Original Article

Effects of Thrombopoietic Growth Factors on Megakaryocytopoiesis in Post-splenectomized Thalassemic Patients with Thrombocytosis

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Abstract:

Thrombocytosis is a frequently documented complication among postsplenectomized thalassemic patients (Thal/postsplenec).[1] To elucidate the possible causes of high platelet counts among these patients, growth and development of marrow mononuclear cells to generate colony forming unit-megakaryocytes (CFU-Meg) were investigated. All patients with Thal/postsplenec had notably high platelet counts (> 500 x 10^9/L) similar to those with chronic myeloproliferative disorders (MPD) and reactive thrombocytosis, whereas the platelet counts in nonsplenectomized thalassemic patients (Thal/nonsplenec) were comparable to those of the control. Using plasma clot culture in the absence of exogenous growth factors, the spontaneous growth of CFU-Meg appeared only in MPD whereas none was found in the other groups. In the presence of interleukin -3 (IL-3) and thrombopoietin (Tpo), the morphological features of the growing CFU-Meg in Thal/postsplenec were similar to those in controls and Thal/nonsplenec in which IL-3 stimulated the proliferation of immature megakaryocytes. Tpo alone stimulated differentiation and maturation of megakaryocytes. The response to IL-3 and Tpo stimulation in all groups showed concentration-related effects with a similar extent of responses, indicating that the elevated platelet counts in Thal/postsplenec were not associated with spontaneous growth or hyperresponsiveness of the stem cells to growth factors. However, serum Tpo levels in Thal/postsplenec were significantly elevated. These results suggest that the elevation of Tpo in Thal/postsplenec might be a major factor contributing to the high platelet counts among Thal/postsplenec patients.

Keywords : ● *Megakaryocytopoiesis* ● *Thalassemia* ● *Thrombopoietin* ● *Thrombocytosis* ● *Splenectomy*

J Hematol Transfus Med 2017;27:423-31.

Received 27 February 2017 Accepted 12 April 2017

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นิพนธ์ต้นฉบับ

การศึกษาผลกระทบของธรอมโบพอยติคโกรทแฟคเตอร์ต่อการสร้างเมกาคาริโอไซต์ในผู้ป่วยธาลัสซีเมียหลังตัดม้ามที่มีภาวะเกล็ดเลือดสูง

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บทคัดย่อ

เกล็ดเลือดสูงเป็นภาวะแทรกซ้อนที่พบได้ในผู้ป่วยธาลัสซีเมียหลังตัดม้าม เพื่อค้นหาสาเหตุของความผิดปกติที่ทำให้มีภาวะเกล็ดเลือดสูงในผู้ป่วยกลุ่มดังกล่าวจึงได้ศึกษาถึงความสามารถของเซลล์ไขกระดูกจากผู้ป่วยในการเจริญเติบโตและพัฒนาการ จากเซลล์เดี่ยวที่มีนิวเคลียสเดียวมีพัฒนาการผลิตกลุ่มเซลล์เมกาคาริโอไซต์ (CFU-Meg) ที่มีสมบัติแตกต่างออกไป ผลการศึกษาพบว่าผู้ป่วยธาลัสซีเมียหลังจากตัดม้ามแล้วทุกราย มีปริมาณเกล็ดเลือดสูงกว่าปกติ (> 500 x 10^9/ลิตร) คล้ายกับที่พบในกลุ่มผู้ป่วยที่มีภาวะผิดปกติของการแบ่งตัวของเซลล์ ขณะที่ผู้ป่วยธาลัสซีเมียที่ไม่ได้ตัดม้ามมีปริมาณเกล็ดเลือดอยู่ในเกณฑ์ปกติ จากการเพาะเลี้ยงเซลล์ไขกระดูกจากผู้ป่วยดังกล่าวด้วยวิธีพลาสมาคลอท พบว่าภาวะที่ไม่มีสารกระตุ้นการการเจริญเติบโต เซลล์ของผู้ป่วยธาลัสซีเมียหลังตัดม้ามคล้ายกับเซลล์ของคนปกติและผู้ป่วยธาลัสซีเมียที่ไม่ตัดม้าม กล่าวคือไม่สามารถผลิตกลุ่มเซลล์เมกาคาริโอไซต์ (CFU-Meg) ในขณะที่ผู้ป่วยที่มีความผิดปกติของการแบ่งตัวของเซลล์ สามารถผลิตกลุ่มเซลล์เมกาคาริโอไซต์ได้ (CFU-Meg) นอกจากนี้ยังพบว่าการเจริญเติบโตและการตอบสนองของเซลล์ต่อสารกระตุ้น interleukin-3 (IL-3) หรือ thrombopoietin (Tpo) เมื่อให้ตามลำพัง หรือให้ร่วมกันในผู้ป่วยธาลัสซีเมียตัดม้ามเกือบทุกรายไม่แตกต่างจากเซลล์คนปกติและผู้ป่วยธาลัสซีเมียที่ไม่ตัดม้าม โดยสาร IL-3 มีผลกระตุ้นการแบ่งตัวของเซลล์ที่ยังเยาว์ ส่วนสาร Tpo สามารถกระตุ้นเซลล์ให้มีพัฒนาการไปเป็นเซลล์โตเต็มวัยที่สมบูรณ์ได้ แต่เมื่อให้สารกระตุ้น IL-3 และ Tpo พร้อมกันไม่พบว่ามีการเสริมฤทธิ์กัน จากการศึกษานี้ชี้ให้เห็นว่าภาวะเกล็ดเลือดสูงในผู้ป่วยธาลัสซีเมียที่ตัดม้ามไม่ได้มีสาเหตุหลักมาจากความผิดปกติของเซลล์ในการแบ่งตัวและเจริญ หรือการตอบสนองต่อสารกระตุ้น แต่อย่างไรก็ตามได้พบว่าระดับของสาร Tpo ในซีรั่มของเลือดและของไขกระดูกของผู้ป่วยที่ได้รับการตัดม้ามสูงกว่าผู้ป่วยธาลัสซีเมียที่ไม่ได้ตัดม้าม อาจสรุปได้ว่าระดับ Tpo ซึ่งเป็นสารกระตุ้นการสร้างเกล็ดเลือดอาจจะเป็นปัจจัยที่สำคัญที่มีผลต่อการเพิ่มของเกล็ดเลือดในผู้ป่วยหลังตัดม้าม

คำสำคัญ : ● *Megakaryocytopoiesis* ● *Thalassemia* ● *Thrombopoietin* ● *Thrombocytosis* ● *Splenectomy*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2560;27:423-31.

Introduction

Persistent thrombocytosis has long been documented among a large number of postsplenectomized thalassemic patients (thal/postsplenec).[1,2] However, at present, the mechanism underlying the association of thrombocytosis with splenectomy among these patients is still not clear. Several possibilities can be considered. Thrombocytosis may be due to either a spontaneous growth of megakaryocytes, an increase in sensitivity of megakaryocyte progenitors to cytokines or an overproduction of some thrombopoietic growth factor acting on megakaryocytes.

Changes in circulating levels of blood platelets are determined mainly by a change in the number of megakaryocytes during thrombopoiesis. Although the physiological mechanisms by which the regulation of platelet production by bone marrow megakaryocytes are unclear, several cytokines including interleukin-1 (IL-1), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-11 (IL-11) and thrombopoietin (Tpo) have been reported to play important roles.[3] The injection of Tpo in mice has been demonstrated to induce a marked increase in megakaryocyte and platelet count.[4] Furthermore, Tpo-deficient mice, generated by gene targeting, exhibited a striking decrease in megakaryocytes and platelets whereas other types of blood cells remained unaffected.[5,6] All these reports suggest that Tpo is the major physiologic regulator for platelet production. In reactive thrombocytosis (RT) and chronic myeloproliferative disorder (MPD), Tpo levels have been reported to be elevated despite an increase in megakaryocyte/platelet mass.[7,8] Quite possibly, the high platelet counts in thal/postsplenec may relate to abnormalities of cells such as defective proliferation or responsiveness to growth-stimulating factors or increased Tpo levels.

The present study aimed to investigate the effect of exogenous thrombopoietic growth factors, particularly Tpo and IL-3, on the growth of megakaryocyte progenitor cells or colony forming-unit megakaryocytes (CFU-Meg). The relationship between Tpo level and persistent postsplenectomy thrombocytosis in thal/postsplenec was determined.

Materials and Methods

Patients

Peripheral blood samples by routine venopuncture were obtained from 14 normal adult controls, 17 patients with nonsplenectomized thalassemia/hemoglobin E subjects (thal/nonsplenec), 4 patients with postsplenectomized nonthalassmia, 18 patients with postsplenectomized thalassemia/hemoglobin E (thal/postsplenec), 15 patients with chronic myeloproliferative disorders (MPD) and 13 patients with reactive thrombocytosis (RT). After informed consent was obtained, bone marrow was aspirated from the posterior superior iliac crest of 4 normal adult controls, 5 patients with thal/nonsplenec, 12 patients with thal/postsplenec and 2 patients with chronic MPD.

Blood sample collection

Serum was kept at -70°C for further analysis of Tpo level. Thrombopoietin concentrations were determined using enzyme-linked immunoabsorbent assay (ELISA) with commercially available kits (R&D Systems, Inc., Minneapolis, MN, USA). The limits of sensitivity of this assay were < 15 pg/mL.

Megakaryocyte colony-forming unit (CFU-Meg) assay

Megakaryocyte progenitor cells (CFU-MEG) were assayed using a plasma clot culture system as previously described.[9] Marrow cells were collected in heparin (5 U/mL) and diluted 1:1 with Iscove's modification of Dulbecco's minimum essential medium (IMEM) containing 50 IU/mL preservative-free heparin. Light density cells were separated by centrifugation at 700 x g 20 min over Ficoll-Hypague (FH: 1.077) and T-lymphocytes and adherent cells were further depleted by sheep erythrocyte rosetting and 1-h adherence to plastic. For culture, 4 x 10^5 cells were cultured in 1 mL of IMEM consisting of 10% AB serum supplemented with 150 (μg/mL human iron-saturated transferrin, 1% bovine serum albumin, 1.5 mM epsilon-aminocaproic acid,

10^{-5}M beta-mercaptoethanol, 200 U/mL, penicillin, 20 g/mL streptomycin and 10% fetal calf serum. Clotting was induced by the addition of 2 mg/mL predialyzed human fibrinogen and 0.2 U/mL bovine thrombin. Before clotting, cells and complete medium (0.25 mL) were transferred to wells in a multi-well tissue culture tray. All cultures were performed in quadruplicate and growth factor was added on day-1. After 12-15 days of incubation at 37°C in a humidified atmosphere containing 5% CO_2 cultures were evaluated. A CFU-Meg derived colony was defined as a cluster of three or more cells after staining the clot by an immunoperoxidase method using the monoclonal antibody.[10]

Statistical Analysis

All data were expressed as means ± the standard error of mean (mean ± SEM). One-way analysis of variance and Student Newman Keul's test were used to determine the significance of any difference. The difference of means between two groups was determined using the unpaired Student's *t*-test.

Results

Clinical characteristics of control subjects and patients are summarized in Table 1. The age and sex distribution of thal/nonsplenec, thal/postsplenec, nonthalassemia splenectomy were similar whereas the mean age of RT and MPD were higher. A wide range of time had elapsed since splenectomy in thal/postsplenec patients, ranging from 0.25 to 25 years (11.2 ± 1.9 years). Platelet counts in the thal/postsplenec group were much higher than those in the normal control and thal/nonsplenec, splenectomized nonthalassemia groups ($p < 0.05$), but were significantly lower than those in the RT and MPD groups ($p < 0.05$).

Megakaryocyte precursors or CFU-Meg were identified in the cultures using a specific monoclonal antibody against GPIIb/IIIa, a megakaryocyte surface antigen, as shown in Figure 1. By using this specific marker for megakaryocytes, no other identifiable hematopoietic cells were labeled in our culture. GPIIb/IIIa is specifically expressed on the cell surface of megakaryocytes. In the absence of exogenous growth factors, spontaneous growth of CFU-Meg was observed only among chronic MPD patients whereas none was found in the control, thal/nonsplenec or thal/postsplenec groups.

The responsiveness of bone marrow mononuclear cells to either IL-3 or Tpo or their combination was investigated. By this staining method, IL-3 stimulated

Table 1 Clinical characteristics of subjects

Parameters	Normal control	Thalassemia/ nonsplenectomy	Thalassemia/ postsplenectomy	MPD	RT	Non-thalassemia /splenectomy
No. of subjects	14	17	18	15	13	4
Age (years)	27 ± 2	26 ± 1.7	29 ± 2	57.3 ± 4.7	46 ± 4	28.5 ± 6
Male/Female	9/4	9/8	11/7	7/7	7/6	2/2
β-thalassemia/HbE	-	17	18	-	-	-
Blood transfusion	-	1	6	-	-	3
Desferrioxamine	-	-	11	-	-	-
WBC (x 10^9/L)	5.8 ± 0.3	7.4 ± 0.5	9.6 ± 0.5	25.9 ± 13.1	13.6 ± 1.6	13 ± 2.7
Hb (g/dL)	14 ± 0.4	7.1 ± 0.4	6.7 ± 0.2	11.5 ± 0.7	10.4 ± 0.5	11.9 ± 0.1
Hct (%)	42.1 ± 0.9	23.6 ± 1.3	23 ± 0.6	35.2 ± 2.1	33.3 ± 1.5	37.4 ± 0.5
Platelet count (x 10^9/L)	268 ± 22	249 ± 30	687 ± 33*$^+$	905 ± 58*	869 ± 96*	349 ± 59*

Values of blood biochemistry are means ± SEM; MPD and RT are myeloproliferative disorders and reactive thrombocytosis, respectively; *$p < 0.05$ significantly different from normal control; $^+p < 0.05$ significantly different from MPD and RT

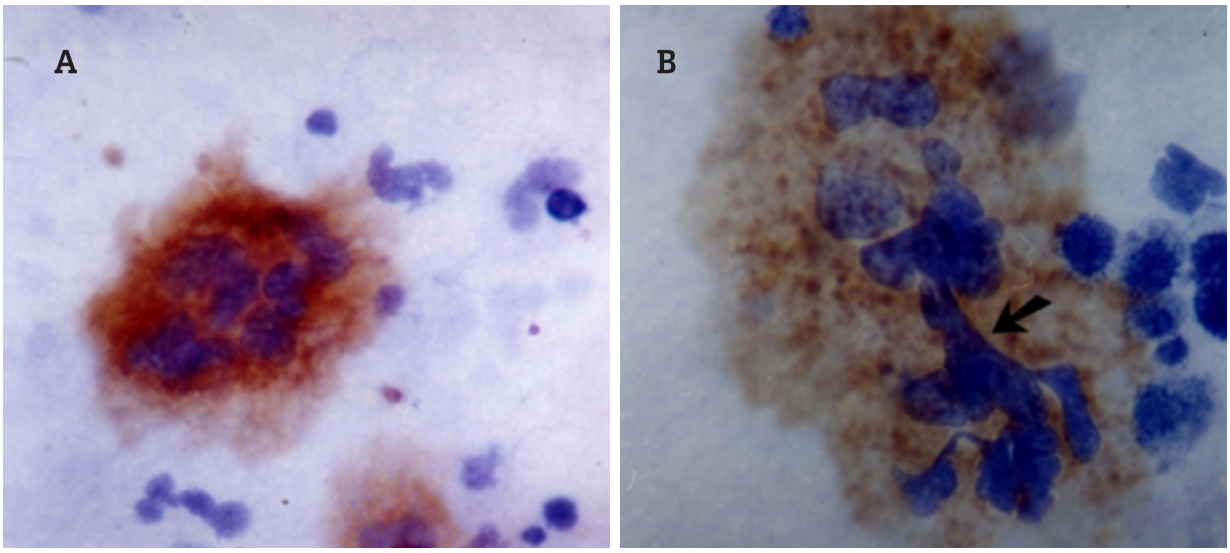

Figure 1 Light micrographs showing individual megakaryocytes (Meg) within the colony from a normal control subject in plasma clot culture in the presence of interleukin-3 (IL-3) 400 ng/mL (**A**) and thrombopoietin (Tpo) 100 ng/mL (**B**) The cultures were fixed and Meg colonies identified by immunocytochemical staining of CD41+. Counterstain is with hematoxylin. Multilobular nuclei (arrow) were seen in cultures containing Tpo (x 1,000).

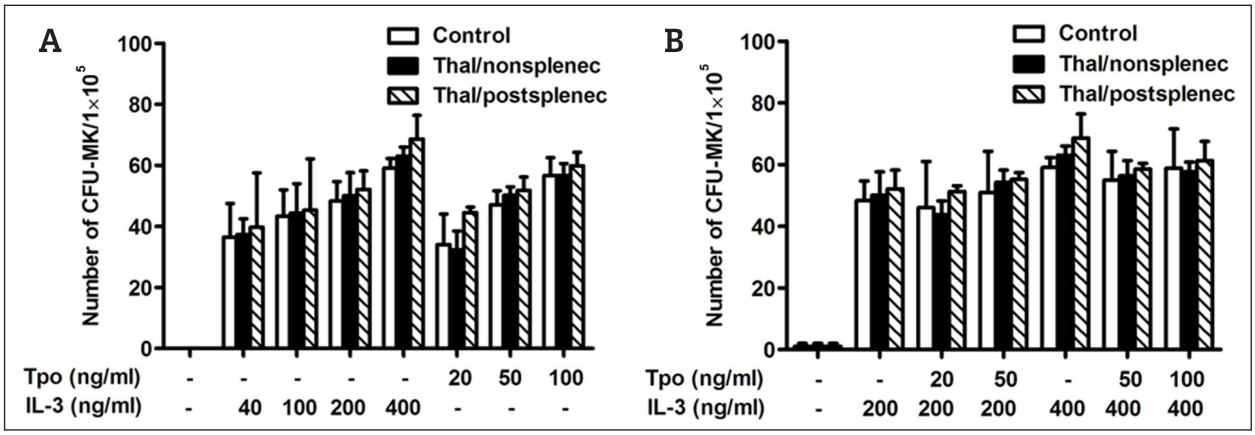

Figure 2 Effect of thrombopoietin (Tpo) and IL-3, added either alone (**A**) or in combination of two growth factors (**B**), on megakaryocyte colony formation by mononuclear cells, 1 x 10^5 per well. They were obtained from normal control subjects, patients with nonsplenectomized β-thalassemia/hemoglobin E (thal/nonsplenec), and postsplenectomized β-thalassemia/hemoglobin E (thal/postsplenec) patients.
Values are means ± 2sd SEM obtained from 4-12 experiments.

the growth of immature megakaryocytes in which the cells were small and contained a single round nucleus staining for CD41+ in spot fashion; however, a few cells with a small number of lobular nuclei were also observed. On the other hand, Tpo stimulated differentiation, and cytoplasmic and nuclear maturation of megakaryocytes in which the megakaryocytic cell appeared larger in size with multilobular nuclei and widely stained for CD41+ (Figure 1).

The formation of CFU-Meg colonies in response to IL-3 or Tpo stimulation in all groups showed concentration-related effects (Figure 2). In the presence of IL-3 (40-400 ng/mL) and Tpo (20-100 ng/mL), the CFU-Meg numbers and morphological features of the growing CFU-Meg in thal/postsplenec were similar to those in thal/nonsplenec and the controls (Figure 2a). In the presence of different combinations of IL-3 and Tpo, CFU-Meg formation was similar to cultures containing

IL-3 alone, but larger colonies with multilobular nuclei were more prominent (Figure 2b). The extent of response in most of thal/postsplenec was similar to those in thal/nonsplenec and the controls, except that two cases of thal/postsplenec showed apparently greater response.

To evaluate any abnormalities in the production of thrombopoietic growth factors, serum Tpo levels were measured among controls and patients (Figure 3). In thal/postsplenec, the serum Tpo was significantly elevated when compared with that in normal controls ($p < 0.05$), but did not significantly differ from that in thal/nonsplenec, MPD groups. Because of the wide variation between individuals and the limited number of patients, Tpo levels among MPD patients did not significantly differ from that among normal control subjects, although a trend toward a higher Tpo level was observed in the MPD group. Tpo levels in bone marrow serum was also determined and found to be elevated in most of thal/postsplenec (20-260 pg/mL) whereas those in thal/non-splenec and MPD were very low and less than 20 mg/mL.

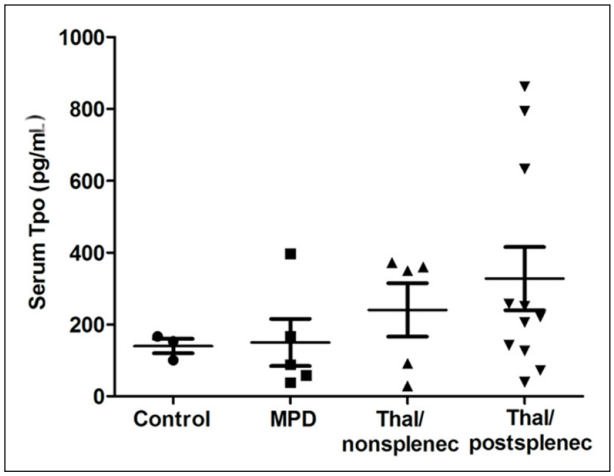

Figure 3 Serum thrombopoietin (Tpo) levels in normal control subjects, patients with myeloproliferative disorders (MPD), patients with nonsplenectomized β-thalassemia/hemoglobin E (thal/nonsplenec) and patients with splenectomized β-thalassemia/hemoglobin E (thal/posesplenec).
Horizontal bars indicate the mean value in the group.

Discussion

The present study demonstrated that thrombocytosis in thal/postsplenec is not due to spontaneous proliferation of megakaryocytes. The study showed that when megakaryocytes were cultured in the absence of growth stimulating factors, the spontaneous colony formation appeared only in MPD and none were found in the other groups, suggesting that thrombocytosis in thal/postsplenec is unrelated to the intrinsic defect of spontaneous proliferation of stem cells. In addition, thrombocytosis in thal/postsplenec was unrelated to a defect at the growth and differentiation stages of megakaryocytes. Hyperresponsiveness to cytokines also did not generally occur in thal/postsplenec. However, the high level of Tpo in the circulation in thal/postsplenec suggests that elevation of Tpo might be a major factor responsible for thrombocytosis. To our knowledge, this is the first study of *in vitro* growth and development of megakaryocytes from thalassemic patients both with and without splenectomy.

Thrombocytosis has been found in patients who were splenectomized from other causes; however, their elevated platelet counts shortly returned to the normal range[11]. In contrast, in the present study, thrombocytosis remained in postsplenectomy for up to 25 years. In addition, no correlation was detected between the extent of thrombocytosis and the amount of time since splenectomy. Therefore, we suggest that the asplenic condition itself was not the cause of thrombocytosis among thal/postsplenec patients. Therefore, other factors must be contributing to the high level of platelet counts.

Development of human megakaryocytes is under the influence of several thrombopoietic growth factors. However, none of these growth factors is reported to be effective in promoting megakaryocyte colony formation when present alone in culture.[12,13] Results of our studies showed that IL-3 initiated megakaryocyte progenitor cell development but did not complete the process of maturation, whereas Tpo acted on both proli-

feration and differentiation of megakaryocytopoiesis. Tpo influenced both endo-replication and complete cytoplasmic maturation. These results are consistent with the earlier reports showing that IL-3 is a potent stimulant of CFU-Meg proliferation but has little effect on megakaryocytes maturation.[14,15] In the present study, the responsiveness of megakaryocyte from patients with thal/postsplenec to IL-3 and/or Tpo did not differ from those from normal control subjects and thal/nonsplenec. We suggest that the high platelet count in thal/postsplenec is not due to hypersensitivity of megakaryocyte to IL-3 or Tpo. Although the responsiveness of mononuclear cells of thal/postsplenec to either IL-3 or Tpo alone or in combination did not differ from that of normal control subjects and thal/nonsplenec. Interestingly, 2 of 18 cases of thal/postsplenec generated higher megakaryocyte colonies than both normal control subjects and thal/nonsplenec. It seems that there is a variation of marrow CFU-Meg characteristics vary in the thal/postsplenec population.

Abnormalities in the production of various endogenous growth-stimulating factors could also be responsible for the high platelet count in postsplenec/thal. In our thal/postsplenec samples, Tpo levels in both serum and bone marrow were markedly elevated. At present, the relationship between serum Tpo and the number of platelets in circulation is not clearly understood. In our normal control subjects, an inverse relationship between serum Tpo levels and platelet counts was observed, whereas, this was not found among thalassemic patients with both nonsplenec and postsplenec. Previously, Kuter and Rosenberg (1995)[16] described the relationship between the Tpo level and platelet counts, noting that the basal level of Tpo is usually low and its circulating level varies reciprocally and proportionally to changes in the platelet mass. The direct regulation of Tpo by the platelet mass was further explained by uptake of Tpo via Tpo receptors on platelets, which was then internalized and degraded.[17]

The increase in serum Tpo along with the great increase in platelet counts as found in thal/postsplenec subjects, might be related to a defect in the feedback mechanism controlling Tpo levels. Two possibilities might be considered to account for a defect in the feedback mechanism. First, it might be due to abnormalities of platelet function as reported by several studies[18,19], where the postsplenectomized group had a higher incidence of platelet hyperaggregation. Moreover, changes in the morphology of thalassemia platelets in postsplenectomized thalassemic patients with lower platelet pseudopodia reversibility compared with nonsplenectomized cases where platelet counts were normal have been reported.[20-24] Therefore, the abnormality in platelet functions and/or morphological changes of platelets might alter the ability of platelets to clear Tpo from circulation. These changes were also reported in the MPD model.[22] Moreover, the elevation of serum Tpo might be related to an over expression of Tpo mRNA in organs which are Tpo production sites. Currently, Tpo is reported to be produced in several organs throughout the body such as the liver and kidneys.[25,26] However, it has been reported that the level of Tpo mRNA expression in these tissues did not vary with circulating Tpo concentrations or platelet numbers.[27] Therefore, it can be concluded that the serum Tpo level is regulated not only by Tpo gene expression or only by platelet number in the circulation, but by both the total counts of megakaryocytes in bone marrow and the spleen and of platelets in circulation.[28]

Interestingly, in the present study, bone marrow Tpo levels in most of the thal/postsplenec were higher than those in MPD and thal/nonsplenec. These results might indicate an overproduction of Tpo in the bone marrow, which is the major site of megakaryocytopoiesis. This might be one of the major causes of thrombocytosis in thal/postsplenec. The increase in Tpo levels might influence megakaryocyte maturation in the pool, resulting in an increase in expression in the number of completely mature megakaryocytes and polyploidies, even-

tually leading to an increase in platelet production per megakaryocyte.

It can be concluded that thrombocytosis in thal/postsplenec is not associated with spontaneous growth or hyperresponsiveness of the stem cells to the growth factors. The marked elevation of serum Tpo levels in thal/postsplenec is suggested to be a major factor contributing to the high platelet counts among patients. Further study to understand the regulation of megakaryocyte maturation and platelet formation among these patients will throw some light on the detailed mechanism and medical therapy.

Authors' Contribution

Suporn Chuncharunee initiated, and collected samples from patients, analyzed the data and composed the manuscript. Napaporn Archararit conducted the biochemical assay and plasma clot culture experiments. Ladda Leungratanamart conducted the experiments, analyzed the data and drafted the manuscript. Pawinee Piyachaturawat coordinated the project, interpreted the data and revised the manuscript. All authors approved of the final version of the manuscript.

Disclosure of conflict of interest

All authors have no conflict of interest to report.

Acknowledgements

This study was supported by a grant for joint research between the Faculty of Medicine, Ramathibodi Hospital and the Faculty of Science, Mahidol University.

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