Red Blood Cell Vesicles in Thalassemia

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Abstract

Vesicles are part of the red blood cells membrane which can be found in a small number in normal apoptotic process and increased in some diseases. In the present study, the authors measured the percentage of red blood cell vesicles in healthy subjects (n = 7), patients with α-thalassemia or Hemoglobin (Hb) H disease (n = 7), β-thal/Hb E with nonsplenectomized (n = 5) and splenectomized (n = 7) before and after induction heated at 48.6°C by using flow cytometry. It was found that the percentage of vesicles in every group were not statistically significantly different ($p > 0.05$) between pre and post incubation at 5 min. The percentage of vesicles of healthy subjects, β-thal/Hb E non-splenectomized patients and splenectomized patients were highest when induced by heating for 60 min. For patients with Hb H disease, the percentage of vesicles was maximum at 30 min when compared with healthy subjects, β-thal/Hb E nonsplenectomized patients and splenectomized patients, respectively. In the present study, the authors report the significant increase of the percentage of vesicles in Hb H disease, β-thal/Hb E nonsplenectomized and splenectomized after induction by heat when compared with healthy subjects. These findings may support the different pathology of the red blood cells found in α- and β-thalassemia.

Key word : Vesicles, Thalassemia, Flow Cytometry

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Loss of red blood cell membrane material in the form of spectrin-free vesicles occur during cell aging[1] and in disease states such as sickle-cell anemia [2], hereditary elliptocytosis[3], and α-thalassemia or Hemoglobin (Hb) H disease[3]. *In vitro*, vesiculation of red blood cells can be induced by ATP depletion [4], spectrin oxidation[5], Ca^{2+}-loaded[6], heated[7,8], and storage of normal red blood cells[9]. All of these manipulations produce stress to cytoskeleton which is the membrane protein that maintains the stability of phospholipid in the lipid bilayers of red blood cell membranes. The exact mechanism of vesiculation is not known, several explanations have been proposed to account for Ca^{2+}-induced phospholipid scrambling [10,11], denaturing spectrin by heat-induction[7,8]. The clinical significance of vesicles in normal hemostasis and hematologic disorders remain unknown. Isolated vesicles can shorten Russell's viper venom clotting time by 55 per cent to 70 per cent of control values[3]. Thus, the vesicles may play a role in the hypercoagulation in some hemolytic disorders and the process of vesiculation itself may contribute to increased rigidity of the red blood cells which subsequently remove them from the circulation[3]. Recently, it was found that patients with meningococcal sepsis who generally suffer from disseminated intravascular coagulation had an elevated number of circulating microparticles[12].

Thalassemia (thal) is a heterogeneous group of genetic hemoglobin disorders resulting from reduced synthesis of α- and β-globin chain[13]. In Thailand, α- and β-thalassemia and abnormal Hb E are common[13-16]. The pathophysiology of diseases related to the degree of anemia is caused by both intramedullary hemolysis and red blood cells destruction in peripheral blood. Chronic hypercoagulable state is also observed in thalassemia, particularly in splenectomized β-thalassemia who receive regular blood transfusions [17]. Evidence has shown that the β-thalassemic red blood cell membranes are less stable and fragment easier than α-thalassemia[18]. The α-thalassemic red blood cells also had relatively better deformability, increased susceptibility to phagocytosis, reduced sialic acid content compared to β-thalassemic red blood cells [19]. Oxidative damage of cytoskeleton in thalassemia may occur from excess globin chains and result in the abnormal cell membranes and deformability[20-23]. The excess α- or β-globin chains might cause membrane damage by different mechanisms, which result in a different number of vesicles in two types of thalassemia. In the present study, the authors determined the red blood cell vesicles in Hb H disease and β-thal/Hb E before and after heating at 48.6°C by using flow cytometry.

MATERIAL AND METHOD

Samples of venous blood were collected in K_2EDTA from healthy volunteers (n = 7), Hb H disease (n = 7), splenectomized (n = 7) and non-splenectomized (n = 5) β-thal/Hb E patients. Their ages range from 17-60 years. The diagnosis of thalassemia was performed on the basis of clinical and laboratory findings as previously described[24]. All patients are in a steady state and had not received a blood transfusion for at least 3 months before blood collection.

For the preparation of heated-induced vesicles [7], the blood was heated at 48.6°C for exactly 5, 10, 20, 30 and 60 min, respectively. The vesicles produced before and after heat induction were stained with phycoerythrin (PE) -labeled anti-glycophorin A (Becton Dickinson, San Jose, USA) and fluorescein-isothiocyanate (FITC) -labeled anti-CD41 (Becton Dickinson, San Jose, USA) for platelets. 1 μl of blood samples were mixed with an equal volume with the above monoclonal antibodies, followed by 16 μl of isotonic buffered, Hemaline (Becton Dickinson, San Jose, USA), then incubated in the dark at room temperature for 15 min. Subsequently, 81 μl of Hemaline was added and then 50 μl of samples were fixed with 600 μl of 1 per cent paraformaldehyde. The samples were analyzed by FACSort flow cytometer with Cell Quest software (Becton Dickinson, San Jose, USA). Both forward scatter and sideward scatter were set at logarithmic gain. The red blood cell vesicles were identified on the scatter with positive binding of PE-labeled anti-glycophorin A and negative FITC-labeled anti-CD41 (Fig. 1).

Data were analyzed with SPSS for Windows, release 7.0. Difference was considered statistically significant at $p < 0.05$. For comparison of the number of vesicles in blood samples, the nonparametric tests were used.

RESULTS

The mean percentage of red blood cell vesicles before and after heat induction at 48.6°C among each group are shown in Table 1. There were no statistically

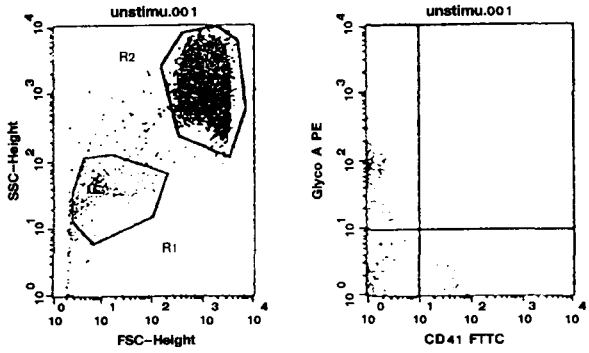

Fig. 1. Representative FACSort dot plots of unstimulated whole blood of β-thal/Hb E subject. A) R1 gate represents platelets and red blood cell vesicles, and R2 gate represents red blood cells. B) 1.59 per cent of red blood cell vesicles are in the upper left (gated on R1) which PE-labeled anti-glycophorin A positive and FITC-labeled anti-CD41 negative.

significant differences between healthy and thalassemic patients before and after heating at 5 min, though the mean percentage of the vesicles of the patients was slightly higher. Significant differences were observed in the number of vesicles of Hb H and β-thal/Hb E splenectomized patients when compared with healthy subjects ($p < 0.05$) if heated at 10 min. In addition, the percentage of vesicles of all thalassemic groups heated at 20 and 30 min were more highly significant than healthy subjects, especially in Hb H patients (Fig. 2). However, when the whole blood was heated at 60 min, the percentage of vesicles of all groups, except the Hb H red blood cells, showed increased results.

DISCUSSION

The red blood cell undergoes spontaneous vesiculation *in vivo* under unknown mechanisms. The authors studied *in vitro* vesiculation of red blood cells under extreme conditions. Heating at 48.6°C for 10 min induced red blood cells to vesiculate as vesicles of various sizes of 1-2 microns. These red blood cell vesicles could be detected by light microscope[7]. However, in the present study the authors enumerated the number of red blood cell vesicles more precisely by using flow cytometry. Our experiment of vesiculation by mean of heat, which denatured spectrin[5], demonstrated the different number and different pattern of change when compared between red blood cell of healthy subjects and thalassemic patients of different genotypes (Fig. 2). In Hb H, marked increase of the percentage of vesicles was found and maximum at 30 min, whereas in β-thal/Hb E nonsplenectomized and splenectomized had a lower percentage at the same time. In addition, heating at 48.6°C for 60 min, the percentage of vesicles in Hb H disease was still higher than normal red blood cells and β-thal/Hb E nonsplenectomized and splenectomized red blood cells, respectively. Furthermore, in Hb H disease, the presence of a high level of red blood cell vesicles can be explained by the excess β-globin chains which is unstable and could precipitate more as β_4 which damage the red blood cell membrane resulting in vesiculation[25]. Unlike α-thalassemia, the excess α-globin chain, cannot form such homo-tetramers, and upon synthesis bind the cytoplasmic surface of the membrane where they produce oxidative damage. Although the clinical picture of β-thalassemic red blood cells was more severe than α-thalassemic red blood cells, the level of vesicles were lower when heated. This suggests that the degree of membrane skeletal protein defects when heated is different be-

Table 1. The means ± SD of percentage of red blood cell vesicles before and after incubation at 48.6°C of healthy and thalassemic subjects.

Subjects	Mean percentage of vesicles					
	0 min*	5 min	10 min	20 min	30 min	60 min
Healthy (n = 7)	0.94 ± 0.26	2.71 ± 0.72	2.52 ± 0.98	2.80 ± 0.88	3.28 ± 1.73	13.10 ± 5.19
Hb H disease (n = 7)	1.56 ± 1.65	4.84 ± 2.50	11.55 ± 9.30	20.43 ± 12.69	26.87 ± 12.77	24.90 ± 15.34
β-thal/Hb E (ns) (n = 5)	3.30 ± 2.96	2.78 ± 1.26	3.89 ± 0.94	5.06 ± 1.87	6.32 ± 3.66	8.84 ± 5.28
β-thal/Hb E (s) (n = 7)	2.29 ± 1.43	4.39 ± 2.59	7.04 ± 5.00	6.90 ± 4.85	7.45 ± 5.43	8.58 ± 6.78

* unstimulated, ns = nonsplenectomized, s = splenectomized

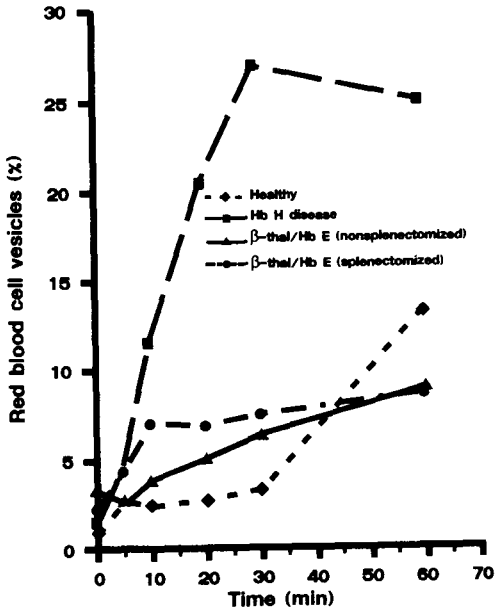

Fig. 2. Comparison of mean percentage of red blood cell vesicles before and after incubation at 48.6°C of healthy and thalassemic subjects.

tween β- and α-thalassemic red blood cells. The homotetramers of β_4 in α-thalassemia in the form of inclusion bodies could precipitate more when heated, causing more vesiculation. Whereas the unstable excess α-globin chain might not be changed when exposed to heat, this will not damage the membrane any further, leading to lower levels of vesicles than α-thalassemia.

It is known that red blood cells undergo spontaneous vesiculation *in vivo* under a variety of conditions. Normal red blood cells vesiculate during physiologic aging[3,5], therefore, the higher percentage of red blood cell vesicles seen in thalassemia blood than in normal blood after heating suggested that, apart from red blood cells senescence, other mechanisms characterized by intrinsic membrane defects are likely to be involved in the vesiculation process. Indeed, several pathologic red blood cell known to associate with membrane damage such as sickle cell anemia, spherocytosis, hemolytic elliptocytosis, band 4.1 deficiency, and Hb H diseases have a tendency toward fragmentation and microvesiculation[3,5,26]. *In vitro* manipulations that are known to disrupt the membrane protein network including spectrin cross-link with diamide treatment, spectrin aggregation under pH 5.4 or heating at 49°C, are further evidence for marked vesiculation of blood[3,5,26,27]. It is therefore likely that in thalassemia, precipitation of unstable globin chains and heat-denatured on red blood cells during Hb H crisis could lead to disruption of protein-lipid interaction which will eventually induce red blood cell to vesiculate.

In summary, there is a significant increase of the percentage of vesicles in Hb H disease, β-thal/Hb E nonsplenectomized and splenectomized after induction by heat when compared with healthy subjects. These findings may support the different pathology of the red blood cells found in β- and α-thalassemia due to excess β- and α-globin chains which had highly specific effects to membrane stability.

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vesicles ของเม็ดเลือดแดงในธาลัสซีเมีย

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vesicles คือส่วนของเยื่อหุ้มเซลล์เม็ดเลือดแดงที่หลุดออกมา ซึ่งพบได้เล็กน้อยในภาวะปกติระหว่างการตายของเซลล์และเพิ่มจำนวนสูงขึ้นในบางโรค ในการศึกษานี้ได้วัดจำนวน vesicles ของผู้มีสุขภาพดี (7 ราย), ผู้ป่วยอัลฟา-ธาลัสซีเมียหรือโรคฮีโมโกลบิน เอช (7 ราย), เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้าม (5 ราย) และเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ตัดม้าม (7 ราย) ก่อนและหลังการกระตุ้นเม็ดเลือดแดงด้วยความร้อน 48.6°C โดยใช้วิธีโฟลไซโตเมทรี พบว่าก่อนการกระตุ้นและหลังการกระตุ้น 5 นาที จำนวนร้อยละของ vesicles ที่เกิดขึ้นในกลุ่มต่าง ๆ ไม่แตกต่างอย่างมีนัยสำคัญทางสถิติ ($p > 0.05$) จำนวนร้อยละของ vesicles ของผู้มีสุขภาพดีและเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้ามและตัดม้ามจะเพิ่มมากที่สุดเมื่อกระตุ้นด้วยความร้อนนาน 60 นาที แต่ในฮีโมโกลบิน เอช จะมากที่สุดเมื่อกระตุ้นนาน 30 นาที เมื่อเทียบกับผู้มีสุขภาพดี, เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้ามและตัดม้าม ตามลำดับ การศึกษานี้ได้รายงานการเพิ่มสูงขึ้นของจำนวน vesicles ในผู้ป่วยโรคฮีโมโกลบิน เอช, เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้าม และตัดม้ามหลังจากการกระตุ้นด้วยความร้อนเมื่อเทียบกับผู้มีสุขภาพดี และข้อมูลนี้ช่วยสนับสนุนว่าพยาธิสภาพของเม็ดเลือดแดงมีความแตกต่างกันระหว่าง อัลฟา- และ เบต้า-ธาลัสซีเมีย

คำสำคัญ : vesicles, ธาลัสซีเมีย, โฟลไซโตเมทรี

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