A mechanism of ineffective erythropoiesis in β-thalassemia/Hb E disease

Pathrapol Lithanatudom,[1] Amporn Leecharoenkiat,[1] Tirawat Wannatung,[1] Saovaros Svasti,[2] Suthat Fucharoen,[2] and Duncan R. Smith[1]

[1]Molecular Pathology Laboratory and [2]Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Thailand

Citation: Lithanatudom P, Leecharoenkiat A, Wannatung T, Svasti S, Fucharoen S, and Smith DR. A mechanism of ineffective erythropoiesis in β-thalassemia/Hb E disease. Haematologica 2010;95:716-723. doi:10.3324/haematol.2009.015701

Online Supplementary Appendix: Design and Methods

Isolation and culture of CD34+ hematopoietic progenitor cells

Peripheral blood samples from 10 β-thalassemia/Hb E patients and 10 normal volunteers were collected in heparin-treated tubes. $CD34^+$ cells were selected and cultured as described previously, with the exception that cells were cultured for ten days.[1] Differentiation and cellular morphology of cells were observed using Wright-Giemsa's staining and differential counting. To induce cellular stress day 10 erythroblasts were cultured in either serum-free medium prepared as described elsewhere[2] for four, eight and 12 h, or cultured in complete medium in the presence of tunicamycin (2 μg/mL of medium, Sigma Chemical, St. Louis, MO, USA) in normal medium for 12 hours. For calcium modulation studies, β-thalassemia/Hb E erythroblasts were treated with ethyleneglycoltetraacetic acid (EGTA; Sigma-Aldrich, St. Louis, MO) to chelate intracellular calcium on day 7 prior to analysis on day 10.

Detection of XBP-1 mRNA splicing

XBP-1 mRNA splicing was detected by RT-PCR analysis. RNA extraction and cDNA synthesis were as described elsewhere.[3] Both un-spliced and spliced XBP-1 products were amplified with the specific primers XBP-1 (XBP1f: 5'-CCTTGTAGTTGAGAACCAGG-3' and XBP1r: 5'-GGGGCTTGGTATATATGTGG-3') which give expected sizes of 442 and 416 bp, respectively. First strand cDNA templates were denatured at 94°C, 10 secs, annealed at 55°C, 20 secs and extended at 72°C, 30 secs for 30 cycles. PCR products were separated on 2% agarose gels by electrophoresis and bands visualized by ethidium bromide staining.

Quantitative and semi-quantitative UPR transcript analysis

At appropriate time points, 1 μg of RNA was extracted from normal and thalassemic erythroblasts using Trizol (Molecular Research Center, Cincinnati, OH) and first-strand cDNA was synthesized using an oligo-$(dT)_{17}$ primer and the AMV RT cDNA synthesis kit (Promega, Madison, WI). Quantitative real time RT-PCR (qRT-PCR) was performed based on the SYBR green technique to detect the expression of four UPR associated gene transcripts (*ERdj4*, *EDEM*, *CHOP*, and *GRP78*). PCR amplification reaction was performed using SYBR green master mix reaction containing 2X master mix, dNTP, Taq polymerase, $MgCl_2$ (iQ™ SYBR® GREEN Supermix; Bio-Rad, Hercules, CA), and primers. First strand cDNA templates were denatured at 94°C, 10 secs, annealed at 55°C, 30 secs and extended at 72°C, 40 secs for 45 cycles. The primer sequences used are ERdj4 (ERdj4f: 5'-TTAGAAATGGCTACTCCCCAGTCA-3' and ERdj4r: 5'CTGTCCTGAACAGTCAGTGTATGTAG-3') EDEM-1 (EDEM-1f: GCCTCCTTTCTGCTCACAGAATAATAA-3' and EDEM-1r: 5'-CTCCTTCTCCTTCATTGCAGGCT-3"), CHOP (CHOPf: CAGAACCAGCAGAGGTCACA-3' and CHOPr: 5-CCAATTGTTCATGCTTGGTG-3'), GRP78 (GRP78f: 5'-GTATTGAAACTGTAGGAGGTGTC -3' and GRP78r: 5'-TATTACAGCACTAGCAGATCAG -3') and Actin (Actf: 5'-GAAGATGACCCAGATCATGT-3' and Actr: 5'-ATCTCTTGCTCGAAGTCCAG-3'). The expression levels of four gene transcripts were normalized against actin. The $2^{\Delta\Delta CT}$ method was used to calculate relative changes in gene expression. A standard curve of each gene was constructed using the threshold cycle CT versus ten-fold serial dilutions of cDNA (1:2, 1:20, and 1:200) to evaluate the efficiency of the amplification. The specificity of the reaction is given by detection of the melting curve of the amplification products immediately after the last reaction cycle. PCRs were performed in the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Additionally, semi-quantitative RT-PCR analysis was performed to provide visual confirmation of the expression of the four UPR-associated genes using the same sets of primers and PCR profile as used for the real time RT-PCR. PCR conditions were optimized for amplification in the exponential phase and PCR products were separated on 2% agarose gels by electrophoresis and visualized by ethidium bromide staining.

Western blot analysis

Western analysis was undertaken as described previously[1] using the following primary antibodies: a rabbit anti-phospho eIF2a polyclonal antibody (#9721; Cell Signaling Technologies, Beverly, MA), a rabbit anti-eIF2a polyclonal antibody (#9702; Cell Signaling Technologies, Beverly, MA), a rabbit anti-GRP78 polyclonal antibody (sc-13968; Santa Cruz Biotechnology, Santa Cruz, CA) and a goat anti-actin polyclonal antibody (sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used were a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000) and a horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:3000) (both from Pierce, Rockford, IL, USA). Detection of signal was performed using the Enhanced Chemiluminescense Plus system (ECL plus; GE Healthcare, Buckinghamshire, UK) following the manufacturer's guideline.

Indirect immunofluorescence and confocal imaging

Approximately 2.5×10^4 erythroblasts from normal controls or β-thalassemia/Hb E patients grown under normal conditions or serum

deprived for 12 h were spun down onto glass cover slips using a Statspin (Iris Sample Processing, Westwood, MA) at 600rpm for six minutes and subsequently processed and imaged as described elsewhere.[4] Primary antibodies used were a goat anti-PERK polyclonal antibody (sc-9481; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit anti-GRP78 polyclonal antibody (sc-13968; Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse anti-ATF6 monoclonal antibody (IMG-273; Imgenex, San Diego, CA) while secondary antibodies used included a Rhodamine Red X conjugated goat anti-rabbit IgG polyclonal antibody (111-295-144, Jackson, West Grove, PA), a Cy5 conjugated rabbit anti-goat IgG polyclonal antibody (81-1616; Invitrogen, Carlsbad, CA), a FITC conjugated donkey anti-rabbit IgG polyclonal antibody (sc-2090, Santa Cruz Biotechnology, Inc) and a FITC conjugated goat anti-mouse IgG polyclonal antibody (02-18-06; KPL, Guilford, UK). Fluorescent confocal microscope images were captured using Olympus FluoView 1000 (Olympus Corporation, Shinjuku-ku, Tokyo) equipped with Olympus FluoView software v. 1.6 exactly as described elsewhere.[4] Image analysis and calculation of Pearson's correlation coefficients and Confidence Intervals were undertaken as previously described.[4]

Flow cytometry for erythroid marker expression and apoptosis detection

1×10^5 erythroblasts derived from normal and thalassemic patients were used to determine the state of erythroid differentiation, as well as the degree of apoptosis induction in day 10 erythroblasts in either normal medium or treated medium as appropriate. To assess erythroid differentiation, cells were stained with an Allophycocyanin (APC)-conjugated monoclonal mouse anti-human Glycopholin A (CD235a) antibody (BD Bioscience, Pharmingen, San Diego, CA) and a fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse anti-human transferrin receptor (CD71) (BD Bioscience, Pharmingen, San Diego, CA) antibody. To assess the induction of apoptosis, cells were incubated with FITC-conjugated Annexin V and subsequently stained with propidium iodide (PI) using the ApoAlert® apoptosis kit (Clontech, Mountain View, CA). Fluorescence signals were detected using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA).

Intracellular calcium measurement

3×10^5 erythroblasts obtained from normal healthy donors and thalassemic patients were used to determine intracellular calcium levels as described elsewhere.[1]

Statistical analysis

All band intensities from both RT-PCR and Western blot analysis were quantitated and normalized against actin expression using the ImageJ 1.41 program (National Institutes of Health, USA). Results in each experiment are statistically presented as the mean value together with the standard error of the mean (SEM) calculated by the GraphPad Prism5 software (GraphPad Software, La Jolla, CA). Statistical significance of mean differences was determined using the non-parametric t-test with a significant P value less than either 0.05 or 0.01.

Supplementary references

1. Wannatung T, Lithanatudom P, Leecharoenkiat A, Svasti S, Fucharoen S, Smith DR. Increased erythropoiesis of beta-thalassaemia/Hb E proerythroblasts is mediated by high basal levels of ERK1/2 activation. Br J Haematol. 2009;146(5):557-68.
2. Choi I, Muta K, Wickrema A, Krantz SB, Nishimura J, Nawata H. Interferon gamma delays apoptosis of mature erythroid progenitor cells in the absence of erythropoietin. Blood. 2000;95(12):3742-9.
3. Susantad T, Smith DR. siRNA-mediated silencing of the 37/67-kDa high affinity laminin receptor in Hep3B cells induces apoptosis. Cell Mol Biol Lett. 2008;13(3):452-64.
4. Panyasrivanit M, Khakpoor A, Wikan N, Smith DR. Co-localization of constituents of the dengue virus translation and replication machinery with amphisomes. J Gen Virol. 2009;90(Pt 2):448-56.

Online Supplementary Table S1. Clinical parameters of 10 β-thalassemia/Hb E patients.

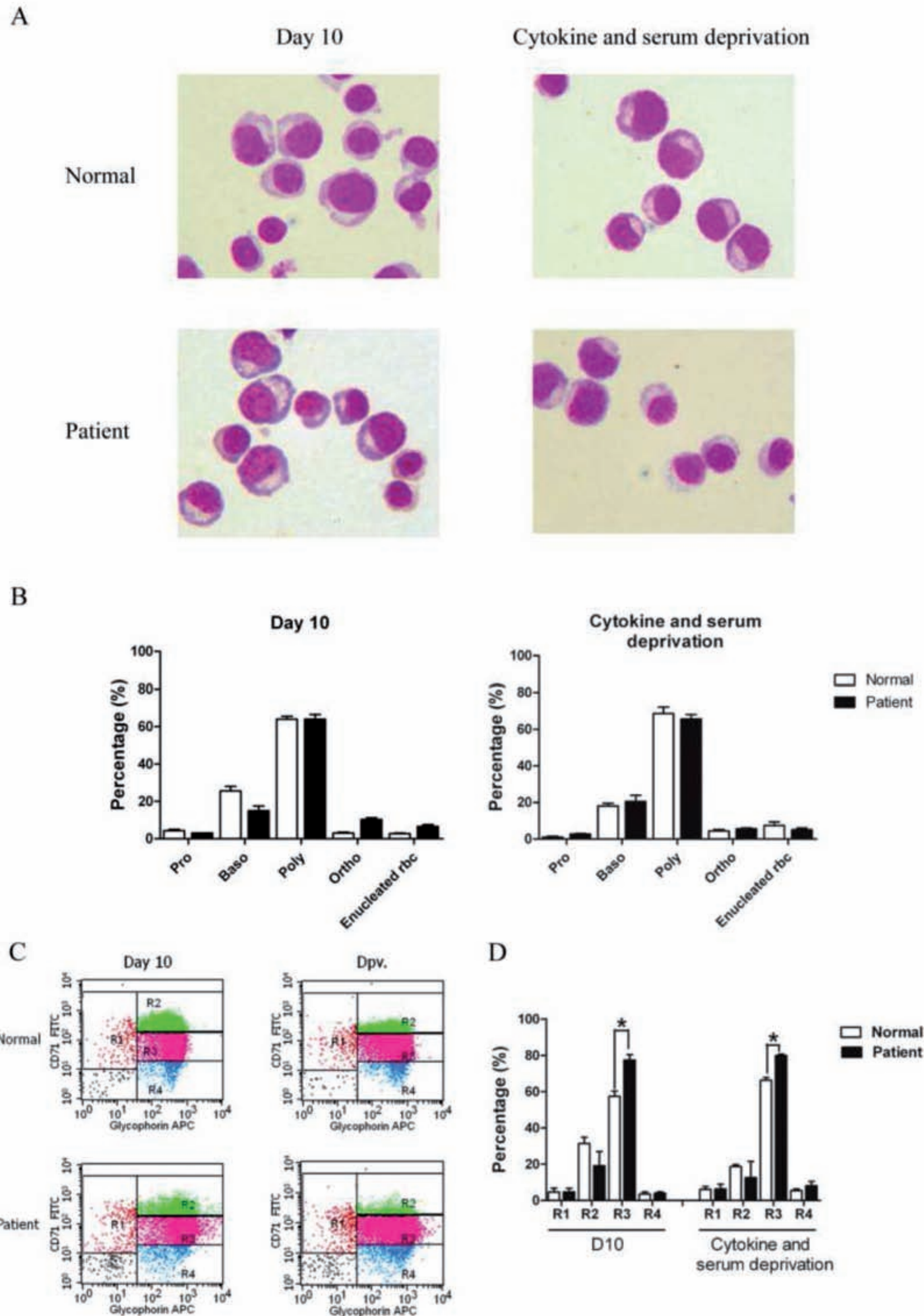
Patient	Sex	Hb (g/l)	Hct (%)	MCV (fl)	MCH (pg)	MCHC (g/l)	RBC ($\times 10^6$ cells/μL)	Genotype (mutation)	Severity
1	M	69	22.9	59.3	18.0	304	3.85	β^E/β^0 (codon 41/42; -TTCT)	Mild
2	M	78	24.2	72.9	23.6	323	3.32	β^E/β^0 (codon 41/42; -TTCT)	Mild
3	M	65	23.9	54.1	14.8	274	4.94	β^E/β^0 (codon 41/42; -TTCT)	Mild
4	F	91	26.5	53.7	16.7	311	4.47	β^E/β^0 (codon 17; A→T)	Mild
5	F	95	29.8	66.6	21.1	316	4.41	β^E/β^0 (codon IVSI-1; G→T)	Mild
6	M	69	24.1	60.6	19.2	293	3.98	β^E/β^0 (codon 41/42; -TTCT)	Severe
7	M	58	24.1	66.3	18.2	274	3.63	β^E/β^0 (codon 41/42; -TTCT)	Severe
8	F	50	17.4	79.7	22.7	285	2.19	β^E/β^0 (codon 41/42; -TTCT)	Severe
9	F	54	22.3	68.5	20.0	292	2.26	β^E/β^0 (codon 41/42; -TTCT)	Severe
10	F	66	23.4	75.6	22.9	304	3.09	β^E/β^0 (codon 17; A→T)	Severe

Hb: total hemoglobin; Hct: hematocrit ; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; RBC: red blood cell.

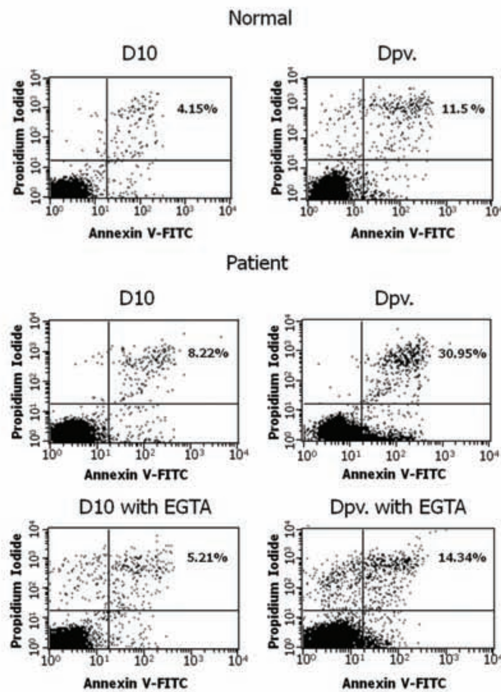
Online Supplementary Table S2. Summary of average Pearson's correlation coefficients.

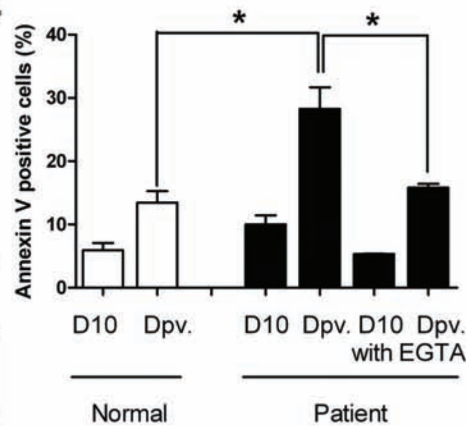
Colocalization markers	Pearson's correlation coefficients		
	Normal	Patient	*P* value
ATF6 – GRP78 (Control D10)	0.92±0.01	0.89±0.01	*P*=0.0003
ATF6 – GRP78 (Deprivation)	0.11±0.02	0.90±0.01	*P*<0.0001
ATF6 – GRP78 (Tunicamycin)	0.12±0.01	0.13±0.01	*P*=NS
ATF6 – GRP78 (D10 with EGTA)	-	0.89±0.03	-
ATF6 – GRP78 (Deprivation with EGTA)	-	0.16±0.03	-
PERK – GRP78 (Control D10)	0.87±0.02	0.92±0.01	*P*=0.0001
PERK – GRP78 (Deprivation)	0.49±0.06	0.92±0.01	*P*<0.0001
PERK – GRP78 (Tunicamycin)	0.46±0.01	0.51±0.01	*P*=NS
PERK – GRP78 (D10 with EGTA)	-	0.84±0.02	-
PERK – GRP78 (Deprivation with EGTA)	-	0.41±0.01	-

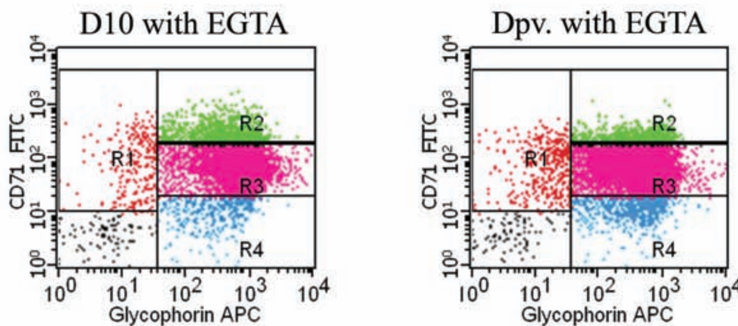
P<0.05 is considered significant

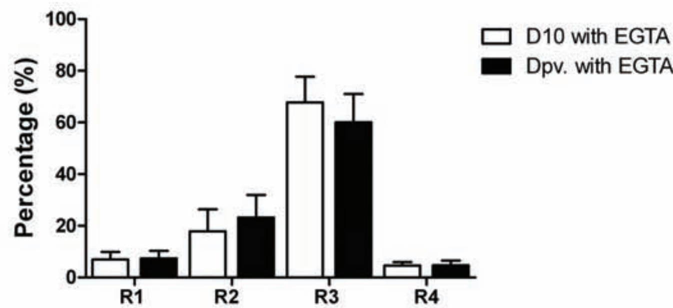


Online Supplementary Figure S1. Determination of erythroid maturation stage in normal and β-thalassemia/Hb E erythroblast. (A) Wright-Giemsa's staining of normal and thalassemic erythroblasts (x1000). (B) Stages of erythroid differentiation of normal and thalassemic erythroblasts on day 10 of normal culture and after 12 h of serum and cytokine deprivation (Dpv). (C) Representative flow cytometry analyses from one normal control and one patient after staining with glycophorin A (CD235a) and CD71 to determine the stages of erythroid differentiation on day 10 and after serum deprivation. Quadrants are designated as R1 (CD71 high, glycophorin A low), R2 (CD71 high, glycophorin A high), R3 (CD71 medium, glycophorin A high) and R4 (CD71 low, glycophorin A high). (D) Quantitation analysis of erythroid maturation stages as shown in (C) from 3 normal and 4 β-thalassemia/Hb E patients. Data is displayed as mean +S.E.M.









Online Supplementary Figure S2. Serum withdrawal induced apoptosis in day 10 erythroblasts. (A) Representative flow cytometry analyses of day 10 erythroblasts from one control and one β-thalassemia/Hb E patient under conditions of normal growth (D10), serum withdrawal for 12 hours (Dpv) and (for patient sample) cells grown for three days (days 7 to 10) in the presence of 1mM EGTA. Cells were stained with Annexin V-FITC and propidium iodide (PI) prior to flow cytometry analysis. Percentage indicates total of solely Annexin V+ cells (lower right) and AnnexinV+/PI+ cells (upper right). (B) Graphical analysis of total Annexin V positive cells treated as (A) for 6 normal controls and 6 β-thalassemia/Hb E patients. The mean values (± SEM) are shown. An asterisk (*) indicates *P*<0.05. (C) Representative flow cytometry analysis of erythroid markers, glycophorin A (CD235a) and CD71 to monitor differentiation of day 10 β-thalassemia/Hb E erythroid cells upon EGTA treatment (days 7 to 10) with and without serum deprivation. Quadrants are as in Figure 1C. (D) Quantitation analysis of the stage of erythroid differentiation as in (C). Means (± SEM) values of 4 β-thalassemia/Hb E patients are shown.