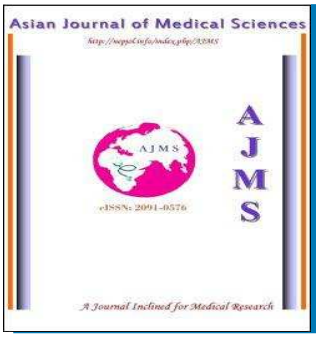

INTERLEUKIN-1 INVOLVED IN APOPTOSIS OF BETA-THALASSEMIA/HEMOGLOBIN E ERYTHROID PROGENITOR CELLS

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"Cytokines play essential role in apoptosis of thalassemic red cells"

ABSTRACT

Objective: The major pathophysiological features of β-thalassemia are anemia and ineffective erythropoiesis. Ineffective erythropoiesis has been shown by an intense marrow erythroid hyperplasia and increased apoptosis during basophilic to orthochromatic normoblast stages. Some cytokines like interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) have been found to involved in apoptosis. Although the pro-apoptotic activity of IFN-γ and TNF-α is well documented, there are only few studies on IL-1, especially on erythroid lineage. In this *in vitro* study, the role of cytokine IL-1α and IL-1β in apoptosis of erythroid progenitor cells from β-thalassemia/HbE patients was assessed.

Methods: Erythroid progenitor cells were isolated from peripheral blood of healthy subjects and β-thalassemia/HbE patients. Cells were then cultured, with and without 20 ng/ml IL-1α and IL-1β. Total cells and percent cell viability were performed by using trypan blue staining. Percent cell apoptosis was analyzed by using flow cytometer.

Results: Both IL-1α and IL-1 β were significantly decreased erythroid progenitor cells. IL-1 at 20 ng/ml reduced the glycophorin A positive cells and percent cell viability of erythroid progenitor cells from β-thalassemia/HbE patients, while there was increased apoptosis in this group. The highest percent apoptosis was observed in 20 ng/ml IL-1β treated β-thalassemia/HbE erythroid progenitor cells.

Conclusion: IL-1β could be involved in apoptosis of erythroid progenitor cells from β-thalassemia/HbE patients which might be related with ineffective erythropoiesis of the disease.

Key words: Ineffective erythropoiesis, apoptosis, IL-1, β-thalassemia/HbE

INTRODUCTION

The β-thalassemias are a heterogeneous group of congenital anemia characterized by the impaired β-globin chain synthesis, which occurs from any of more than 200 point mutations and, rarely by the gene deletion.[1] Ineffective erythropoiesis is one of the major pathophysiological features of this disease.[2] The mechanism includes the increased intramedullary erythroid death and arrested proliferation of erythroid progenitors.[3] In such a case decreased production of mature RBC has been observed due to increased apoptosis at basophilic to orthochromatic erythroblast stages.[3,4,5,6] Cytokines like interferon-γ (IFN-γ), tumor necrosis factor α (TNF α) and interleukin-1 (IL-1) were found to involve in the inhibition of erythropoiesis.[7] This plays a crucial role in the pathophysiology of the hematopoietic disorder associated with the bone marrow failure and anemia. [8] This study was focused on the *in vitro* role of both Interleukin IL-1α and IL-1β in apoptosis related to ineffective erythropoiesis by using the liquid culture system. It has been found that both type of interleukin-1 (IL-1α and IL-1β) can inhibit the action of erythropoietin on EPO responsive cells.[9] Macrophage derived IL-1 has found to indirectly suppressed the erythropoiesis *in vivo* and *in vitro* by the help of tumor necrosis factor (TNF), and the IFN-γ.[10, 11, 12] Moreover, TNF shares many biological activities with interleukin-1. [13] In β-thalassemic patients, the increased number of activated macrophages have been observed, which is the main source of many pro-apoptotic cytokines, especially IL-1.[14] These cytokines especially TNF-α and IFN-γ were also found to be involved in the up regulation of Fas expression on $CD34^+$ cells, showing the possible Fas mediated apoptosis of erythroid progenitors cells.[15] The increased level of TNF-α and IL-1β in the serum of β-thalassemic patients is also an indication of increased apoptosis involved in the ineffective erythropoiesis of β-thalassemia/HbE patients. [16] However, there have been inconclusive information on roles and mechanisms of IL-1 in erythropoiesis especially in thalassemic red cells. Then, the aim of this study is to investigate the effect of IL-1α and IL-1β on apoptosis of erythroid progenitor cells from β-thalassemia/HbE patients.

MATERIALS AND METHODS

1. Hematological Profiles:

Peripheral blood was collected from five of healthy subjects and six β-thalassemia/HbE patients. Normal hematological parameter and normal hemoglobin typing A_2A was found in all healthy subjects. The low level of red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), and increased levels of reticulocyte count were found in β-thalassemia/HbE patients (Table1). Informed consent was obtained according to the protocol approved by the Ethical clearance committee on Human Rights related to Research Involving Human Subjects Research, Mahidol University; Bangkok, Thailand. Diagnosis was conformed on the basis of clinical manifestation, family history, red cell indices, and Hb typing by high performance liquid chromatography (HPLC). Age of β-thalassemia/HbE patients was between 10-60 years old.

2. Erythroid progenitor cell culture:

Peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation in Histopaque® (density1.077g/dl, Sigma, USA).Contaminated red blood cells were removed by incubating in red blood cell lysis buffer and Platelets were removed by cell centrifugation through Phosphate-buffered saline (PBS). [17,18] CD34 positive cells were selected by LS separation column from the suspension of PBMC with FcR blocking reagent and anti-CD34 antibody labeled immunomagnetic micro beads (Miltenyi Biotech, Auburn,CA,USA).[6]

CD34 positive cells (1-2x10^5 cells/ml) was cultured in Iscove's Modified Dulbeco's Medium IMDM(GIBCO-BRL, NY, USA) supplemented with 15% human AB serum, and 15% FBS. The cytokines were added at the concentration, interlukin-3 (10 ng/ml), stem cell factor (20 ng/ml) and erythropoietin (2 U/ml). Cell suspension was divided into different wells of culture dishes for the experiment with and without treatment of different concentration of rhIL-1α and IL-1β. Cells were then incubated at 37^0C, in 5% CO_2, with high humidity of 95%. On day 3, cells were collected and centrifuged to change the media, and then growth factors were added to the cell suspension as in day-0 and incubated under the same condition until day-7.

3. Total cell count and cell viability assay

20µl of cell suspension was mixed with 20µl of 0.4% Trypan blue solution. Viable cells and number of total cells were counted under hemocytometer, and then percent of cell viability was calculated.

4. Apoptosis assay

Apoptotic cells were analyzed by using flow cytometer. Annexin V labeled FITC apoptosis kit was used to detect externalization of PS, which occurs in the ongoing process of apoptosis. Glycophorin A labeled PE staining was also used simultaneously because of its ability to stain the maturing erythroid cells. First erythroid cultured cells were washed in Dulbeco's phosphate-buffered saline (DPBS) and then resuspended in 100µl of 1X annexin V binding buffer conjugated solution. 2 µl of annexin V FITC and 5µl of glycophorinA labeled PE antibody were mixed in the cell suspension then incubated for 15 minutes in dark. Finally the cells were analyzed using the FACSort flow cytometer (BD Biosciences, Mountain View, San Jose CA). At least 10,000 cells were counted, in order to determine the percent of apoptosis.

5. Statistical analysis

Mean ± SE of the data was reported. Wilcoxon signed rank test was used for statistical analysis of the effect of IL-1α and IL-1 β on paired samples. A p-value of less than 0.005 was considered statistically significant.

RESULTS

1. Various dose effects of IL-1α and IL-1β on erythroid progenitor cell culture

In order to choose the optimal concentration of IL-1 on erythroid progenitor cells, cells were cultured with 5, 20 and 50 ng/ml of IL-1α and IL-1 β. At culture day-7, erythroid cells from healthy subjects and β-thalassemia/HbE patients were incubated with glycophorin A-PE and annexin V-FITC and then percentage of apoptosis was analyzed by flow cytometer. We found that 20 ng/ml of IL-1α and IL-1β showed the highest percentage of apoptosis cell death (Figure1).

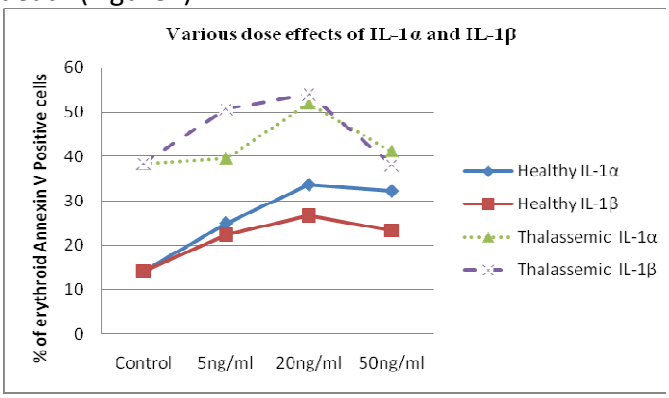

Figure 1 Effect of various doses of IL-1α and IL-1β on cell death of erythroid progenitor cells from healthy subjects and β-thalassemia/Hb E patients.

2. Effect of IL-1 on erythroid progenitor cell proliferation

The total cell count in the healthy subjects was increased to more than 10 fold when erythroid progenitor cells were cultured for 7 days. In the presence of 20ng/ml of IL-1α and IL-1β, the total cell counts of healthy subjects and β-thalassemia/HbE patients were statistic significantly decreased

Table-1 Hematological data of Healthy subjects and β-thalassemia/HbE patients.

Healthy									
Sample No.	Age/sex	WBC ($x10^3/\mu l$)	RBC ($x10^6/\mu l$)	Hb (g/dl)	Hct (%)	MCV (fl)	RDW (%)	Plt ($x10^3/\mu l$)	Hb Typing
1	27/M	10.6	5.4	16.2	44.6	82.3	13.9	317	A_2A
2	24/M	3.6	4.8	15.2	42.7	88.7	13.5	192	A_2A
3	22/F	9.1	4.2	13.2	37.5	88.8	13.3	336	A_2A
4	23/F	8.2	4.3	12.9	37.9	87.3	15	362	A_2A
5	25/M	5.4	5.6	17.3	48.3	86.2	13.8	277	A_2A
Mean		7.3	4.8	14.9	42.2	86.6	13.9	296.8	
SD		2.8	0.6	1.8	4.5	2.6	0.6	66.2	

β-thalassemia/HbE										
Sample No.	Age/sex	WBC ($x10^3/\mu l$)	RBC ($x10^6/\mu l$)	Hb (g/dl)	Hct (%)	MCV (fl)	Retics (%)	RDW (%)	Plt ($x10^3/\mu l$)	HbE (%)
1	45/F	29.8	3.1	7.8	23.3	74.5	7.7	23.3	461	51.0
2	20/F	108.0	2.5	6.1	19.8	76.4	13.6	25.7	761	64.8
3	31/M	46.8	3.0	5.7	18.4	61.5	18.1	25.8	786	68.9
4	30/M	17.5	2.7	6.8	20.5	75.5	23.9	22.2	786	56.3
5	32/M	24.7	2.9	6.9	22.6	76.8	19.2	33.1	614	65.0
6	31/F	91.1	4.8	8.6	27.3	65.2	10.4	25.5	903	58.0
Mean		53.0	3.1	6.9	21.9	71.6	14.7	25.9	718.5	60.6
SD		37.7	0.8	1.0	3.1	6.5	6.4	3.8	156.3	6.6

compared with untreated cells (Figure 2).

3. Effect of IL-1 on cell viability of erythroid progenitor cells

The percentage of cell viability of erythroid progenitor cells treated with 20 ng/ml IL-1α and IL-1β from healthy subjects and β-thalassemia/HbE patients were lower than untreated cells. The lowest cell viability was found in 20 ng/ml IL-1β treated erythroid progenitor cells from β-thalassemia/HbE (Figure 3).

4. Effect of IL-1 on apoptotic cell death of erythroid progenitor cells

The percentage of annexin V positive cells is a marker of apoptotic cell death was performed in groups of healthy subjects and β-thalassemia/HbE patients on day-7 of culture.

The results showed that the percent apoptosis was increased in IL-1α or IL-1β treated cells of both groups. The highest percentage of apoptosis was observed in IL-1β treated cells of β-thalassemia/HbE (56.7%). While the lowest percentage of apoptosis was observed in the healthy subjects (24.8%). These results suggested that IL-1 especially IL-1 β increased the apoptosis in erythroid progenitor cells (Figure 4).

DISCUSSIONS

In this study we investigated the role of IL-1 in apoptosis of thalassemic erythroid progenitor cells using hematopoietic stem cell culture technique. Erythroid cells from peripheral blood were

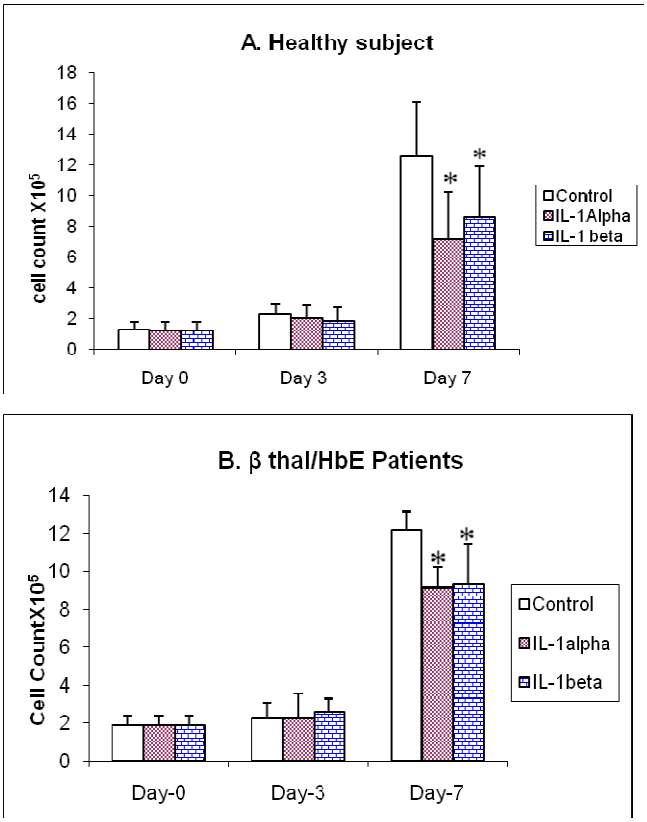

Figure 2 Total cells of erythroid progenitor cell cultured with and without 20 ng/ml IL-1α and IL-1β from healthy subjects (A) and β-thalassemia/Hb E (B). * p< 0.005, compared between IL-1 treated cells and untreated (control)

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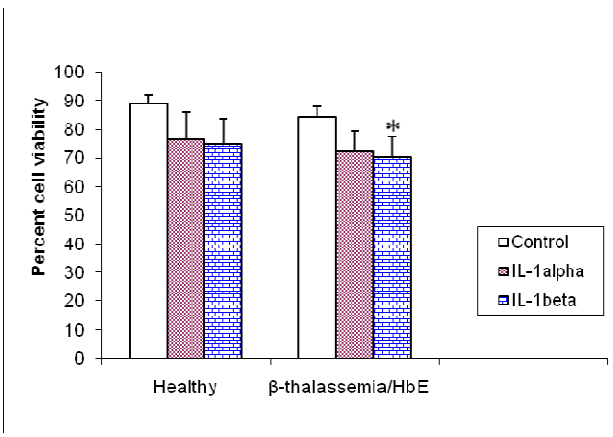

Figure 3 Percentage of cell viability of erythroid progenitor cells treated with and without 20ng/ml IL-1α or IL-1β from healthy subjects and β-thalassemia/HbE patients.* p< 0.005, compared between IL-1 treated cells and untreated (control).

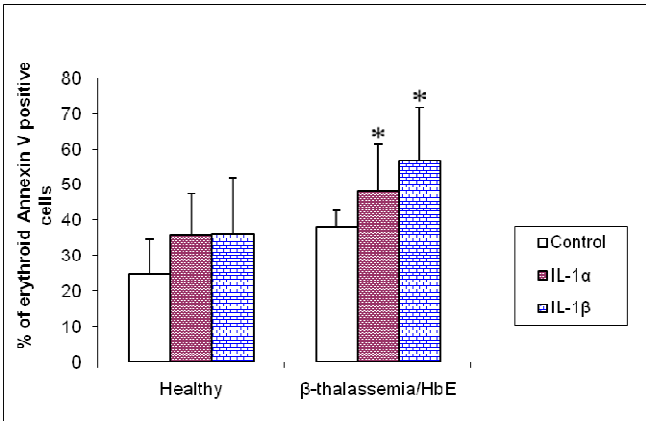

Figure 4 Percentage of cell erythroid annexin V positive cells represents apoptosis in cell treated with and without IL-1α or IL-1β from healthy subjects and β-thalassemia/HbE patients. * p< 0.005, compared between IL-1 treated cells of healthy subjects and patients.

in liquid culture system containing the essential growth factors including interlukin-3 (10 ng/ml), stem cell factor (20 ng/ml) and erythropoietin (2 U/ml) as mention by Muta *et al* 1995. [17] But instead of using CD34 negative cell selection method, CD34 positive cell selection technique was performed by using Mini-MACS magnetic cell sorting isolation kit as mentioned by Zamai *et al*, 2004.[6]

The effect of IL-1α and IL-1β on cell viability was performed on day-7 culture, which showed that both IL-1α and IL-1β treated cell had the lower percentage of cell viability than untreated as control in both groups. This result was point out the suppressive effect of both IL-1α and IL-1β in cell survival during erythropoiesis. This evidence was confirmed by apoptosis analysis using flow cytometry. Interestingly, the highest number of erythroid annexin V positive cells was noted in the culture treated with 20 ng/ml of IL-1β.The statistically significant difference of annexin V positive cells were observed in both IL-1α and IL-1β treated cell culture when compared to untreated cells. In erythropoiesis, FAS and FAS ligand signaling pathway plays an important role in

apoptosis whose cross linking is effective in less mature cells, particularly at basophilic level. An increased apoptosis during basophilic to orthochromatic normoblast has also been reported. [4, 6] Interaction of FAS with FAS-L has a feedback role in normal erythropoiesis, likely involving caspase 3, 7 and 8, which in turn degrade the important transcription factor GATA-1, a factor required for normal erythroid differentiation and this is not common in myeloid cell line.[20] Comparatively annexin V positive cells in cell culture from β-thalassemia/HbE was higher than in healthy subjects, which suggested that there is high apoptosis during erythroid differentiation in β-thalassemia/HbE. This may be due to the presence of activated and increased number of macrophages observed in thalassemic patients which is the source of pro-apoptotic cytokines such as IL-1β, Moreover its significantly high level was also observed in peripheral blood of thalassemic patient more than in healthy subjects.[14, 16]

Ineffective erythropoiesis is one of the main pathophysiology of β-thalassemia and its mechanism includes increased erythroid cells death and arrested proliferation. From results of this study suggested that IL-1 especially IL-1β may play an important role in apoptosis of β-thalassemia/HbE patients and could be related with ineffective erythropoiesis of the disease.

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