ROLE OF INTERLEUKIN-3 AND SIGNALING PATHWAYS ON β-THALASSEMIA/HbE ERYTHROID PROGENITOR CELL IN CULTURE

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Abstract. In order to study the role of the cytokine interleukin-3 (IL-3) and its signaling pathways in erythropoiesis of β-thalassemia/HbE erythroid progenitor cells, CD34 positive cells were isolated from peripheral blood of patients and healthy subjects. After culturing the cells in the presence or absence of IL-3, cell viability was measured by trypan blue staining and apoptotic cells were analyzed by flow cytometry. After 7 days of culture the highest percent erythroid progenitor cell viability was obtained with cells from healthy subjects, while the lowest percentage was found in those from splenectomized β-thalassemia/HbE. Viability of β-thalassemia/HbE erythroid progenitor cells in the presence of IL-3 was higher than that of nonsupplemented cells. In addition, specific inhibitors of protein kinase C (Ro-318220), phospholipase C (U-73122) and Janus kinase 2 (AG-490) were used to investigate the involvement of signaling pathways in erythropoiesis. Percent apoptosis of erythroid progenitor cells from splenectomized β-thalassemia/HbE subjects treated with RO-318220 was higher than those of nonsplenectomized β-thalassemia/HbE and healthy subjects. Treatment with U-73122 resulted in enhanced percent apoptotic cells from normal and β-thalassemia/HbE subjects. All these effects were independent of IL-3 treatment.

INTRODUCTION

β-thalassemia/HbE patients have a severe clinical disorder and cause a major public health in Southeast Asia (Weatherall and Clegg, 2001). There is marked ineffective erythropoiesis and shortened red cell survival, comparable with that observed in homozygous β-thalassemia. β-thalassemia/HbE patients also can have varying degrees of severity. Understanding of the reason for clinical heterogeneity of the disease is still incomplete, but there are evidences showing that patients who have mild clinical symptoms may have a co-inheritance of α-thalassemia, or an increased propensity for synthesizing HbF, or a variety of other factors.

The main cause of ineffective erythropoiesis could be due to apoptosis as it has been shown that marrow erythroid precursor cells in β-thalassemic patients have accelerated apoptosis (Yuan *et al*, 1993; Centis *et al*, 2000). In addition, erythrokinetic studies of patients with moderate and severe forms of α- and β-thalassemias show a relationship between ineffective erythropoiesis and marrow erythroid apoptosis (Pootrakul *et al*, 2000). In erythropoiesis, hematopoietic growth factors, such as interleukin-3 (IL-3), act on numerous targets within the hematopoietic system. IL-3

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stimulates growth of colonies containing mixtures of myeloid and erythroid cells *in vitro* (Iscove *et al*, 1989; Iscove and Yan, 1990). In addition, erythropoiesis from adult but not fetal blood-derived CD133+ stem cells depends strongly on interleukin-3 (Bohmer, 2004). In the signaling pathway of IL-3, one of the earliest detectable changes after binding of IL-3 to its receptor is the phosphorylation of tyrosine of a set of Janus kinase 2 proteins (Ferris *et al*, 1988). However, the precise mechanisms of activation of Janus kinase or other kinases by IL-3 still remain unknown.

We report the role of IL-3 and signaling pathways on the viability in hematopoietic cell culture of erythroid progenitor cells from both splenectomized and nonsplenectomized β-thalassemia/HbE patients in comparison with healthy subjects.

MATERIALS AND METHODS

Patient samples

Peripheral blood samples were collected in heparin from splenectomized (n = 6) and nonsplenectomized (n = 7) β-thalassemia/HbE subjects. Diagnosis of thalassemia was based on family history, red cell indices and hemoglobin typing. All patients were in a steady state and had not received blood transfusion for at least 1 month before blood collection.

Inhibitors

Protein kinase C inhibitor Ro-318220, phospholipase C inhibitor U-73122 and Janus kinase 2 inhibitor AG-490 were purchased from Calbiochem, USA.

Hematological investigation

Red blood cell indices and hemoglobin typing were analysed by Coulter counter (Model ZX6) and Automated HPLC (Variant^{TM}, Bio-Rad, CA, USA).

Isolation of CD34-positive cells

Blood samples were diluted in phosphate-buffered saline (PBS) containing 0.5% diaminetetraacetic acid (EDTA). Mononuclear cells were isolated on Ficoll-Hypaque (Histopaque, Sigma, USA) and washed twice with PBS/0.5% EDTA solution. CD34-positive cells were isolated on MIDI-MACS immunoaffinity column according to the manufacturer's instruction (Miltenyi Biotech, CA, USA).

Erythroid progenitor cell culture

Approximately 10^5 to 10^6 CD34-positive cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco, NY) supplemented with fetal calf serum, human AB serum and stem cell factor (Muta and Krantz, 1993). Cell suspension was divided into two sets, and to one set of cells was added 0.01 U/ml IL-3 (Chemicon, USA). Then, both sets of cells were incubated under an atmosphere containing 5% CO_2 at 37°C. Culture medium was changed at day 3 and cells culture was continued for 7 days.

Cell viability determination

Trypan blue staining was performed before and after CD34-positive selection for determination of cell viability. At days 3 and 7 of culture, 20 μl aliquot of cell culture suspension was mixed with an equal volume of 0.4% trypan blue stain and viability count was performed by using a hemocytometer. Then 200 μl aliquot of cell culture suspension was cytospun and stained with Wright stain for examination of cell morphology.

Detection of apoptosis

Percent apoptotic cell was determined using annexin V labeling. Cells (10^5) were washed in PBS and then resuspended in 1x annexin V binding buffer (0.1 M HEPES/NaOH, 1.4 M NaCl, 25 mM $CaCl_2$) and 2 μl of FITC-conjugated annexin V and 2 μl of propidium iodide solution (1 mg/ml). Samples were kept in the dark at room temperature for 15 minutes and promptly analyzed by flow cytometry (Vermes *et al*, 1995).

Statistical analysis

Data were analysed with SPSS for Win-

dows release 10.0. Difference is considered statistically significant at $p < 0.05$. For comparison of percent cell viability in blood samples, nonparametric tests were used.

RESULTS

The hematological data of healthy subjects and β-thalassemia/HbE patients are shown in Table 1. β-thalassemia/HbE patients have lower hemoglobin, hematocrit, mean cell volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration than healthy subjects.

In order to study the role of the cytokine, interleukin-3, and signaling pathways involved in erythropoiesis of β-thalassemia/HbE erythroid progenitor cells, CD34-positive cells were isolated from healthy subjects and β-thalassemia/HbE patients after mononuclear cell separation. The mean percent cell viability using trypan blue staining before and after selection for CD34-positive cells showed no statistical difference between healthy and thalassemic patients. Erythroid cultures from β-thalassemia/HbE patients showed that IL-3 supplemented cells had a higher percentage of cell viability than nonsupplemented cells after culture for 3 and 7 days (Table 2). The lowest percent cell viability was found in splenectomized β-thalassemia/HbE cells cultured without IL-3 for 7 days, while the highest percentage of cell viability was found in cells of healthy subjects after culturing with IL-3 for 3 days. Both IL-3 supplemented and nonsupplemented cells from splenectomized and nonsplenectomized β-thalassemia/HbE subjects had lower percent cell viability in comparison to those of healthy subjects.

The mean percent apoptotic cells, which are positive with annexin V and propidium iodide staining, were measured at 7-day culture

Table 1
Hematological data of healthy subjects, and nonsplenectomized β-thal/HbE and splenectomized β-thal/HbE patients.

Sample no.	Sex	Age (years)	Hb (g/dl)	Hct (%)	MCV	MCH	MCHC
Healthy (n=3)	M	21	12	37	80	26	33
	M	23	14	43	90	30	43
	F	33	16	47	84	29	34
Nonsplenectiomized	M	42	7	22	56	17	30
β-thal/HbE (n=7)	M	38	6	22	60	18	25
	M	35	8	25	83	27	33
	F	39	6	18	58	18	32
	F	14	6	20	64	19	31
	F	40	7	22	62	20	32
	F	20	8	22	64	20	31
Splenectomized	F	12	6	20	72	21	29
β-thal/HbE (n=6)	F	50	5	17	55	16	30
	F	35	6	20	77	22	29
	M	38	7	20	73	23	32
	M	28	7	21	70	22	32
	F	32	7	21	73	21	30

M= Male, F= Female, Hb = Hemoglobin, Hct = Hematocrit, MCV= Mean Cell Volume, MCH= Mean Corpuscular Hemoglobin, MCHC= Mean Corpuscular Hemoglobin Concentration

Table 2
Viability of erythroid progenitor cells cultured with and without IL-3 for 3 and 7 days.

Subjects	Day 3		p-value	Day 7		p-value
	+IL3	-IL3		+IL3	-IL3	
Healthy control	91.4 ± 4.0	88.2 ± 2.2	0.29	89.4 ± 5.5	86.8 ± 1.0	0.47
Nonsplenectomized β-thal/HbE	85.3 ± 2.8	78.2 ± 5.5	0.03	84.7 ± 2.8	75.4 ± 3.4	<0.01
Splenectomized β-thal/HbE	80.3 ± 5.3	74.4 ± 2.2	0.01	79.9 ± 1.0	74.0 ± 2.5	<0.01

Percent cell viability was measured by trypan blue staining and expressed as mean ± SD.

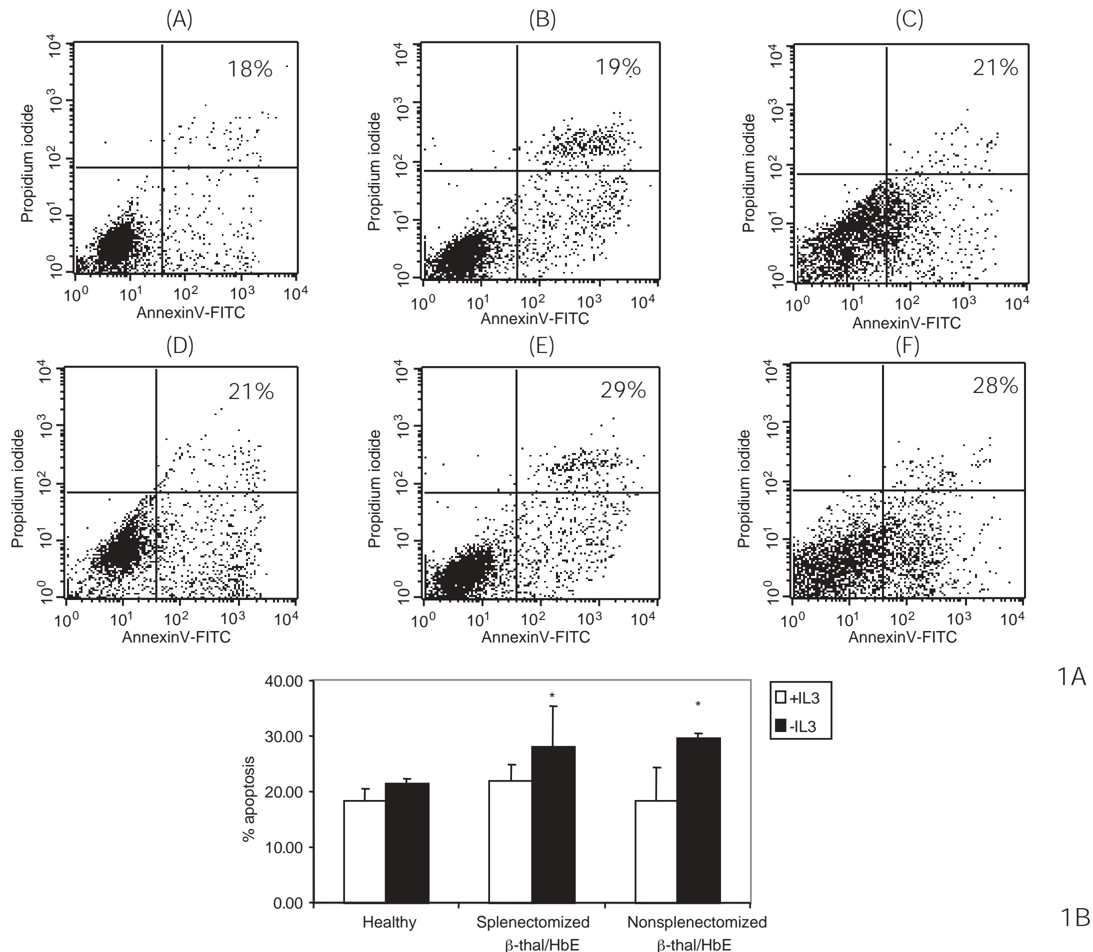

Fig 1–Apoptosis of erythroid progenitor cells from healthy subjects, nonsplenectomized β-thal/HbE and splenectomized β-thal/HbE patients. CD34-positive cells were cultured for 7 days with or without IL-3. Apoptotic cells were analyzed by flow cytometry. Numbers in upper and lower right quadrants (IA) represent the percent apoptosis from each group: IL-3 treated cells from healthy subjects (A), nonsplenectomized β-thal/HbE (B), splenectomized β-thal/HbE (C) and non IL-3 treated cells from healthy subjects (D), nonsplenectomized β-thal/HbE (E), and splenectomized β-thal/HbE (F). Percent apoptotic cells from healthy subjects, nonsplenectomized β-thal/HbE and splenectomized β-thal/HbE are shown in 1B. *$p < 0.05$ compared with non IL-3 treated cells.

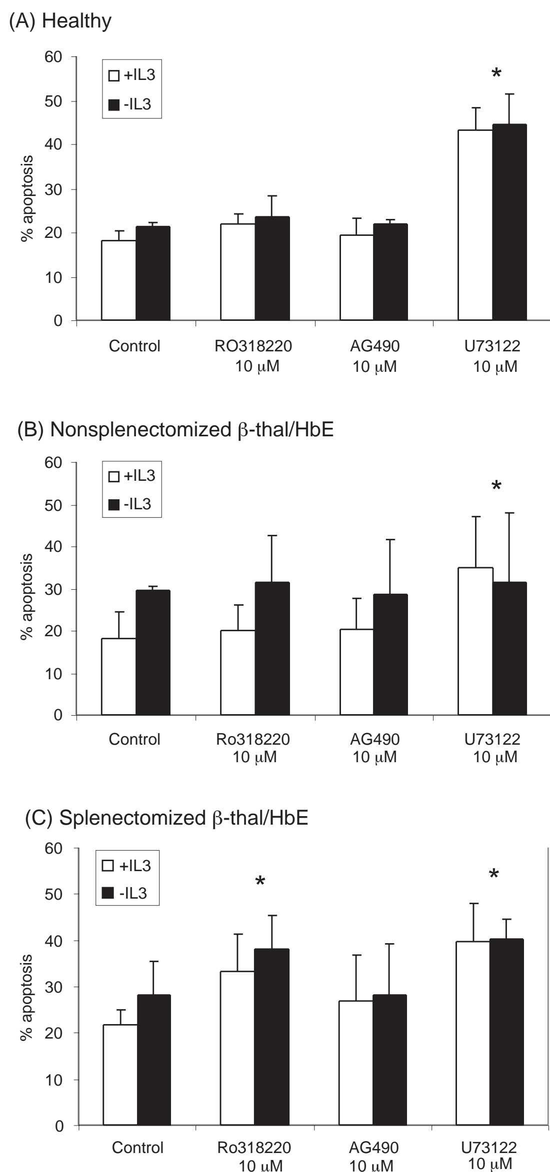

Fig 2–Effects of inhibitors of signaling pathways on erythroid progenitor cells culture. CD34-positive cells were cultured with or without IL-3 for 7 days. Each group was treated with 10 μM Ro-318220 (PKC inhibitor), 10 μM AG-490 (Jak2 inhibitor) and 10 μM U-73122 (PLC inhibitor) for 1 hour at 37ºC. The mean percent apoptotic cells from healthy (A), nonsplenectomized β-thal/HbE (B) and splenectomized β-thal/HbE (C) subjects were determined by flow cytometer. *$p < 0.05$ compared with control.

of erythroid progenitor cells. In the healthy subjects, IL-3 had no effect on apoptosis of erythroid progenitor cells ($p > 0.05$) but percent apoptosis in IL-3 nonsupplemented cells was higher than IL-3 supplemented cells from both nonsplenectomized ($p = 0.02$) and splenectomized β-thalassemia/HbE patients ($p = 0.01$) (Fig 1).

Erythroid progenitor cells were cultured with or without IL-3 for 7 days, and then each group of cells were incubated with 10 μM RO-318220 [protein kinase C (PKC) inhibitor], 10 μM AG-490 [Janus kinase 2 (JAK2) inhibitor] or 10 μM U-73122 (phospholipase C (PLC) inhibitor) for 1 hour at 37ºC. Erythroid progenitor cells from healthy subjects in presence and absence of IL-3, treated with U-73122 only showed percent apoptotic cells comparable to those from β-thalassemia/HbE patients (Fig 2A). U-73122 treatment enhanced percent apoptotic cells, in presence and absence of IL-3, of splenectomized and nonsplenectomized β-thalassemia/HbE patients (Fig 2B, C). Ro-318220 treatment was only able to elevate percent apoptosis in erythroid precursor cells, with and without IL-3 treatment, of splenectomized β-thalassemia/HbE patients (Fig 2C).

DISCUSSION

The results of this study showed that there was a significant decrease in percent erythroid cell viability in culture (3- and 7-day) from both splenectomized and nonsplenectomized β-thalassemia/HbE subjects in comparison with those from healthy subjects. As percent cell viability in IL-3 supplemented cells was higher than IL-3 nonsupplemented cells and absence of IL-3 resulted in high level of apoptosis of cells from patients, these data suggest that IL-3 plays a role in regulating erythropoiesis and in the prevention of apoptosis in erythroid cells from thalassemic patients.

There have been a number of many re-

ports of apoptosis in erythroid cultures (Zamai *et al*, 2004; Sae-ung *et al*, 2005). However, there is a report from Kittikalayawong *et al* (2005) showing no evidence of apoptosis in accelerated maturation of β-thalassemia/HbE erythroid precursor cells. This may due to difference in the culture system employed. CD34-positive selection for hematopoietic progenitor cells and growth in the presence of stem cell factor, erythropoietin and cytokine were used in this study (Muta and Krantz, 1993; Mathias *et al*, 2000).

Recently, there are evidences showing that IL-3 could induce inhibitor of DNA-binding protein-1 in hemopoietic cells (Leeanansaksiri *et al,* 2005) and is involved in stimulation of B-Raf/MEK/Erk signaling pathway to regulate cell proliferation, apoptosis and adhesion (Jin *et al*, 2006). In addition, JAK-2 is essential for signaling through a variety of cytokine receptors including that of IL-3 (Parganas *et al*, 1998; James *et al*, 2005). Many studies have shown that phospholipase C (PLC) and protein kinase C (PKC) can be activated by IL-3 (Rao and Mufson, 1994). In this study, signaling pathways involving phospholipase C, protein kinase C and Janus kinas 2 were investigated by using specific inhibitors. Percent apoptosis of erythroid cells treated with U-73122 (PLC inhibitor) from healthy subjects and thalassemic patients were enhanced, but this effect was independent of IL-3 treatment. A possible explanation is that inhibitor of PLC affects downstream phosphorylation, mediated by diacylglycerol, that is required to prevent cells from entering apoptosis pathways. Treatment with Ro-318220, an inhibitor of PKC, induced a higher level of apoptosis only in erythroid cells of splenectomized β-thalassemia/HbE patients, but this was independent of IL-3 presence. A defect in PKC signaling pathway could lead to abnormal erythropoiesis and hematological disease of β-thalassemia/HbE patients, particulary after splenectomy. PKC beta II acts together with Bcr-Abl in mediating the molecular mechanism for apoptotic suppression in multipotent hemopoietic cells (Xenaki *et al*, 2004).

In summary, in the present study we have shown that IL-3 and PLC are (independently) involved in regulating apoptosis of cultured erythroid precursor cells from β-thalassemia/HbE patients. Elucidation of their molecular mechanisms will require further study.

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