

Restrictive Lung Disease and Serum TGF- β 1 in Thalassemia Major Children

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SUMMARY A cross sectional study was performed in 21 thalassemia major (TM) children at King Chulalongkorn Memorial Hospital during March to August, 2003 to determine whether restrictive lung disease (RLD) was related to serum transforming growth factor-beta 1 (TGF- β 1). All studied patients (57% female, age 11.2 ± 2.6 yrs, duration of transfusion 7.7 ± 4.1 yrs) never had desferoxamine treatment and their pulmonary function, serum ferritin and serum TGF- β 1 were evaluated. Five (24%) had RLD. RLD patients had significantly longer durations of transfusion and higher serum ferritin levels than non-RLD patients (9.1 ± 1.9 vs 5.5 ± 3.2 yrs; $p = 0.03$ and $3,816.6 \pm 1,715.9$ vs $2,084.5 \pm 1,504.8$ ng/ml; $p = 0.04$, respectively). TM children had lower serum TGF- β 1 levels than normal children (7.9 vs 78.8 pg/ml; $p < 0.001$). The serum TGF- β 1 level was not different between RLD and non-RLD patients (13.3 vs 4.2 pg/ml; *ns*), concluding that RLD was related to longer duration of transfusion and higher serum ferritin but not related to serum TGF- β 1 levels.

Thalassemia major is an inherited disorder of the hemoglobin synthesis. Clinical presentations include severe anemia and transfusion dependence. Frequent hemolysis and frequent blood transfusions as well as increased iron absorption from the gut result in an iron overload of the tissues of various organs and lead to multiple organ dysfunctions.¹ Pulmonary function abnormalities, especially restrictive lung disease (RLD), have been reported in many thalassemic patients. The prevalence of RLD varied from 0-80%, depending on the definition of RLD and the method of pulmonary function testing used in each study. However, the nature and pathogenesis of the abnormal lung functions are still controversial.²⁻¹⁵

Several studies demonstrated the association between increased body iron storage and lung dysfunctions in thalassemic patients.^{3,4,7,12,15} Radiologic evidences of interstitial lung disease were reported in

some thalassemia major patients who had RLD.^{12,16} However, whether increased fibrous tissue in the lungs contributes to RLD in these patients has not been well established.

Transforming growth factor- β 1 (TGF- β 1) is an inflammatory cytokine that plays a role in promoting fibroblastic proliferation and matrix accumulation in tissues. It is secreted by antigen-stimulated T cells, lipopolysaccharide-activated mononuclear phagocytes and many other cell types including platelets, epithelial cells and fibroblasts. The major biologic action is to promote the synthesis of ex-

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tracellular matrix protein such as collagen.¹⁷ TGF- β 1 was found to be increased in the circulation and alveolar fluid of patients who had fibrotic diseases.¹⁸⁻²¹ However, its level in thalassemia major patients, especially in those who have RLD, has not been reported yet. We hypothesized that thalassemia major children who had RLD would have increased TGF- β 1 in the circulation if this RLD was related to pulmonary fibrosis.

This study was designed to determine the prevalence of RLD in thalassemia major children and its relationship with the duration of blood transfusion, serum ferritin and serum TGF- β 1 levels. Since desferoxamine, an iron chelating agent, could be the potential cause of pulmonary dysfunction²² and could affect serum ferritin levels, we included only those who never had iron chelation therapy.

PATIENTS AND METHODS

Thalassemia major children aged 9-15 years who required at least 1 blood transfusion per month and never had iron chelation therapy were enrolled from the Hematology Clinic, King Chulalongkorn Memorial Hospital, during March to August, 2003. Study patients included those who 1) had no current infection, 2) had no neuromuscular or respiratory illness that could contribute to pulmonary dysfunction, 3) were currently not on any medications except for folic acid, 4) never had bone marrow transplantation, and 5) demonstrated good effort and ability in performing pulmonary function tests.²³

Informed consents were obtained from the caregivers prior to the study. The study protocol was approved by the Ethics Committee for Human Research Study of King Chulalongkorn Memorial Hospital. Demographic data including age and gender as well as duration of blood transfusion were recorded. All patients had 10 ml of blood collected prior to their regular blood transfusion on the study day. The blood specimens were allowed to clot for 1 hour at room temperature and then incubated overnight at 4°C prior to centrifugation. After centrifugation, the sera were aliquoted and stored at -70°C. At the end of the patient enrollment, all stored sera were thawed and evaluated for ferritin and TGF- β 1 levels. The stored sera of normal children (age and gender matched with the studied patients) were used as con-

trols for serum TGF- β 1 levels. These anonymous sera were prepared from the blood specimens collected from normal children who were enrolled in a vaccination research project of the Center of Excellence Viral Hepatitis Research Unit, King Chulalongkorn Memorial Hospital. The procedures of sera preparation and storage were the same between the control and the study groups.

Pulmonary function testing including spirometry and body plethysmography were performed in the studied patients by using Vmax 22 and Auto-box SensorMedics[®] machines (SensorMedics Corporation; Yorba Linda, CA) on the same day of blood collection and prior to blood transfusion. The best of 3 attempts was used for the analysis. Lung volumes (total lung capacity [TLC], residual volume [RV], RV/TLC ratio) and spirometry parameters (forced vital capacity [FVC], forced expiratory volume in 1 second [FEV₁], FEV₁/FVC ratio, and forced expiratory flow rate from 25% to 75% of vital capacity [FEF_{25-75%}]) were recorded. All pulmonary function parameters except for FEV₁/FVC and RV/TLC ratio were expressed as the percentage of predicted value based on the normal value of the Asian population (European Respiratory Society 1993, Update). RLD was defined if the patients demonstrated a TLC < 80% of the predicted value.²⁴ Pulmonary air trapping was defined if the patients had a RV > 135% of the predicted value and a RV/TLC ratio > 35%.²⁴ The patients were suspected to have large airway obstruction if they demonstrated a FEV₁ < 80% of the predicted value and a FEV₁/FVC ratio < 80%.²³ Medium to small airway obstruction was suspected if the patient had FEF_{25-75%} < 65% of the predicted value²⁵ with normal TLC.

Serum TGF- β 1 in the study and control sera were quantitated at the same time by the same technician, using a TGF- β 1 capture enzyme-linked immunosorbent assay (ELISA [Quantikine[®] TGF- β 1 Immunoassay; R&D System, Inc; Minneapolis, MN]). Activation reagent preparation, TGF- β 1 sample activation and assay procedures were performed according to the manufacturer's advices.

Statistical analysis

The continuous variables including age, duration of blood transfusion, serum ferritin level, se-

rum TGF- β 1 level and pulmonary function variables were expressed as mean \pm SD for normal distribution data and as median for non-parametric data. The means or medians of continuous variables were compared between 2 groups of data by using unpaired Student's t-test for normal distribution data and Mann-Whitney U-test for non-parametric data. The correlation between 2 groups of continuous variables was tested by using the Pearson correlation test for normal distribution data and the Spearman rank sum test for non-parametric data. A *p* value of less than 0.05 was considered statistically significant. GraphPad InStat Program[®] (GraphPad Software Inc; San Diego, CA) was used for statistical analysis.

RESULTS

Twenty one thalassemia major children (57% female) who never had iron chelation therapy and met the inclusion criteria were studied. Demographic data, duration of blood transfusion, serum ferritin level, serum TGF- β 1 level and pulmonary function variables are shown in Table 1. Medium to small airway obstruction was suspected in 13 (62%) patients. RLD and pulmonary air trapping were found in 5 (24%) and 2 (9%) patients, respectively. Splenectomy was performed in 12 (57%) of all studied patients and in 4 (80%) RLD patients.

The duration of blood transfusion correlated

with the serum ferritin levels ($r = 0.7$; $p < 0.001$) and was longer in the RLD group when compared to the non-RLD group (Table 2). Those who had RLD demonstrated higher serum ferritin levels than those who had no RLD (Table 2). There was no difference in age at the time of the study, age at the time of diagnosis and gender distribution between these 2 groups of patients (Table 2).

Serum TGF- β 1 levels were measured in 19 patients and compared to those of 19 normal children (age and gender matched). Thalassemia major children demonstrated lower serum TGF- β 1 levels when compared to normal children (7.9 vs 78.8 pg/ml; $p < 0.001$). The median level of serum TGF- β 1 was not different between RLD and non-RLD groups (Table 2), and did not correlate with the duration of blood transfusion ($r = -0.1$; $p = 0.6$) or TLC ($r = -0.1$; $p = 0.5$). There was no correlation between serum ferritin and serum TGF- β 1 levels ($r = -0.2$; $p = 0.5$).

DISCUSSION

RLD was demonstrated in 24% of our thalassemia major children who never had iron chelation therapy. However, the most common pulmonary function abnormality found in our study was decreased FEF_{25-75%} (without an associated low lung volume) which was suggestive for medium to small airway obstruction. Since we did not perform a

Table 1 Demographic and clinical data of 21 thalassemia major children who never had iron chelation.*

Age at the time of study (yrs)	11.2 \pm 2.6 (10.2, 12.3)
Age at diagnosis (yrs)	4.4 \pm 3.3 (2.9, 5.9)
Duration of blood transfusion (yrs)	7.7 \pm 4.1 (6.0, 9.4)
Serum ferritin level (ng/ml)	2,019.3 \pm 1,812.0 (1,287.2, 2,751.3)
Serum TGF-β1 level (pg/ml)**	7.9 (0 – 40.5)
TLC (% predicted value)	72.7 \pm 31.5 (60.0, 85.4)
RV (% predicted value)	97.9 \pm 23.1 (87.4, 108.4)
RV/TLC ratio (%)	29.2 \pm 6.6 (26.2, 32.2)
FVC (% predicted value)	82.4 \pm 8.2 (78.7, 86.2)
FEV₁ (% predicted value)	88.5 \pm 10.6 (83.7, 93.4)
FEV₁/FVC ratio (%)	91.9 \pm 4.3 (90.0, 93.8)
FEF_{25-75%} (% predicted value)	54.9 \pm 17.0 (47.2, 62.7)

*All data were presented as mean \pm SD (95% confidence interval) except for serum TGF- β 1 level which was presented as median (range).

**Serum TGF- β 1 levels were evaluated in 19 patients.

Table 2 Comparison of clinical data and serum TGF- β 1 levels between thalassemia major patients who had restrictive lung disease (RLD) and those who had no RLD

Clinical data	RLD (n = 5)	non-RLD (n = 16)	p value
Age at the time of the study (yrs) (mean \pm SD)	10.3 \pm 1.8	10.9 \pm 2.7	0.6
Age at the time of diagnosis (yrs) (mean \pm SD)	3.3 \pm 3.2	4.7 \pm 3.3	0.4
% female	60	62	1.0
Duration of blood transfusion (yrs)(mean \pm SD)	9.1 \pm 1.9	5.5 \pm 3.2	0.03*
Serum ferritin level (ng/ml) (mean \pm SD)	3,816.6 \pm 1,715.9	2,084.5 \pm 1,504.8	0.04*
Serum TGF-β1 levels (pg/ml)** (median)	13.3	4.2	0.2

* p value < 0.05 was considered statistically significant.

** Serum TGF- β 1 levels were evaluated in 19 patients (5 in the RLD group and 14 in the non-RLD group).

bronchodilator response test in these patients, sub-clinical asthma that could not be detected by the history and physical exam might be included in this group of patients.

The mechanism of RLD in thalassemic patients is not clearly known. The proposed mechanisms include lung parenchyma abnormalities secondary to iron deposition, chest wall restriction due to hepatosplenomegaly and poor muscle function, the underdevelopment of alveoli, and desferoxamine-induced pulmonary toxicity.^{1,2,7,10,22} Several studies demonstrated the correlation between serum ferritin or the body iron burden and pulmonary dysfunctions.^{4,7,15} However, this was not found in many other studies.^{2,5,6,11,13,14} The discrepancy of the results among the studies could be due to the multifactorial causes of pulmonary dysfunctions in thalassemic patients. In addition, serum ferritin may not be a good marker for demonstrating iron deposition in tissues. Despite this limitation, serum ferritin evaluation is still used in many centers as the standard method to evaluate the body iron burden in patients who are transfusion dependent. We used serum ferritin as an indirect marker to evaluate the body iron burden in thalassemia major children in our study since it is a non-invasive method. We found that those who had RLD had higher serum ferritin levels and longer durations of blood transfusion than those who had no RLD. The higher serum ferritin levels in the RLD group could be secondary to the longer duration of

blood transfusion and, subsequently, higher body iron burden. However, it could also be implied that RLD in these patients might be related to iron.

It has been thought that iron induces inflammatory and fibrotic responses in the lungs of thalassemic patients, similar to other organs such as the liver.¹ However, a relationship between free-radical production from iron and tissue damages in the lungs of thalassemic patients has never been demonstrated.²⁶ Despite having some reports regarding radiological evidences of interstitial pneumonitis and characteristics of lymphocytic alveolitis in alveolar fluids of some thalassemic patients,^{12,16} several necropsy studies in thalassemia and other transfusion-dependent patients failed to demonstrate an increase of fibrous tissues in the lung parenchyma.^{10,27,28} In contrast, iron deposition was found predominantly in bronchial epithelial cells and bronchial glands.²⁸ However, most of these histological studies were performed in thalassemic patients who died from congestive heart failure or sepsis without evaluation of their pulmonary function parameters. Therefore, it could not be postulated that iron had no role in inducing pulmonary fibrosis and RLD in these patients.

Pulmonary biopsy is the definite method to demonstrate fibrotic responses in the lungs. However, the invasiveness of the procedure limits its use in research studies. A less invasive method would be

more practical to document this particular process in the lungs. TGF- β 1 is a cytokine that can be detected in the blood as well as in some body fluids such as alveolar fluids and has been reported to be increased in many fibrotic diseases.¹⁸⁻²¹ However, our study could not demonstrate the relationship between serum TGF- β 1 levels and RLD in thalassemia major children. Serum TGF- β 1 levels did not correlate with TLC, serum ferritin levels and duration of blood transfusion. We could not demonstrate a statistical difference in serum TGF- β 1 levels between the RLD and non-RLD groups, even though there was a trend towards higher levels in the former group. This might be partly due to the limitation of our sample size.

We also found that thalassemia major children had a lower serum TGF- β 1 level than normal children. Iron deposition in reticuloendothelial and epithelial cells may effect the production and regulation of TGF- β 1 in thalassemic patients and result in lower levels of this cytokine in the circulation. Findings from our study were suggestive that the TGF- β 1 level in the circulation may not be a good marker for fibrotic responses in thalassemia major children unlike other fibrotic diseases even though there was a trend of higher levels in the RLD group. Salsaa *et al.*²⁹ reported a particular pattern of some cytokine responses in thalassemia major patients. They found lower than normal unstimulated production and higher than normal induced production of some cytokines in the circulation. The pattern of TGF- β 1 production in thalassemic patients is not well known yet. Further studies regarding this particular cytokine in these patients are still required.

In conclusion, RLD was demonstrated in 24% of our thalassemia major children who never had iron chelation therapy. This RLD was related to longer duration of blood transfusion and a higher serum ferritin level. We could not demonstrate the relationship between RLD and serum TGF- β 1 levels but found that thalassemia major children had lower serum TGF- β 1 levels than normal children. It could be implied from this study that iron might play a role in inducing RLD in thalassemia major but it would still be questionable if this RLD was related to pulmonary fibrosis. Further studies with a larger sample size and studies concerning the pattern of TGF- β 1 production in thalassemia major children as well

as its levels in the alveolar fluid (which will be a more invasive procedure) or other respiratory secretions such as sputum would be helpful in defining the etiology of RLD in these patients.

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