by Oxidized Lipoprotein from β-thalassemia/Hemoglobin E

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Background: β-thalassemia/Hemoglobin E (β-thal/Hb E) is a congenital hemolytic anemia that is prevalent in Thailand. Pulmonary arterial occlusion is the cause of morbidity and mortality in these patients. Abnormality of platelets has been implicated as pathogenesis of this condition. However, the blood-borne factors that induce platelet activation are not identified. Recently, oxidized low-density lipoproteins (ox-LDLs) had been identified in thalassemic blood.

Objective: Identify whether oxidized LDL is the blood bone factor that induce platelet activation in β-thal/Hb E patients.

Material and Method: Platelet activation was measured by monitoring platelet shape change parameter using plasma-free human platelets. The shape change parameter was monitored following exposure to normal LDL, oxidized LDL, and thalassemic LDL.

Results: Oxidized LDL, but not the native LDL and thalassemic LDL, showed platelet activation activity. Oxidation of thalassemic LDL with copper give rise to oxidized LDL with platelet activating activity. However, less copper was needed by LDL from splenectomized β-thal/Hb E patients than those from nonsplenectomized β-thal/Hb E patients.

Conclusion: LDL from splenectomized β-thal/Hb E patients is more susceptible for oxidation and gives rise to oxidized-LDL that plays an important role in thrombosis event in these patients.

Keywords: Blood platelets, Cell shape, Hemoglobin E, Oxidized low density lipoprotein, Lipoproteins, LDL, Thalassemia

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β-thalassemia/Hemoglobin E (β-thal/Hb E) is a congenital hemolytic anemia that is prevalent in Thailand(1,2). Common causes of death in severe patients, particularly those with inadequate treatment are heart failure and infection, which occur while they are still young(3). Chronic hypoxia with and without abnormality pulmonary dysfunction is another complication observed in thalassemia(4). Pulmonary arterial occlusion was noted in a series of autopsy findings of splenectomized thalassemia patients with heart failure(5). Evidence in abnormality of platelets in splenectomized thalassemia has been demonstrated(6,7). Therefore, abnormality of platelet might play an important role in pulmonary arterial thrombosis and contributes to hypoxia in the splenectomized β-thalassemias. However, the precise nature of blood borne factors that modified the in vivo responsiveness of platelets in splenectomized β-thalassemia/Hemoglobin E (splenectomized β-thal/Hb E) has not been clearly defined.

Oxidative damage has been implicated in thalassemia patients(8-10). Recently, oxidized LDLs had
been identified in thalassemia blood\(^{11-13}\). Oxidized lipoproteins have been shown to affect platelet activity, both \textit{in vitro} and \textit{in vivo} in the pathogenesis of atherosclerosis and thrombosis\(^{14-18}\). In addition, supplementation of vitamin E can modulate the hyperactivity of platelets in thalassemia patients to some extent\(^{7}\). Therefore, oxidative stress has been implicated as an important factor causing functional abnormalities of the circulating platelet in splenectomized \(\beta\)-thal/Hb E. However, the role of oxidized LDL on platelet in thalassemia is still not known. Therefore, the aim of the present study is to identify whether oxidized-LDL is a cause of platelet hyperactivity in these patients.

**Material and Method**

**Subjects**

Normal healthy donors from Ramathibodi’s blood bank were included as normal controls. Patients with \(\beta\)-thalassemia/Hb E at the hematology clinic of the Department of Medicine, Siriraj Hospital participated. They were asked not to take any medication. The thalassemic patients, were asked not to take their daily folic acid supplement for at least two weeks before joining the present study. All participants were briefed of the scope and objective of the present study before getting their informed consents.

**Materials**

Thiobarbituric acid, butylated hydroxytoluene, bovine serum albumin, Sepharose 2B-CL, standard bovine serum albumin solution, HEPES, potassium bromide, apyrase, and thrombin were obtained from Sigma Chemical Co. (St. Louis, USA).

**Methods**

1. **Isolation of LDL:** Blood was drawn from fasting volunteers and thalassemia patients into 0.1% EDTA. The plasma was separated by centrifugation at 2,000 g for 15 min at 4°C. The lipoproteins were separated by sequential ultracentrifugation in a Beckman TL-100 Beckman Tabletop UC with a fixed angle TLA-100.2 rotor\(^{19}\). The LDL was dialyzed overnight against PHS (pH 7.4) with at least two changes of dialysate. The amount of protein was determined by a modified Lowry method\(^{20}\), using BSA as a standard. LDL was stored at -70°C and used within 2 weeks.

2. **Oxidation of LDL:** Oxidation of LDL was conducted in a shaking water bath at 37°C. One hundred \(\mu\)l of LDL was incubated with CuSO\(_4\) in 10 mM PBS (pH 7.4). Oxidation was terminated by the addition of 0.1 mM EDTA and immediately frozen. The extent of lipoprotein oxidation represented by ratios of thiobarbituric acid reactive substances (TBARs) in the incubation mixture before and after incubation with CuSO\(_4\)\(^{21}\).

3. **Preparation of platelets:** Blood was obtained by venipuncture from healthy volunteers who had not been on medication for 2 weeks before experimentation. The blood was anticoagulated with ACD (6:1 v/v) and centrifuged at 250 x g for 12 min to obtain platelet-rich plasma. Platelets were first separated from plasma by the albumin-cushion method, purified further by gel filtration using a Sepharose 2B-CL column and resuspended in platelet buffer (HEPES 3.5 mM, NaCl 137 mM, KCl 2.7 mM, glucose 5.5 mM, MgCl\(_2\) 1 mM, 0.35% BSA, pH 7.4)\(^{22}\). The platelet suspension was incubated 37°C for approximately 30 min at to restore platelets to resting state.

4. **Shape-change analysis:** Based on the light-scattering theory the shape-change parameter has been used to assess platelet responses to low dosage of strong stimulants or to weak stimulants\(^{23-26}\). Platelet shape change is determined by the light transmission. The light transmission of suspension of asymmetric particles (discoid platelets) increased with stirring, whereas that of spherical, symmetric particles with or without uniformly distributed pseudopods is insensitive to stirring, assuming that the total volumes do not change upon stimulation.

In the present investigation, platelet shape change parameter was monitoring by Chrono-Log Corp Whole Blood Aggregometer Model 550. 500 \(\mu\)l of \(10^5\) cells/\(\mu\)l was used as test sample and 600 \(\mu\)l of \(5-7 \times 10^4\) cells/\(\mu\)l was used as control sample. Before and after the incubation of platelets with oxidized LDL at 37°C for 5 minutes, the light transmission was recorded by an aggregometer. The electronic stirrer was turned on and off about every 30 seconds (alternatively). The shape-change parameter was calculated as (1 - (test defection/control defection) x 100%). This procedure is as described by Beaumont et al\(^{23}\).

**Results**

**Effect of thalassemic LDL on platelet shape change**

Extent of platelet activation can be measured in platelet suspension by monitoring platelet shape change parameter. Oxidized LDL dose-dependently (1-20 \(\mu\)g/ml) increased shape-change parameters from 4% to 85% respectively (Fig. 1). Platelet shape change,
which was induced by 20 μg/ml ox-LDL, was comparable to those induced by 0.1 U/ml of thrombin. On the contrary, native LDL (20 μg/ml) did not cause any change in platelet shape change parameter (Fig. 1). LDLs from both nonsplenectomized and splenectomized β-thal/Hb E patients like native LDL, did not cause platelet shape change when incubated with the gel-filtrated platelets (Fig. 2)

![Fig. 1](image1.png)  
**Fig. 1** Effect of native LDL and oxidized-LDL (Ox-LDL) on shape-change parameters. Ox-LDL was prepared by incubation with 50 μM CuSO₄ for 24 hr in PBS pH 7.4. Values represent means ± SD (n = 5)

![Fig. 2](image2.png)  
**Fig. 2** Effect of oxidized LDL, normal LDL, nonsplenectomized-thal LDL (Nsp-thal LDL) and splenectomized-thal LDL (Sp-thal LDL) on gel-filtrated platelet suspension. 20 μg/ml of oxidized-LDL significantly increased shape change parameter while 60 μg/ml of normal LDL, Nsp-thal LDL and Sp-thal LDL did not. Normal LDL (LDL from healthy volunteer, n = 4), Nsp-thal LDL (LDL from nonsplenectomized β-thal/Hb E, n = 3), Sp-thal LDL (LDL from splenectomized β-thal/Hb E, n = 5). Values represent mean ± SD of n subjects

Table 1. The oxidizability of normal-, nonsplenectomized and splenectomized b-thalassemia/Hb E LDL to generate platelet-activating activity

<table>
<thead>
<tr>
<th>Subjects</th>
<th>LDL protein (μg/ml)</th>
<th>Copper requirement (μM)*</th>
<th>Maximum SCP** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-1</td>
<td>656.20</td>
<td>30</td>
<td>92.00</td>
</tr>
<tr>
<td>N-2</td>
<td>648.95</td>
<td>25</td>
<td>82.62</td>
</tr>
<tr>
<td>N-3</td>
<td>544.80</td>
<td>25</td>
<td>70.59</td>
</tr>
<tr>
<td>Nsp-1</td>
<td>227.45</td>
<td>25</td>
<td>30.77</td>
</tr>
<tr>
<td>Nsp-2</td>
<td>455.55</td>
<td>25</td>
<td>38.46</td>
</tr>
<tr>
<td>Nsp-3</td>
<td>445.60</td>
<td>25</td>
<td>60.00</td>
</tr>
<tr>
<td>Sp-1</td>
<td>305.70</td>
<td>10</td>
<td>25.00</td>
</tr>
<tr>
<td>Sp-2</td>
<td>291.65</td>
<td>10</td>
<td>31.25</td>
</tr>
<tr>
<td>Sp-3</td>
<td>189.40</td>
<td>15</td>
<td>26.67</td>
</tr>
</tbody>
</table>

LDLs were incubated with various concentrations of CuSO₄ (10-30 μM) for 6 hours (100 μl). Aliquots 200 ml of final suspension were used to determine platelet shape change parameters

N = normal, Nsp = nonplenectomized β-thal/Hb E, Sp = splenectomized b-thal/Hb E

* = The least concentration of copper that could induce platelet-activating activity in LDL

** = The maximum shape-change parameter

The sensitivity of normal- and thalassemia-LDL to CuSO₄, for inducing their platelet-activating activity

Oxidized LDL is the product of incubation of native LDL with various concentration of CuSO₄ for 6 hours. The concentrations of Cu²⁺ that generate platelet activating activity of LDL were 25-30 μM, 25 μM and 10-15 μM for native LDL, nonsplenectomized-thal LDL, and splenectomized-thal LDL respectively. Oxidized-LDL caused platelet shape change with 70%-92% SCP. Oxidation of LDLs from nonsplenectomized-thal and splenectomized-thal resulted in LDL with can cause platelet shape change similar to oxidized-LDL. However, the maximal% SCP caused by oxidized-LDL from nonsplenectomized-thal and from splenectomized-thal is 30%-60% and 25%-31%, respectively, which is less than those caused by oxidized-LDL.

Discussion

β-thalassemia/Hb E is an oxidative stress disease that is a consequence of the disease process(10). This is evident as an increase of plasma lipid peroxidation products and protein peroxidation products in these patients(8,9). Iron overloading is also demonstrated in β-thalassemia/Hb E(27). The presence of high concentration of plasma lipid peroxidation
products in β-thalassemia/Hb E suggests that lipids are extensively modified. Many studies found that lipoproteins have been modified in these patients. It is also showed that thalassemia LDL was cytotoxic to cultured fibroblasts and endothelials, pointing to its role in the atherogenesis-related vascular disease. Due to high oxidative stress in these patients, the authors suspect that LDL of patients may be extensively oxidized and become major factors induces platelet hyperactivity in these patients. To assay for direct effects of LDL from β-thal/Hb E on platelet, the authors measured the initial platelet response, i.e., platelet shape change. Using shape-change parameters was established by Beaumont et al. This technique is shown to be sensitive for assess platelet responses to low dosage or weak stimulants. In the present study, the authors found that LDLs isolated from both non-splenectomized and splenectomized β-thalassemia/Hb E patient did not induce platelet shape change. Thus, LDL isolated from thalassemic patients like LDL from a healthy subject, has no direct effect on platelet. However, this result does not indicate that LDL in thalassemic circulation did not play a role in activation of platelet in vivo. Because the effect of extracted LDL on platelet function in vitro may not be represent the dynamic effect of circulating LDL in vivo. In circulation, thalassemic platelets were continuously exposed to LDL, which might lead to activate those platelets.

Among the methods used to quantify the susceptibility of LDL to oxidation, the determination of LDL oxidation after exposure to copper ion is widely used. It has been shown that the susceptibility of LDL to oxidation by copper ions is closely related to its proneness to biological modification. Even though, in a thalassemia patient, where an imbalance of iron metabolism occurs, a metal ion-dependent mechanism may effectively contribute to LDL oxidation in vivo. After oxidized LDL from both splenectomized- and nonsplenectomized-thal patients with copper, we found that LDL from splenectomized β-thalassemia/Hb E required less amount of copper to initiate the production of bioactive compounds in LDL when compared with LDL from normal and nonsplenectomized patients. Thus, it is suggested that thalassemic LDL may play a role in the activation of the circulating platelet by oxidative stress in β-thalassemia/Hb E patients.

Acknowledgements
This study was supported by the Medical Scholars Program, Mahidol University, Thailand. The technical support of Robert I Grossman, Jeerawan Dangdoungjai and staff at the Blood Bank, Ramathibodi Hospital, Bangkok, Thailand are gratefully acknowledged.

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การเปลี่ยนรูปร่างของเกล็ดเลือดโดยไลโปโปรตีนจากผู้ป่วยธาลัสซีเมีย

วิระศักดิ์ สุทธิพุทธวงศ์, สุภีนันท์ อัญเชิญ, อุดม จันทรารักษ์ศรี, สุทัศน์ ฟู่เจริญ

โรคบีตาธาลัสซีเมีย/ฮีโมโกลบินอีพบได้บ่อยในประเทศไทย การมีภาวะหลอดเลือดใหญ่ที่ปอดอุดตันเป็นสาเหตุของการเสียชีวิต ที่พบบ่อยในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอี เซื่อของการทำงานที่ผิดปกติของเกล็ดเลือดทำให้เกิดภาวะหลอดเลือดใหญ่ แต่ยังไม่มีการศึกษาถึงผลของการไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีต่อการทำงานของเกล็ดเลือด ดังนั้นการศึกษาครั้งนี้จึงทำขึ้นเพื่อศึกษาผลของไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีต่อการทำงานของเกล็ดเลือดโดยวิธีวัดการทำงานเพื่อสรุปการเปลี่ยนรูปร่างของเกล็ดเลือด การทดลองพบว่าไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีไม่สามารถกระตุ้นการทำงานเพื่อสรุปการเปลี่ยนรูปร่างของเกล็ดเลือดได้ แต่พบว่าไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีที่ได้รับการย้อมม้ามแล้วมีความเหมือนกันกว่า ขาดการย้อมม้ามแล้วทำให้เกิดคุณสมบัติในการกระตุ้นการทำงานเพื่อสรุปการเปลี่ยนรูปร่างของเกล็ดเลือดในตัวไลโปโปรตีนเมื่อมีการย้อมม้ามแล้วไลโปโปรตีนจากผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีที่ไม่ได้รับการย้อมม้าม ดังนั้นการทดลองนี้เสนอว่าไลโปโปรตีนผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีจำเป็นต้องมีบทบาทในการเกิดภาวะของเกล็ดเลือด.