ENHANCED MATURATION AND PROLIFERATION OF β-THALASSEMIA/Hb E ERYTHROID PRECURSOR CELLS IN CULTURE

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Abstract. Upon erythroid cell maturation *in vivo*, β-thalassemic erythroid cells accumulate unmatched unstable α-globin chains that are believed to be a causal factor in such cell destruction. This study showed that β-thalassemia/Hb E erythroid precursor cells from peripheral blood had accelerated maturation, and could mature to the terminal erythroid stage. During the early period of cell culture, erythroid precursor cells derived from subjects with the more severe form of β-thalassemia/Hb E had higher rate of erythroid maturation. In addition, peripheral blood mononuclear cells from β-thalassemia/Hb E subjects had higher erythroid proliferative potential than cells derived from normal controls. Erythroid proportion in the more severe β-thalassemia/Hb E cases was less than that of the milder cases. Premature apoptosis was not observed during the 15 days of erythroid cell culture from both β-thalassemia/Hb E and normal subjects.

INTRODUCTION

β-Thalassemia major and compound heterozygous β-thalassemia/Hb E (β-thal/Hb E) are hereditary anemias characterized by the absence or reduced synthesis of β-globin chains, leading to an excess of unmatched α-globin chains in erythroid cells. Accumulation of such unmatched α-globin chains leads to shortened red blood cell survival in the peripheral blood and premature destruction of erythroid precursors in the bone marrow (ineffective erythropoiesis) (Weatherall, 1998; Pootrakul *et al*, 2000). The excess α-globin chains are unstable and their denaturation and degradation products include membrane bound-iron (Tavazzi *et al*, 2001) and hemin (Phumala *et al*, 2003), which cause the generation of reactive oxygen species (ROS) even in the erythroid precursor cells synthesizing hemoglobin. With increasing cell maturation, there is increasing synthesis of globin chains, and thus the likelihood of more α-globin accumulation leading to more erythroid cell destruction (Mathias *et al*, 2000; Pootrakul *et al*, 2000). The more severe thalassemia cases have shown more ineffective erythropoiesis (Pootrakul *et al,* 2000).

We hypothesized that in the more severe β-thalassemic patients there should be more abnormal characteristics of their erythroid cells. Studies on thalassemic erythroid cells have used peripheral blood stem cells instead of bone marrow cells (Chen *et al*, 1992; Fibach and Rachmilewitz, 1993). Using the two-phase liquid culture of peripheral blood-derived erythroid precursor cells, we investigated erythroid maturation (via morphological assessment) and erythroid expansion (via erythroid proportion) in comparison with the nonerythroid cells in the culture system.

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MATERIALS AND METHODS

Patients

Heparinized peripheral blood was collected from 8 non-splenectomized patients with β-thal/

Hb E, 4-19 years of age. Informed consent and approval from institutional ethics committee of human experimentation in compliance with the ICH/GCP were obtained. The clinical data of the β-thal/Hb E patients are shown in Table 1. β-Thal/Hb E patients are allocated as being severe and moderate cases based on anemic severity and such other factors as age at disease presentation, age at first blood transfusion, requirement of blood transfusion, splenomegaly, and growth retardation (Winichagoon *et al,* 1993). In this study, β-thal/Hb E patients were allocated as severe based on hemoglobin (Hb) level of below 7.0 g/dl. Subject number 5, although having Hb level of 8.5 g/dl, was placed in the severe group due high transfusion rate, splenomegaly and growth retardation.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation in Histopaque® (density of 1.077 g/ml, Sigma, USA) and cultured at $5x10^5$ - 10^6cells/ml in the two-phase liquid culture (TPLC) system (Fibach *et al,* 1989; Fibach and Rachmilewitz, 1991). In brief, PBMCs were seeded in α-MEM medium (GIBCO BRL, USA) supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, USA), 1 μg/ml of cyclosporine A (Sandoz, Switzerland) and 10% conditioned medium collected from the culture of the bladder carcinoma 5637 cell line (ATCC, USA). Cultures were incubated at 37ºC in an atmosphere of 5% CO_2 in humid air. After 7 days, the nonadherent cells were harvested, washed twice in incomplete α-MEM medium, and recultured in phase II α-MEM medium containing 30% FBS, deionized bovine serum albumin (BSA) (Roche Diagnostics, Germany) at a concentration of either 1% or 10%, 10 mM β-mercaptoethanol (Sigma, USA), 1.5 mM L-glutamine (GIBCO BRL, USA), 1 μM dexamethasone (Sigma, USA), 0.2 U/ml of human recombinant erythropoietin (rHuEPO) (Cilag AG, Switzerland). The cultures were continued for another week and then the floating cells were harvested, washed twice in incomplete α-MEM medium and cultured in phase II α-MEM medium containing 2.0 U/ml of rHuEPO. On day 3, 5 and 8 (termed as day 7+3, day 7+5, and day 7+8) cells were collected for morphology examination and differential and total cell counting.

Morphological assessment

One hundred μl of cell suspension were sedimented onto glass slide by using Cytospin 3 (Shandon, UK). Morphological features of the erythroblasts were examined under a light microscope after staining with Wright's eosine methylene blue stain (MERCK, Germany). Between 1,000-2,000 cells per slide were observed under a light microscope (Olympus BH2, Japan), for differential counting of erythroid precursor cells based on their characteristic morphological appearances. Late erythroid precursor cells consisted of orthochromatic and polychromatophilic erythroblasts.

Maturation index

Maturation index (MI) is defined as the percentage of late erythroid precursor cells compared with total erythroid precursor cells.

Erythroid proportion index

Erythroid proportion index (EPI) is defined as the percentage of erythroid cells compared with total nucleated cells.

Cell count

Two hundred μl of samples from the cell cultures were analyzed by automated cell counter (Technicon H*3 RTC, Bayer, Germany).

Statistical analysis

Mean ± SE of the data was reported. Wilcoxon signed ranks test was used for statistical analysis of the effect of BSA on paired samples. Student's *t*-test was used for statistical comparisons of the data derived from β-thal/Hb E patients and normal control samples, and a p-value of < 0.05 is considered statistically significant.

RESULTS

The two-phase liquid culture (TPLC) procedure for growing human erythroid cells *in vitro* is divided into two phases (Fibach *et al*, 1989; Fibach and Rachmilewitz, 1991). In the primary phase, an erythropoietin (EPO)-independent phase, cells are first cultured in the presence of a combination of growth factors, excluding EPO, allowing early erythroid progenitors, burst forming units (BFU-Es), to proliferate and differentiate into colony forming units (CFU-E)-like progenitor cells. The CFU-E-like progenitor cells are then cultured in the secondary phase, an EPO-

Table 1
Clinical data of β-thal/Hb E patients.

Subject	Age (y)	Sex	Age at disease presentation	Age at first blood Tx	Mean Hb (g/dl)	PRC/bwt/year (ml/kg/y)	Spleen (cm)	Growth retardation
1	4	M	1 y 6 m	-	8.3	0	0	No
2	10	M	6 y	7 y 6 m	7.6	0	3	No
3	11	F	3 m	2 y	6.4	182.6	5	No
4	11	F	4 y	7 y	7.8	35.6	0	No
5	12	F	6 m	4 y 10 m	8.5	147.5	5	yes
6	12	M	1 y 9 m	1 y 9 m	6.9	93.8	2	yes
7	15	M	6 m	3 y 9 m	6.3	146.6	5	yes
8	19	M	4 y	4 y 7 m	6.5	33.7	6	No

Hb: hemoglobin; Tx: transfusion; PRC: packed red cell; bwt: body weight

Table 2
Effect of bovine serum albumin (BSA) on erythroid cells derived from β-thal/Hb E subjects and normal controls.

Conc. of BSA	MI			EPI		
	Day 7+3	Day 7+5	Day 7+8	Day 7+3	Day 7+5	Day 7+8
β-thal/Hb E subject (n = 5)						
10% BSA	60.51±7.01	81.44±7.14	96.38±1.32	69.22±8.06	72.11±4.44	70.37±7.87
1% BSA	73.49±6.01	86.28±4.55	97.94±0.78	55.19±9.56	58.92±6.06	43.17±9.78
Normal control (n = 2)						
10% BSA	34.40±1.40	55.80±1.80	74.42±0.71	3.00±1.00	10.14±0.04	22.23±0.98
1% BSA	38.99±1.21	53.14±0.54	71.97±3.64	4.85±1.75	9.98±0.88	28.20±1.80

The results are shown as mean ± SE; MI: percent late stage erythroid precursor cells compared with total erythroid precursor cells; EPI: percent erythroid cells compared with total nucleated cells.

dependent phase, in which the CFU-E-like progenitor cells continue to proliferate and mature to orthochromatic erythroblasts and finally enucleated erythrocytes. Cultures in the secondary phase in the presence of 2.0 U/ml of rHuEPO yielded predominantly erythroid cells and a small number of nonerythroid cells (data not shown). However, in order to obtain an erythroid proportion index (EPI) required for this study, the culture system was modified by using 0.2 U/ml of rHuEPO in the first 7 days of EPO-dependent phase, followed by 2.0 U/ml of rHuEPO for another week, yielding a considerable amount of nonerythroid cells.

In addition, when 10% BSA was used rather than 1% BSA, the original concentration used in the cell culture, a delay in β-thal/Hb E erythroid maturation was observed (Table 2). Ten percent BSA significantly decreased MI of β-thal/Hb E samples, but not of normal control samples, on day 7+3 ($p = 0.043$). Ten percent BSA also caused a significant increase in EPI on every observed day ($p = 0.043$) (Table 2). The high BSA concentration also produced 1.5 fold increase in total cell counts of the cultured cells compared with 1% BSA (data not shown). Consequently all TPLC experiments were performed with 10% BSA in the secondary phase medium.

PBMCs from 8 β-thal/Hb E patients and 4 normal individuals were cultured using the high BSA TPLC system. Cells from patients with β-thal/Hb E showed significantly higher values of MI (Table 3) and EPI (Table 4) than those from normal cells on every observed day (p-values

Table 3
Maturation index of erythroid cells from β-thal/Hb E subjects and normal controls.

Subject	MI			
	Day 7	Day 7+3	Day 7+5	Day 7+8
Severe (n = 5)	40.92 ± 13.12	74.07 ± 8.99	83.57 ± 7.87	94.78 ± 1.27
Moderate (n = 3)	24.19 ± 16.51	59.92 ± 7.80	87.63 ± 3.95	98.12 ± 0.98
Normal (n = 4)	0.06 ± 0.06	29.16 ± 3.09	51.25 ± 2.82	77.95 ± 4.10

The results are shown as mean ± SE; MI: percent late stage erythroid precursor cells compared with the total erythroid precursor cells.

Table 4
Erythroid proportion index of cultured cells from β-thal/Hb E subjects and normal controls.

Subject	EPI			
	Day 7	Day 7+3	Day 7+5	Day 7+8
Severe (n = 5)	33.73 ± 14.49	67.40 ± 9.57	69.93 ± 7.00	61.60 ± 7.43
Moderate (n = 3)	39.33 ± 9.85	80.43 ± 3.27	80.27 ± 5.51	86.33 ± 4.92
Normal (n = 4)	1.80 ± 0.74	7.61 ± 2.77	12.61 ± 2.11	29.77 ± 4.42

The results are shown as mean ± SE; EPI: percent erythroid cells compared with total nucleated cells.

ranged from 0.000 to 0.038). Late stage erythroid precursor cells from β-thal/Hb E samples appeared earlier (day 7) than those of normal controls (day 7+3) (Fig 1). EPI values in the more severe β-thal/Hb E cases were lower than in the moderate cases (Fig 1). During the early period of phase II (day 7 to day 7+3), the percentage of late stage erythroid precursor cells appeared to be higher in the more severe β-thal/Hb E cases than the moderate cases (Fig 1), but this is not statistically significant. At the end of the culture period (day 7+8), almost all of the β-thal/Hb E erythroid precursor cells matured to the orthochromatic stage, but early stage erythroid precursor cells were still found in cultures from normal controls (Fig 2).

DISCUSSION

In this study, the two-phase liquid culture (TPLC) (Fibach *et al*, 1989; Fibach and Rachmilewitz, 1991) was employed to generate erythroid precursor cells from peripheral blood for investigation of erythroid cell expansion and maturation of PMBC-derived erythroid precursor cells from β-thal/Hb E subjects in comparison with normal controls. Normally, 1% BSA is used in the culture condition, but we have found that 10% BSA was necessary to obtain MI and EPI values that allowed the studies to be carried out. In the high BSA-containing TPLC system, erythroid maturation of β-thal/Hb E samples was retarded compared to that in 1% BSA condition, resulting in an increased percentage of early stage erythroid precursor cells, which have a higher proliferative potential with a peak of cells in the S phase of the cell cycle in the transition from proerythroblast to basophilic erythroblast (Wojda *et al*, 2002). This may be due to the presence of factors present in BSA that affect erythroid cells (Congote, 1985, 1987; Congote and Esch, 1987).

PBMC-derived erythroid precursor cells from β-thal/Hb E subjects have higher MI and EPI values compared to normal controls. The increased maturation of thalassemic erythroid cells could have resulted from the presence of hemin, which is known to be capable of accelerating erythroid maturation (Fibach *et al,* 1995; Kamano *et al,* 1994; Kollia *et al,* 1997) and is elevated in serum of β-thal/Hb E subjects (Phumala *et al,* 2003). Consistent with this notion was the observation that erythroid maturation was higher in samples of severe (and pre-

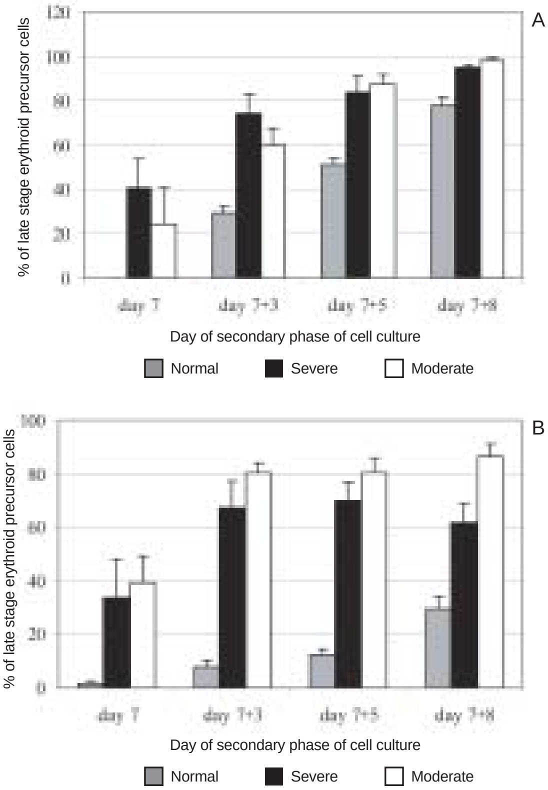

Fig 1–Percentage of late stage erythroid precursor cells (A) and erythroid precursor cells (B) in culture. PBMC-derived erythroid cells were cultured in the presence of 0.2 U/ml of rHuEPO for 7 days and then in 2.0 U/ml of rHuEPO for additional 8 days. β-Thal/Hb E patients were allocated as being severe and moderate cases as described in Materials and Methods.

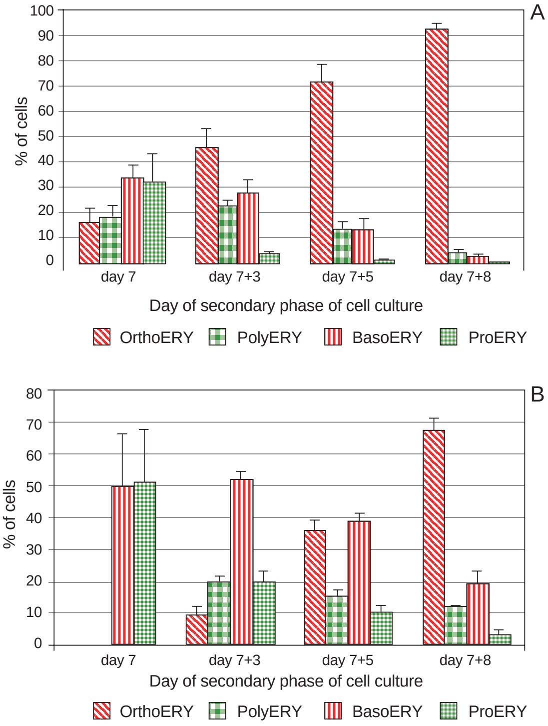

Fig 2–Percentage of various stages of erythroid precursor cells cultured from peripheral blood of β-thal/Hb E patients (A) and normal individuals (B). Cells were cultured in the presence of 0.2 U/ml of rHuEPO for 7 days and then in 2.0 U/ml of rHuEPO for additional 8 days. Cells were stained with Wright's eosin methylene blue stain and observed by light microscopy. OrthoERY: Orthochromatic erythroblast; PolyERY: Polychromatophilic erythroblast; BasoERY: Basophilic erythroblast; ProERY: Proerythroblast.

sumably having erythroid cells with more unmatched α-globin chains) compared with those from moderate β-thal/Hb E cases.

Interestingly, PBMC-derived erythroid precursor cells from β-thal/Hb E subjects were capable of maturing to orthochromatic erythroblasts, with no evidence of apoptosis at the polychromatophilic erythroblast stage as reported for cells derived from bone marrow (Mathias *et al,* 2000). A similar observation has been reported by Paiboonsukwong (2004). Thus apoptosis of β-thalassemic erythroid cells may not be due to the direct effect of unmatched α-globin chains, but due to the interaction of these oxidatively damaged cells with the bone marrow milieu. Schrier *et al* (2003) have recently reported apoptotic death of β-thalassemia major erythroid cells from Italian patients triggered by FAS/FAS-ligand interaction.

Although the EPI of β-thal/Hb E samples was higher than that of normal control, this value dropped in the final days of incubation. A possible explanation is that once the thalassemic erythroid cells have reached full maturation, the accumulation of the unmatched α-globin chains may result in cell toxicity and death (possibly by necrosis).

In summary, we have developed a modified two-phase liquid culture system that permits study of β-thal/Hb E erythroid cell expansion and maturation, and have shown that PMBC-derived erythroid precursor cells from such subjects have elevated maturation index values and can reach late maturation with no evidence of premature apoptosis, contrary to observations of erythroid cells derived from the bone marrow (Mathias *et al*, 2000). These finding suggest that with appropriate manipulation of the environment to which the thalassemic erythroid cells are exposed, it may be possible to correct the ineffective erythropoiesis seen in thalassemic patients.

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