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

Werasak Sutipornpalangkul PhD, MD*,
Supeenun Unchern PhD**, Yupin Sanvarinda PhD**,
Udom Chantharaksri PhD**, Suthat Fucharoen MD***

** Department of Orthopaedic Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
*** Department of Pharmacology, Faculty of Science, Mahidol University, Bangkok, Thailand*
**** Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Nakhonpathom, Thailand*

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 nonsplencectomized 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*

J Med Assoc Thai 2009; 92 (4): 504-9
Full text. e-Journal: *http://www.mat.or.th/journal*

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

Correspondence to: Sutipornpalangkul W, Department of Orthopaedic, Faculty of Medicine Siriraj Hospital, Bangkok 10700, Thailand. Phone: 0-2419-7965, Mobile: 081-751-2428, E-mail: tewsv@mahidol.ac.th

been identified in thalassemia blood[11-13]. Oxidized lipoproteins have been shown to affect platelet activity, both *in vitro* and *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 β-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 β-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 μl 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 anticoagulanted 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 μl of 10^5 cells/μl was used as test sample and 600 μl of 5-7 x 10^4 cells/μl 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 μg/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)

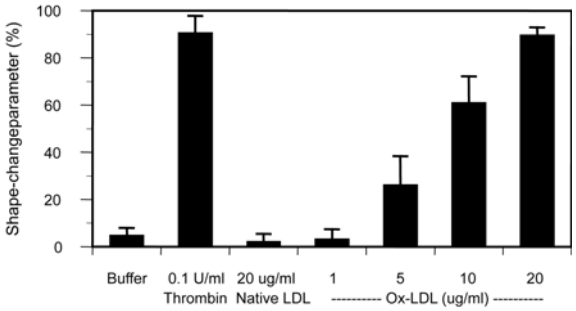

Fig. 1 Effect of native LDL and oxidized-LDL (Ox-LDL) on shape-change parameters. Ox-LDL was prepared by incubation with 50 μM $CuSO_4$ for 24 hr in PBS pH 7.4. Values represent means ± SD (n = 5)

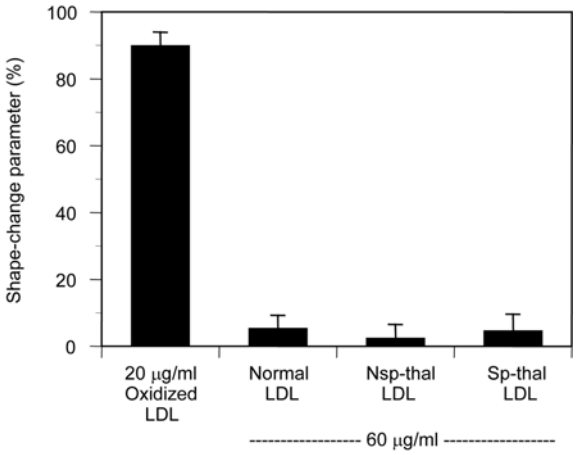

Fig. 2 Effect of oxidized LDL, normal LDL, nonsplenectomized-thal LDL (Nsp-thal LDL) and splenectomized-thal LDL (Sp-thal LDL) on gel-filtrated platelet suspension. 20 μg/ml of oxidized-LDL significantly increased shape change parameter while 60 μg/ml of normal LDL, Nsp-thal LDL and Sp-thal LDL did not. Normal LDL (LDL from healthy volunteer, n = 4), Nsp-thal LDL (LDL from non-splenectomized β-thal/Hb E, n = 3), Sp-thal LDL (LDL from splectomized 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

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

LDLs were incubated with various concentrations of $CuSO_4$ (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 $CuSO_4$ for inducing their platelet-activating activity

Oxidized LDL is the product of incubation of native LDL with various concentration of $CuSO_4$ for 6 hours. The concentrations of Cu^{2+} 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[11-13,28,29]. It is also showed that thalassemia LDL was cytotoxic to cultured fibroblasts and endothelials, pointing to its role in the atherogenesis-related vascular disease[11,30]. 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, *ie.*, platelet shape change. Using shape-change parameters was established by Beaumont et al[23]. This technique is shown to be sensitive for assess platelet responses to low dosage or weak stimulants[18,25]. 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 irons is closely related to its proneness to biological modification[31]. 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.

References

1. Wasi P, Na-Nakorn S, Pootrakul S, Sookanek M, Disthasongchan P, Panich V, et al. Alpha- and beta-thalassemia in Thailand. Ann N Y Acad Sci 1969; 165: 60-82.
2. Fucharoen S, Winichagoon P. Hemoglobinopathies in Southeast Asia. Hemoglobin 1987; 11: 65-88.
3. Fucharoen S, Piankijagum A, Wasi P. Deaths in beta-thalassemia/Hb E patients secondary to infections. Birth Defects Orig Artic Ser 1987; 23: 495-500.
4. Youngchaiyud P, Suthamsmai T, Fucharoen S, Udompanich V, Pushpakom R, Wasi P. Lung function tests in splenectomized beta-thalassemia/Hb E patients. Birth Defects Orig Artic Ser 1987; 23: 361-70.
5. Sonakul D, Pacharee P, Laohapand T, Fucharoen S, Wasi P. Pulmonary artery obstruction in thalassaemia. Southeast Asian J Trop Med Public Health 1980; 11: 516-23.
6. Winichagoon P, Fucharoen S, Wasi P. Increased circulating platelet aggregates in thalassaemia. Southeast Asian J Trop Med Public Health 1981; 12: 556-60.
7. Unchern S, Laoharuangpanya N, Phumala N, Sipankapracha P, Pootrakul P, Fucharoen S, et al. The effects of vitamin E on platelet activity in beta-thalassaemia patients. Br J Haematol 2003; 123: 738-44.
8. Matayatsuk C, Lee CY, Kalpravidh RW, Sirankapracha P, Wilairat P, Fucharoen S, et al. Elevated F2-isoprostanes in thalassemic patients. Free Radic Biol Med 2007; 43: 1649-55.
9. Matayatsuk C, Poljak A, Bustamante S, Smythe GA, Kalpravidh RW, Sirankapracha P, et al. Quantitative determination of ortho- and meta-tyrosine as biomarkers of protein oxidative damage in beta-thalassemia. Redox Rep 2007; 12: 219-28.
10. Rachmilewitz EA, Weizer-Stern O, Adamsky K, Amariglio N, Rechavi G, Breda L, et al. Role of iron in inducing oxidative stress in thalassemia: Can it be prevented by inhibition of absorption and by antioxidants? Ann N Y Acad Sci 2005; 1054: 118-23.
11. Livrea MA, Tesoriere L, Maggio A, D'Arpa D, Pintaudi AM, Pedone E. Oxidative modification of

low-density lipoprotein and atherogenetic risk in beta-thalassemia. Blood 1998; 92: 3936-42.
12. Morales NP, Charlermchoung C, Luechapudiporn R, Yamanont P, Fucharoen S, Chantharaksri U. Lipid fluidity at different regions in LDL and HDL of beta-thalassemia/Hb E patients. Biochem Biophys Res Commun 2006; 350: 698-703.
13. Phumala MN, Cherlermchoung C, Fucharoen S, Chantharaksri U. Paraoxonase and platelet-activating factor acetylhydrolase activities in lipoproteins of beta-thalassemia/hemoglobin E patients. Clin Chem Lab Med 2007; 45: 884-9.
14. Weidtmann A, Scheithe R, Hrboticky N, Pietsch A, Lorenz R, Siess W. Mildly oxidized LDL induces platelet aggregation through activation of phospholipase A2. Arterioscler Thromb Vasc Biol 1995; 15: 1131-8.
15. Takahashi Y, Chiba H, Matsuno K, Akita H, Hui SP, Nagasaka H, et al. Native lipoproteins inhibit platelet activation induced by oxidized lipoproteins. Biochem Biophys Res Commun 1996; 222: 453-9.
16. Chen LY, Mehta P, Mehta JL. Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: relevance of the effect of oxidized LDL on platelet function. Circulation 1996; 93: 1740-6.
17. Liapikos TA, Antonopoulou S, Karabina SP, Tsoukatos DC, Demopoulos CA, Tselepis AD. Platelet-activating factor formation during oxidative modification of low-density lipoprotein when PAF-acetylhydrolase has been inactivated. Biochim Biophys Acta 1994; 1212: 353-60.
18. Sutipornpalangkul W, Unchern S, Sanvarinda Y, Chantharaksri U. Modification of platelet shape change parameter by modified phospholipids in oxidized LDL. J Med Assoc Thai. 2009; 92: 229-35.
19. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955; 34: 1345-53.
20. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 1978; 87: 206-10.
21. Smith JB, Ingerman CM, Silver MJ. Malondialdehyde formation as an indicator of prostaglandin production by human platelets. J Lab Clin Med 1976; 88: 167-72.
22. Grossman I. The effect of dengue-2 virus on the function of normal human platelets separated from plasma [Doctor of medicine thesis]. Bangkok, Thailand: Mahidol University; 1993.
23. Beaumont DO, Whitten KW, Mock RG, Slattery CW. Measuring platelet function with platelet shape change, an early event in aggregation. Thromb Res 1989; 53: 109-27.
24. Latimer P, Born GV, Michal F. Application of light-scattering theory to the optical effects asso ciated with the morphology of blood platelets. Arch Biochem Biophys 1977; 180: 151-9.
25. Ozaki Y, Jinnai Y, Yatomi Y, Kume S. Protein kinase C inhibitors suppress disc-sphere changes of human platelets, as assessed with the shape-change parameter. Eur J Pharmacol 1993; 235: 255-65.
26. Patscheke H, Dubler D, Deranleau D, Luscher EF. Optical shape change analysis in stirred and unstirred human platelet suspensions. A comparison of aggregometric and stopped-flow turbidimetric measurements. Thromb Res 1984; 33: 341-53.
27. Pootrakul P, Huebers HA, Finch CA, Pippard MJ, Cazzola M. Iron metabolism in thalassemia. Birth Defects Orig Artic Ser 1988; 23: 3-8.
28. Brizzi P, Isaja T, D'Agata A, Malaguarnera L, Malaguarnera M, Musumeci S. Oxidized LDL antibodies (OLAB) in patients with beta-thalassemia major. J Atheroscler Thromb 2002; 9: 139-44.
29. Maioli M, Vigna GB, Tonolo G, Brizzi P, Ciccarese M, Donega P, et al. Plasma lipoprotein composition, apolipoprotein(a) concentration and isoforms in beta-thalassemia. Atherosclerosis 1997; 131: 127-33.
30. Banjerdpongchai R, Wilairat P, Fucharoen S, Bunyaratvej A. Morphological alterations and apoptosis of endothelial cells induced by thalassemic serum in vitro. Southeast Asian J Trop Med Public Health 1997; 28 (Suppl 3): 149-54.
31. Frostegard J, Regnstrom J, Tornvall P, Hamsten A, Nilsson J. The susceptibility of low density lipoprotein to chemical oxidation is closely related to proneness to biological modification. Free Radic Res 1995; 23: 581-92.

การเปลี่ยนรูปร่างของเกล็ดเลือดโดยไลโปโปรตีนจากผู้ป่วยธาลัสซีเมีย

วีระศักดิ์ สุทธิพรพลางกูร, สุภีนันท์ อัญเชิญ, ยุพิน สังวรินทะ, อุดม จันทรารักษ์ศรี, สุทัศน์ ฟู่เจริญ

โรคบีตาธาลัสซีเมีย/ฮีโมโกลบินอีพบได้บ่อยในประเทศไทย การมีภาวะหลอดเลือดแดงใหญ่ที่ปอดอุดตันเป็นสาเหตุการเสียชีวิต ที่พบได้บ่อยในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอี เชื่อว่าการทำงานที่ผิดปกติของเกล็ดเลือดทำให้เกิดภาวะดังกล่าวข้างต้น แต่ยังไม่มีการศึกษาถึงผลของออกซิไดซ์ไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีต่อการทำงานของเกล็ดเลือด ดังนั้นการศึกษาครั้งนี้จึงทำขึ้นเพื่อดูผลของไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีต่อการทำงานของเกล็ดเลือดโดยวิธีวัดการเปลี่ยนรูปร่างของเกล็ดเลือด การทดลองพบว่าไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีไม่สามารถกระตุ้นการเปลี่ยนรูปร่าง ของเกล็ดเลือดได้ แต่พบว่าไลโปโปรตีนที่พบในผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีที่ตัดม้ามแล้วมีความไวมากกว่า ต่อการถูกออกซิเดชั่นแล้วทำให้เกิดคุณสมบัติการกระตุ้นการเปลี่ยนรูปร่างของเกล็ดเลือดในตัวไลโปโปรตีน เมื่อเทียบกับไลโปโปรตีนจากคนปกติและไลโปโปรตีนจากผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีที่ไม่ได้รับการตัดม้าม ดังนั้นการทดลองนี้เสนอว่าไลโปโปรตีนผู้ป่วยบีตาธาลัสซีเมีย/ฮีโมโกลบินอีน่าจะมีบทบาทในการเกิดก้อนของเกล็ดเลือด