Cytokine-Induced Apoptosis of Beta-Thalassemia/Hemoglobin E Erythroid Progenitor Cells via Nitric Oxide-Mediated Process in vitro

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Key Words

Apoptosis · Cytokines · Nitric oxide ·
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Abstract

Background/Aim: β-Thalassemia/hemoglobin E (β-thal/HbE) is a common hereditary anemia in Thailand. Ineffective erythropoiesis due to apoptosis and decreased lifespan of circulating thalassemic red blood cells are the major causes of anemia. Changes to bone marrow microenvironment could contribute to apoptotic events. This study examined the effects of cytokines interleukin-1β, tumor necrosis factor-α and interferon-γ on apoptosis of β-thal/HbE erythroid progenitor cells in vitro, including nitric oxide-mediated apoptotic processes. **Methods:** Percent apoptosis of erythroid progenitor cells from 5 β-thal/HbE patients and 5 normal control subjects was examined using flow cytometry. In addition, the inducible nitric oxide synthase (iNOS) mRNA level and nitrite production were measured using quantitative PCR and the Griess method, respectively. **Results:** Upon cytokine treatment, a higher percent apoptosis was obtained with β-thal/HbE erythroid progenitor cells compared with control, and the maximum effect was observed using 20 ng/ml interferon-γ on day 14 of culture. There was an increase in iNOS mRNA level and a concomitant elevation of nitrite concentration in culture medium. Apoptosis and ni-

trite level were abrogated when β-thal/HbE and control cells were treated with S-methylisothiourea sulfate, an iNOS inhibitor. **Conclusion:** The marked sensitivity of erythroid progenitor cells from β-thal/HbE patients to cytokine-induced apoptosis via an NO-mediated process reflects a proapoptotic status of such thalassemic red blood cells.

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Introduction

Thalassemias are heterogeneous groups of autosomal recessive anemia resulting from reduced or absent synthesis of globin chains that form the heterotetrameric hemoglobin. The syndrome can be divided into two groups: α-thalassemia due to deletion of α-globin genes and β-thalassemia due to mutations of the β-globin gene. Compound heterozygous β-thalassemia/hemoglobin E (β-thal/HbE) is the most common form of β-thalassemia found in Thailand [1, 2].

Anemia in β-thalassemia is due to both ineffective erythropoiesis and reduced lifespan of circulating effete red blood cells, stemming from the presence of toxic unmatched α-hemoglobins, which are believed to cause apoptosis of erythroid precursor cells within the bone marrow and premature eryptosis of the circulating thalassemic red blood cells [3, 4]. In fact, there are other

Table 1. Hematological parameters of healthy subjects and β -thal/HbE patients

Sample	Age years	RBC $\times 10^{12}/l$	Hb g/dl	HCT %	MCV fl	MCH pg	MCHC g/dl	RDW %	RETIC %	WBC $\times 10^9/l$	PLT $\times 10^9/l$	Geno- type
Normal	27.4 $\pm$ 5.1	6.2 $\pm$ 0.7	14.2 $\pm$ 1.5	42.8 $\pm$ 4.3	90.7 $\pm$ 2.4	29.9 $\pm$ 1.2	32.9 $\pm$ 0.7	12.6 $\pm$ 1.1	1.0 $\pm$ 0.4	6.2 $\pm$ 0.7	241 $\pm$ 57.7	β/β
β -thal/HbE	26.7 $\pm$ 7.4	4.3 $\pm$ 0.7	7.5 $\pm$ 1.7	24.1 $\pm$ 3.6	59.2 $\pm$ 3.8	18.0 $\pm$ 1.4	30.4 $\pm$ 1.7	24.7 $\pm$ 1.4	6.66 $\pm$ 2.8	8.6 $\pm$ 0.5	325.8 $\pm$ 130.2	β^0/E

Data are presented as the mean $\pm$ SD. RBC = Red blood cells; Hb = hemoglobin; HCT = hematocrit; MCV = mean cell volume; RDW = red cell distribution width; RETIC = reticulocyte count; WBC = white blood cells; PLT = platelets.

causes of anemia, such as microenvironment factors. Indeed, in vivo and in vitro studies have implicated the roles of interferon (IFN)- γ , tumor necrosis factor- α (TNF- α)-related apoptosis-inducing ligand and interleukin-1 (IL-1) in the inhibition of proliferation and differentiation of erythroid progenitor cells [5]. Moreover, there are significant increases in levels of IL-1 α and TNF- α able to suppress erythropoiesis in bone marrow of thalassemic patients [6].

Nitric oxide (NO) has been shown to be involved in apoptosis signaling pathway in many diseases, such as anemia of chronic diseases [7], aplastic anemia [8] and leukemia [9]. NO is synthesized from L-arginine by NO synthase (NOS), which exists in three isoforms, namely, neuronal NOS, inducible NOS (iNOS) and endothelial NOS [10–12]. Synthesis of iNOS is stimulated by immunostimulatory cytokines, such as TNF- α and IL-1 β [13, 14].

This study investigated the effects of cytokines IL-1 β , TNF- α and IFN- γ on apoptosis of β -thal/HbE erythroid progenitor cells in culture and examined the involvement of the NO-mediated apoptosis process.

Materials and Methods

Blood Samples

Peripheral blood (20 ml) from 5 β -thal/HbE patients and 5 healthy subjects were collected into heparinized tubes. Diagnoses of β -thal/HbE patients were based on clinical manifestation, family history, red cell indices and hemoglobin typing using high-performance liquid chromatography. Hematological profiles of the study groups are summarized in table 1.

Preparation of Peripheral Blood Mononuclear Cells

Fresh heparinized blood was centrifuged at 600 g, and the packed red cells were diluted with phosphate-buffered saline containing 2 mM EDTA, and then were layered onto Histopaque®-1.077 (density 1.077 $\pm$ 0.001 g/dl). Following centrifugation at 600 g, peripheral blood mononuclear cells were isolated from the interface layer.

Erythroid Progenitor Cell Culture

CD34-positive (hematopoietic stem) cells, isolated from peripheral blood mononuclear cells using the EasySep® CD34 selection kit (Stemcell Technologies), following the manufacturer's instructions, were cultured in Iscove's modified Dulbecco's medium supplement with 2 units of erythropoietin (Janssen-Cilag), 15% fetal bovine serum (Invitrogen Corporation), 15% human AB serum, 10 ng/ml stem cell factor (Chemical International), 10 ng/ml IL-3 (Chemical International) and 2% (w/v) penicillin-streptomycin (Invitrogen Corporation) for 14 days at 37°C under a humidified atmosphere of 5% CO₂.

Cytokine Treatment

Erythroid progenitor cells (10⁴) were incubated for 14 days with 2, 20 and 40 ng/ml recombinant human IL-1 β , TNF- α or IFN- γ (Chemical International). In some experiments, in addition to the cytokine, 1 ng/ml S-methylisothiourea sulfate (Chemical International), a selective iNOS inhibitor, was added throughout the culture period.

Total Cell Counting

Total cell counts were determined by staining with 0.4% Trypan blue dye and counted in a hemocytometer under light microscope.

Apoptosis Assay

Cytokine-treated and -untreated erythroid progenitor cells were suspended in Annexin V binding buffer and incubated with Annexin V-FITC (BD Bioscience) and anti-glycophorin A PE-conjugated antibodies (R&D Systems) for apoptosis determination in a flow cytometer (BD Bioscience).

Determination of iNOS mRNA Level by Quantitative PCR

Total RNA was isolated from erythroid progenitor cell culture using Trizol (Invitrogen Corporation) and reverse transcribed using the Superscript First-Strand cDNA Synthesis kit (Invitrogen Corporation) according to the manufacturer's instructions. Quantitative PCR (qPCR) thermal cycling was carried out in an iCycle real-time machine (Bio-Rad) using the iQ SYBR Green Supermix kit as follows: 95°C for 3 min; 35 cycles of 95°C for 30 s, 62.5°C for 30 s and 72°C for 1.5 min. Levels of iNOS mRNA were normalized relative to β_2 -microglobulin mRNA using the 2^{- $\Delta\Delta$ CT} formula.

NO Production Determination

NO production was based on measurement of nitrite concentration in cell culture as determined by the Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihy-

Fig. 1. Mean cell count of erythroid progenitor cells from healthy subjects (a–c) and β -thal/HbE patients (d–f). CD34-positive cells from 5 β -thal/HbE patients and 5 healthy subjects were treated with cytokines for 14 days, and total cell count was determined by Trypan blue staining.

drochloride in 5% H_3PO_4 ; Sigma] [15]. A 50- μl aliquot of culture supernatant was mixed with 100 μl of Griess reagent and the mixture incubated for 5 min at 25°C. Absorbance at 570 nm was measured in an automated plate reader (Beckman Coulter). A standard solution of sodium nitrite was used to generate a calibration curve.

Statistical Analysis

Results are expressed as the mean $\pm$ SD. Statistical analysis was performed using Student's t test, and significant difference is set at a p value <0.05 .

Fig. 2. Percent apoptosis of erythroid progenitor cells from healthy subjects (a) and β -thal/HbE patients (b). CD34-positive cells from 5 β -thal/HbE patients and 5 healthy subjects were treated with cytokines for 14 days and then stained with Annexin V-FITC and anti-glycophorin A PE-labeled antibodies. Percent apoptosis was determined by flow cytometry. * $p < 0.05$ compared with the control group.

Results

Effects of Cytokines on Erythroid Progenitor Cell Counts

Erythroid progenitor cell numbers from healthy subjects and β -thal/HbE patients in culture increased in a time-dependent manner over the 14 days in culture (fig. 1). At day 7 of culture, the majority of cells were pronormoblasts (data not shown). Addition of IL-1 β , TNF- α or IFN- γ to cell cultures resulted in a decrease in erythroid progenitor cell numbers compared with untreated cells, with cytokine-treated cells from β -thal/HbE patients being more affected (fig. 1).

Effects of Cytokines on Apoptosis of Erythroid Progenitor Cells

Apoptosis of erythroid progenitor cells treated with various concentrations of cytokines was determined using Annexin V-FITC and anti-glycophorin A antibody-PE conjugate staining and analysis by flow cytometry. Glycophorin A staining was used as a marker of erythroid cells and that with Annexin V for apoptotic cells. Erythroid progenitor cells of β -thal/HbE patients had a higher proportion of apoptotic cells than those from healthy subjects (fig. 2). Cytokine-treated β -thal/HbE erythroid cells showed a greater percentage of apoptosis than the corresponding treated control cells. The highest percent cell apoptosis (17.22%) was observed with β -thal/HbE cells treated with 20 ng/ml IFN- γ (14 days).

Effects of Cytokines on iNOS mRNA Expression of Erythroid Progenitor Cells and NO Level in Culture Medium

Levels of iNOS mRNA in cytokine-treated erythroid progenitor cells were measured by qPCR (relative to β_2 -microglobulin mRNA). Cytokines do not significantly increase iNOS mRNA levels in cells from healthy subjects, but there are significant increases in β -thal/HbE cells (fig. 3a), with the highest level detected in β -thal/HbE cells treated with 20 ng/ml IFN- γ (14 days).

As an indirect indication of iNOS-induced NO production, nitrite levels in the culture supernatant were measured using the Griess reagent. As expected, the highest nitrite content was detected in culture medium of β -thal/HbE cells exposed to 20 ng/ml IFN- γ for 14 days (fig. 3b), corresponding to the highest level of percent apoptosis.

Effect of iNOS Inhibitor on Cytokine-Induced Apoptosis of Erythroid Progenitor Cells and Nitrite Level in Culture Medium

In order to confirm that cytokines indeed had induced apoptosis of erythroid precursor cells through an NO-mediated process, cytokine-treated cells were simultaneously exposed to 1 ng/ml S-methylisothiourea sulfate, a selective of the iNOS inhibitor, throughout the period of culture. This significantly reduces percent cell apoptosis (fig. 4) as well as nitrite production in culture (fig. 5) for erythroid precursor cells from both β -thal/HbE patients

Fig. 3. iNOS mRNA level (a) and nitrite concentration (b) of erythroid progenitor cells from healthy subjects and β -thal/HbE patients. CD34-positive cells from 5 β -thal/HbE patients and 5 healthy subjects were treated with cytokines for 14 days, and iNOS mRNA expression was determined by qPCR. Nitrite concentration in the culture medium was measured using the Griess method. * $p < 0.05$ compared with the control group.

Fig. 4. Effect of iNOS inhibitor on percent apoptosis of erythroid progenitor cells from healthy subjects (a) and β -thal/HbE patients (b). CD34-positive cells from 5 β -thal/HbE patients and 5 healthy subjects were treated with IL-1 β , TNF- α and IFN- γ , with or without iNOS inhibitor for 14 days. Percent apoptotic cells were measured by flow cytometry as described in the legend to figure 2. * $p < 0.05$ compared with the control group.

and healthy subjects, but the inhibitor does not significantly reduce erythroid cell numbers from healthy subjects or thalassemic patients (data not shown).

Discussion

β -Thalassemia/HbE is a common hereditary anemia in Southeast Asia, and carriers present with a wide range of disease severity, ranging from essentially asymptomatic

to a severe transfusion-dependent state [16]. The underlying pathophysiology of β -thalassemia is associated with deposition of unmatched α -hemoglobin chains, which is believed to lead to ineffective erythropoiesis through induction of apoptosis. The mechanism by which this phenomenon is induced remains unclear, although it has been proposed that both the precipitation of unmatched α -hemoglobin chains as well as an accumulation of unbound iron could lead to oxidative stress and subsequent cell death [16].

Fig. 5. Effect of iNOS inhibitor on nitrite concentration in culture medium of erythroid progenitor cells from healthy subjects (a) and β -thal/HbE patients (b). Experiments were performed as described in the legend to figure 4. Nitrite concentration was measured by the Griess method. * $p < 0.05$ compared with the control group.

The production of hematopoietic cells is under tight control of a group of hematopoietic cytokines [17]. Each cytokine has multiple actions mediated by a variety of membrane receptors whose cytoplasmic domains contain specialized regions initiating various responses including survival, proliferation, differentiation, maturation and functional activity [17]. In anemia, hematopoietic stem/progenitor cells express membrane receptors for proinflammatory cytokines, and several studies have demonstrated that a direct action of the cytokines on hematopoietic cell lines *in vitro* can impair erythroid development and numbers of erythroid progenitor cells [18]. Elevated serum levels of TNF- α , IL-1 β and IFN- γ have been observed in β -thal/HbE patients, and these levels correlate with the severity of clinical symptoms [19]. Cytokines IL-1 and IL-6 have a major inhibitory effect on development of red cell precursors in bone marrow, and the inhibitory effect of TNF- α on the growth of erythroid burst- and colony-forming units is direct or in part mediated by IFN- γ produced by bone marrow stromal cells [20, 21].

However, there is an associated pathway in these pathologies involving free radical-mediated damages dependent on the interplay between the physiological free radicals superoxide anion and NO [22]. The latter is involved in several pathophysiology processes, namely as a toxic effector of nonspecific immune defense against microorganisms and as an immunomodulator. Through the reaction with thiol groups and iron-sulfur proteins, DNA synthesis by iron-dependent enzymes is inhibited, result-

ing in suppression of cell proliferation. Moreover, cytotoxicity as a result of substantial NO formation is established through initiation of apoptosis, characterized by upregulation of tumor suppressor p53, changes in the expression of pro- and antiapoptotic Bcl-2 gene family members, cytochrome c release from mitochondria into cytosol, activation of caspase cascade, chromatin condensation, and DNA fragmentation. The production of high levels of intracellular NO is associated with autocytotoxicity, suppression of tumorigenicity and abrogation of metastasis, which in part is explained by induction of apoptosis [23]. In addition, vascular damage plays an important role in the pathogenesis of vasculitides that characterizes rheumatic diseases [24]. Cytokine-mediated, neutrophil-dependent injury of human umbilical vein endothelial cells is associated with NO production [24]. In hematopoietic progenitor cells, Fas, by causing iNOS expression, evokes apoptosis that in turn is attenuated through suppression of NO formation [25].

This study demonstrated that the cytokines IL-1 β , TNF- α and IFN- γ caused higher levels of apoptosis of β -thal/HbE erythroid progenitor cells in culture compared to those from normal control cells. Apoptosis was mediated by synthesis of iNOS and production of NO (inferred from the presence of nitrite in culture medium). This apoptosis phenomenon could be abrogated by inhibiting iNOS activity, resulting in a concomitant reduction in nitrite formation. Among the three cytokines tested, IFN- γ (20 ng/ml for 14 days) was the most potent. That thalas-

semic cells are sensitive to NO-mediated apoptosis may be due in part to the levels of cytokine receptors in these cells. It has been documented that cytokine receptors are increased in many diseases [25]. For instance, there are significantly higher serum levels of cytokine receptors IL-2R and TNF-R1 in active polymyositis dermatomyositis patients than in controls [25]. Moreover, cancer-induced diabetes mellitus showed a higher level of TNF-R1 than normal subjects [24].

In summary, β -thal/HbE erythroid progenitor cells in culture were more sensitive to cytokine (IL-1 β , TNF- α

and IFN- γ)-induced apoptosis through iNOS-generated NO compared to cells from normal subjects. This may be part of the etiology of ineffective erythropoiesis contributing to anemia in thalassemia.

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