INVOLVEMENT OF NF-κB IN NORMAL AND THALASSEMIC ERYTHROPOIESIS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (MEDICAL TECHNOLOGY) FACULTY OF GRADUATE STUDIES MAHIDOL UNIVERSITY 2004

ISBN 974-04-5081-4

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Doctor of Philosophy (Medical Technology)
on
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ACKNOWLEDGEMENTS

I wish to express my deepest, sincere gratitude to my advisors, Dr. Pranee Fucharoen, Prof. Suthat Fucharoen and Assoc. Prof. Koichiro Muta, for their valuable supervision, guidance and continuous encouragements which have enabled me to carry out my Ph.D. studies successfully. I am indebted to the members of the thesis examination committee, for providing constructive comments and suggestions and also for proofreading of the thesis. I am also indebted to Prof. Dr. Kovit Pattanapanyasat for his helpful suggestion about flow cytometry, Prof. Dr. Naipinich Kochabpakdi for his help on teaching and using of confocal microscopy.

I would like to thank all staffs at the Thalassemia Research Center, Mahidol University, Thailand and at the Internal Medicine, Kyushu University, Japan for their friendship and their guidance and assistance in laboratory work. In addition, I would like to thank Mrs. Surada Noulsri for her help on technical assistance of flow cytometry.

My sincerely thank is extended to my family for their infinite love, understanding and encouragement throughout the entire period of my studies.

Finally, sincere appreciation is expressed to the Thai Royal Affair, the NSTDA, Thailand and Kyushu University, Japan for support of this work.

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ABSTRACT

The mechanism to prevent apoptosis is a key function of the growth factors in regulating erythropoiesis in the bone marrow and in *in vitro* erythroid differentiation study. This mechanism involves many signal transduction molecules, including nuclear transcription factor-κB (NF-κB), which plays an important role in normal erythropoiesis. Many studies have indicated that NF-κB activation can suppress cell death pathway and protect many cell types from apoptosis. NF-κB activation allows freed NF-κB to translocate from cytoplasm to the nucleus and bind to a specific site of DNA to regulate gene expression, such as anti-apoptotic protein Bcl-x.

Accelerated apoptosis of erythroid progenitor cells in thalassemia has been reported. The role of NF-κB in regulating apoptosis in erythroid progenitor cells of normal and β-thalassemia/Hb E was investigated in this study by using cyclosporine A (CsA) to block NF-κB complex dissociation. The correlation of apoptosis, NF-κB expression, Bcl-x expression and mitochondrial function was assessed from the CsA-treated and CsA-untreated cells cultured with or without EPO or IFN-γ. The results showed that EPO retarded apoptosis of both normal and thalassemic erythroid progenitor cells via NF-κB activation and Bcl-x involvement, while IFN-γ retarded apoptosis of normal and thalassemic erythroid progenitor cells via other mechanisms. This information helps to get a better understanding of how erythropoiesis, in both normal and thalassemia, may be regulated.

KEY WORDS: NF-κB / THALASSEMIA / ERYTHROPOIESIS / EPO / IFN-γ

146 pp. ISBN 974-04-5081-4

บทบาทของ NF-κB เกี่ยวข้องกับกระบวนการสร้างเม็ดเลือดแดงปกติและธาลัสซีเมีย
(INVOLVEMENT OF NF-κB IN NORMAL AND THALASSEMIC ERYTHROPOIESIS)

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บทคัดย่อ

การป้องกันการตายแบบอะพอพโตซิสโดยโกรตแฟกเตอร์เป็นกลไกหนึ่งที่เกี่ยวข้องกับการสร้างเม็ดเลือดแดงในไขกระดูกและการเปลี่ยนแปลงของเซลล์เม็ดเลือดแดงในหลอดทดลอง มีรายงานว่า NF-κB เกี่ยวข้องกับการสร้างเม็ดเลือดแดงปกติ การกระตุ้น NF-κB สามารถยับยั้งกระบวนการที่ทำให้เซลล์ตายและช่วยป้องกันการเกิดอะพอพโตซิสของเซลล์หลายชนิด เมื่อ NF-κB ถูกกระตุ้น NF-κB ซึ่งจับกับโปรตีนยับยั้งจะหลุดเป็นอิสระ แล้วเคลื่อนย้ายจากไซโตพลาสมเข้าไปยังนิวเคลียสเพื่อจับกับยีนในตำแหน่งที่จำเพาะ ส่งผลให้ยีนนั้นแสดงออก

การศึกษาที่ผ่านมาพบว่าเซลล์ต้นกำเนิดเม็ดเลือดแดงธาลัสซีเมียเกิดอะพอพโตซิสมากกว่าปกติโดยไม่ทราบสาเหตุ งานวิจัยนี้จึงได้ศึกษาบทบาทของ NF-κB ในการควบคุมการเกิดอะพอพโตซิสของเซลล์ต้นกำเนิดเม็ดเลือดแดงปกติและเซลล์ต้นกำเนิดเม็ดเลือดแดงธาลัสซีเมียโดยใช้ cyclosporine A (CsA) เป็นสารยับยั้งการแยกตัวของ NF-κB เมื่อนำเซลล์ต้นกำเนิดเม็ดเลือดแดงที่เพาะเลี้ยงในหลอดทดลองมาอุ่นในสภาวะที่มีหรือไม่มี CsA ก่อนนำไปเพาะเลี้ยงในสภาวะที่มีหรือไม่มี EPO หรือ IFN-γ แล้วตรวจหาความสัมพันธ์ของการเกิดอะพอพโตซิสและการแสดงออกของ NF-κB การแสดงออกของ Bcl-x ตลอดจนการทำงานของไมโตคอนเดรีย พบว่า EPO ชะลอการตายของเซลล์ต้นกำเนิดเม็ดเลือดแดงปกติและเซลล์ต้นกำเนิดเม็ดเลือดแดงธาลัสซีเมียโดยการกระตุ้น NF-κB และการแสดงออกของ Bcl-x ขณะที่ IFN-γ ชะลอการตายของเซลล์โดยกลไกอื่น ผลการศึกษานี้เป็นข้อมูลที่ช่วยให้เข้าใจกลไกการสร้างเม็ดเลือดแดงปกติและเม็ดเลือดแดงธาลัสซีเมียได้ดียิ่งขึ้น

146 หน้า ISBN 974-04-5081-4

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree celsius
α	Alpha
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis promoting factor-1
APO-1	CD95 or Fas receptor
ATP	Adenosine triphosphate
β	Beta
Bax	Bcl-2 associated x protein
Bcl-x_L	B cell lymphoma-x long
BFU-E	Burst-formining unit-erythroid
BH3	Bcl-2 homology region 3
BH4	Bcl-2 homology region 4
BM	Bone marrow
bp	Base pair
Br-UTP	Bromodeoxyuridine triphosphate
Ca^{2+}	Calcium ion
Caspase	Cysteinyl aspartate-specific proteinase
CD117	Stem cell factor receptor or c-kit
CD71	Transferrin receptor
CD95	Fas receptor or APO-1
CD95L	Fas ligand
CDK	Cyclin dependent kinase
Ced-	Cell death gene
CFU-E	Colony forming unit-erythroid
c-IAP	Cellular inhibitor of apoptosis

LIST OF ABBREVIATIONS (CONT.)

CIP	CDK inhibitor protein
CsA	Cyclosporine A
Cu	Copper
δ	Delta
DCFH-DA	2',7'-dichloro dihydrofluorescein diacetate
DD	Death domain
DED	Death efffector domain
$DiOC_6$	3',3'-dihexiloxa-dicabocyanide
DISC	Death-inducing signaling complex
dL	Deciliter
DNA	Deoxyribonucleic acid
DR3	Death receptor 3
DTT	Dithiothreitol
$\Delta\psi_m$	Mitochondrial transmembrane potential
ε	Epsilon
ECFCs	Erythroid-colony forming cells
ECL	Enhancer chemiluminescence
EDTA	Ethylenediamine-tetra-acetic acid
EGTA	Ethyleneglycol-bis(aminoethylether)-N,N'-tetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
EPOR	Erythropoietin receptor
FADD	Fas associated death domain
FADD	Flavin adenosine dinucleotide
fam-DEVD-*fmk*	Carboxyfluorescein (FAM)-labeled aspatylglutamylvalylaspartic acid fluoromethyl ketone (FMK)

LIST OF ABBREVIATIONS (CONT.)

fam-LEHD-*fmk*	Carboxyfluorescein (FAM)-labeled leucylglutamylhistidylaspartic acid (LEHD) fluoromethyl ketone (FMK)
fam-VAD-*fmk*	Carboxyfluorescein (FAM)-labeled valylalanylaspartic acid (VAD) fluoromethyl ketone (FMK)
Fe	Iron
FeS	Ferrous sulfide
FITC	Fluorescencein isothiocyanate
fL	Femtoliter
FLICE	FADD-like interleukin converting enzyme
FMN	Flavin mononucleotide
γ	Gamma
g	Gram
g/mL	Gram per mililiter
Gadd45β	Growth arrest and DNA-damage-inducible beta
GATA-1	The transcription factor of the GATA family
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPA	Glycophorin A
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
Hb CS	Hemoglobin Constant Spring
HCAM	Homing cell adhesion molecule
HE	Hydroethedium
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HSC	Hematopoietic stem cell
IAP	Inhibitor of apoptotic protein
ICAM	Intracellular cell adhesion molecule

LIST OF ABBREVIATIONS (CONT.)

ICE	Interleukin 1b converting enzyme
IFN	Interferon
IGF-1	Insulin-like growth factor 1
Ig G	Immunoglobulin G
IκB	Inhibitory κB
IKK	Inhibitory κB kinase
IL-1	Interleukin-1
IL-3	Interleukin-3
IL-6	Interleukin-6
IMDM	Isocove's modified Dulbecco's medium
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun kinase
JNKK	JNK kinase
κ	Kappa
kb	Kilobase pair
kDa	Kilodalton
L	Liter
LPS	Lipopolysaccharide
μ	Micro
M	Molar
MAP	Mitogenic activating protein
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MEKK1	MAP/Erk kinae kinase 1
μg	Microgram
mg	Milligram

LIST OF ABBREVIATIONS (CONT.)

μL	Microliter
mL	Mililiter
μm	Micrometer
μM	Micromolar
mM	Milimolar
Mo	Molybdenum
mRNA	Messenger ribonucleic acid
NF-AT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
ng	Nanogram
NIK	NF-κB inducing kinase
NLS	Nuclear localization sequence
nm	Nanometer
nNOS	Neural nitric oxide synthase
NP-40	Noniodet P-40
O_2^-	Superoxide anion
PARP	Poly(adenosine diphospate-ribose) polymerase
PB	Peripheral blood
PE	Phycoerythrin
pH	Negative logarithm of hydrogen ion activity
PI	Proprodium iodide
PI3K	Phosphoinositol-3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RDW	Red cell distribution width

LIST OF ABBREVIATIONS (CONT.)

Rh 123	Rhodamine 123
RHR	Real homology region
RIP	Receppror-interacting protein
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCF	Stem cell factor or kit ligand
SD	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Src homology 2
SHIP	SH2 containing inositol 5-phosphate
STAT	Signal transduction and activation of transcription
TdT	Terminal deoxynucleotidyl transferase
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor
TPO	Trombopoietin
TRADD	TNF receptor associated death domain
TRAF	TNFR associated factor
TRAIL	TNF-related apoptosis-inducing ligand
Tris	Tris (hydroxymethyl)aminomethane)
TUNEL	TdT-mediated dUTP-fluorescein nick end labelling (TUNEL)
UV	Ultraviolet light
VCAM	Vascular cell adhesion molecule

LIST OF ABBREVIATIONS (CONT.)

VLA-4	Very late antigen-4
VLA-5	Very late antigen-5
XIAP	Human X-chromosome-linked inhibitor-of-apoptosis
ψ	Pseudo
ζ	Zeta

CHAPTER 1
INTRODUCTION

The thalassemias are a group of inherited disorders in which globin chain synthesis is impaired. Thalassemia is generally classified into two types according to the defect of globin gene i.e. α-thalassemia and β-thalassemia. As a direct result of genetic defect, thalassemic red cells contain the precipitated inclusion of an excess amount of hemoglobin subunit (1, 2). The main pathophysiologic feature of thalassemia is the accumulation of unpaired globin chains in erythrocyte precursors and red blood cells. This accumulation alters cell membrane function and results in early cell destruction and ineffective erythropoiesis. The evidence of ineffective erythropoiesis in thalassemia was first reported by an electron microscopic study of marrow fragments from patients with homozygous β-thalassemia (3). Recent studies show that the extent of apoptosis is variable in each type of thalassemia, and apoptosis is increased with increasing erythroid maturity (4-6). Thalassemic erythroid precursors underwent apoptosis at a rate that was 3 to 4 times normal. Because thalassemic marrow has between 5- to 6-fold more erythroid precursors than healthy marrow, thus an absolute increase in erythroid precursor apoptosis was about 15-fold above healthy (4). The extreme rates of erythroid hyperplasia and apoptosis might be characteristic of more severely affected patients. Although, the regulation of normal erythropoiesis is extensively studied, there are less information, especially molecular mechanism, about the regulation of erythropoiesis in thalassemia is known.

The hematopoietic pluripotent stem cell undergoes a series of developmental changes that commit to specific cell lineage. The first erythroid-committed progenitor is defined functionally as a burst-forming unit (BFU-E). Following proliferation and differentiation, these cells give rise to colony forming unit (CFU-E). These progenitor cells continue to proliferate and differentiate, becoming mature red cells containing hemoglobin. Erythroid maturation is mediated by combination of regulatory proteins,

acting in concert direct to the development of the progenitor cells into mature erythrocytes. Important growth factors include interleukin-3 (IL-3), erythropoietin (EPO), granulocyte macrophage-colony-stimulating factor (GM-CSF), and stem cell factor (SCF) have been shown to deliver survival/proliferation signals to erythroid progenitor cells. EPO is especially essential to maintain the viability of erythroid progenitor cells by allowing them to undergo the process of proliferation and maturation (7-9). However, it is becoming clear that erythroid cells die not only as a consequence of growth factor withdrawal but also in response to apoptosis inducers, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and Fas ligand (FasL). Although these negative regulators of hematopoiesis are involved in inhibitory effect, IFN-γ has been reported to stimulate growth of hematopoiesis (10-12). Furthermore, in the absence of EPO, IFN-γ delays apoptosis of mature erythroid progenitor cells (13). However, IFN-γ induced Fas expression and activated both caspase 8 and caspase 3 in the absence of EPO. Expression of Bcl-x, which is a member of anti-apoptotic regulator of Bcl-2 family, is also decreased when the erythroid progenitor cells are incubated with IFN-γ without EPO. Thus, IFN-γ produces a stimulating signal for survival of mature erythroid progenitor cells by reducing apoptosis through a mechanism other than modulating Fas and one related to the expression of Bcl-x. Since one of the key proteins that modulate the apoptotic response is NF-κB, the transcription factor that can protect or contribute to apoptosis, the role of NF-κB should be verified.

NF-κB transcription factor is a heterodimer consisting of the 50-kDa (p50) and 65-kDa (p65) subunits. In an inactive state, it binds to an inhibitory protein, IκBα, and is localized in the cytoplasm. Upon activation, NF-κB dissociates from IκBα, translocates to the nucleus and binds to a specific site of the DNA to regulate gene expression (14, 15). Several genes in erythroid progenitor cells, such as *c-myb* and *c-myc* (16), consist of NF-κB binding sites, and their expressions affect erythroid development (17, 18). The activation of NF-κB involves intracellular components that mediate cytokine stimuli such as MAP kinase, JAK, STAT and PI3K. In addition, NF-κB is activated by cytokines known to regulate erythropoiesis such as tumor necrosis factor-α (TNF-α) (19, 20), interferon-γ (IFN-γ) (20), and transforming growth factor-

β1 (TGF-β1) (21). Recently, the erythropoietin receptor (EPOR) has been shown to transmit signals that lead to the activation of NF-κB (22). NF-κB was expressed during early normal erythroid proliferation and its level declined during differentiation (16). It was postulated that NF-κB modulated the erythropoiesis via its downstream target genes, *c-myb* and *c-myc*, which their expressions were required for the erythroid development (17, 23, 24). *c-Myc* and *c-myb* were expressed at a high level in basophilic erythroblasts (23). It also associated with the progression of erythroid progenitor cells into S phase of cell cycle (17).

The possible role of NF-κB in erythroid gene regulation and erythropoiesis has been demonstrated in transient transfection studies of human embryonic fetal/erythroid cell line K562 and mouse adult erythroid MEL cells (25). The relatively high activity of NF-κB in erythroid progenitor cells was involved in the suppression of *NF-E2*, which is known to transactivate erythroid-specific gene. NF-κB motif located at 12 kb 3' to the ζ-globin gene also demonstrated (26). The NF-κB complex formation was a crucial component of ζ-globin gene silencing. A two-base substitution that disrupted NF-κB site protein binding resulted in the loss of the developmental silencing activity (26). The role of NF-κB as the activation of erythropoietic genes, as well as a second messenger for cytokine responses, suggests that it may have an important role in human erythropoiesis. Several studies have also indicated that NF-κB activation is required to protect many cell types from program cell death by suppressing the apoptotic cell death pathway (27-29).

The Bcl-2 family of cell death regulators is critical for determining cell fate in the apoptotic pathway. NF-κB differentially regulates the expression of particular Bcl-2-related death inhibitors. An NF-κB binding site has been demonstrated in the *Bcl-x* gene (30, 31), and it directly activates the expression of Bcl-x_L (32). Bcl-x was strongly increased during the terminal differentiation stages of erythroblasts, reaching maximum transcript and protein levels at the time of maximum hemoglobin synthesis (33). Bcl-x expression in proerythroblasts was highly EPO-dependent (33). Bcl-x involved in erythroid progenitor cell survival via GATA-1, EPO cooperation and mitochondrial function (34, 35).

CHAPTER 2
OBJECTIVES

The aim of this thesis is to clarify the role of NF-κB in regulating apoptosis of erythroid progenitor cells in normal and thalassemia by using cyclosporine A. In the presence of stimuli, active NF-κB translocates from the cytoplasm to the nucleus and binds its specific binding site of target gene, Bcl-x, resulting in the gene expression. Bcl-x could prevent apoptosis by inhibiting the releasing of cytochrome c from the mitochondria. To provide this hypothesis, cyclosporine A was added to block NF-κB complex dissociation, thus the less amount of free active NF-κB would translocate from the cytoplasm to the nucleus. Consequently, the expression of antiapoptotic protein, Bcl-x, was decreased, and the increased apoptosis of erythroid progenitor cells was demonstrated. The mitochondrial function was also determined when apoptotic induction was induced by CsA. The strategies of the study were as follows:

1. Determine concentrations of EPO and IFN-γ, which produce anti-apoptotic effect on erythroid progenitor cells.

2. Investigate the optimal concentration of CsA, which produces apoptotic effect on erythroid progenitor cells in the presence or absence of EPO and IFN-γ.

3. Investigate the expression and the localization of NF-κB in erythroid progenitor cells pre-treated with or without cyclosporine A before culturing cells in the presence or absence of EPO or IFN-γ.

4. Determine the expression of Bcl-x and the mitochondrial function in erythroid progenitor cells pre-treated with or without cyclosporine A before culturing cells in the presence or absence of EPO or IFN-γ.

5. Analyse the relationship of apoptosis and the expression of NF-κB, Bcl-x and the mitochondrial function of erythroid progenitor cells.

CHAPTER 3
LITERATURE REVIEWS

1. Apoptosis

1.1 Apoptosis and necrosis

Apoptosis is an inducible suicide program that occurs at all stages of multicellular life. It is required for normal development, immune system function, tissue remodelling, and to prevent inappropriate cellular proliferation. Apoptosis and necrosis are two modes of cell death with distinct morphological and biochemical features (36). Apoptosis is an active process characterized by cell shrinkage, nuclear and cytoplasmic condensation, chromatin fragmentation and phagocytosis. In contrast, necrosis is a passive form of cell death associated with inflammation, resulting from cellular and organelle swelling, rupture of the plasma membrane and spilling of cellular contents into the extracellular environments.

1.2 Morphological features

There are three distinct phases of apoptosis. In the first phase, the DNA is digested by specific endonucleases into fragments and ultimately packed into vesicles. The changes in DNA include strand breakage and condensation of nuclear chromatin. The endoplasmic reticulum swells and exocytoses its contents. The cell becomes denser as the cytoplasm shrinks and involutes. In the second phase, the cell produces budding, which contain organelles or nuclear fragments, and these break off into multiple membrane-bound vesicles. The remaining cell becomes a round, smooth membrane-bound remnant (apoptotic body). In the third phase, the cell membrane becomes permeable to dyes such as tryphan blue. The apoptotic body and membrane-bound buds may then be phagocytosed by macrophages, epithelial cells, vascular endothelium or tumor cells.

In contrast, necrosis is characterised by cellular and organelle swelling with late nuclear fragmentation and breakdown by lysosomal enzymes. The swelling is caused by a deficit in adenosine triphosphate (ATP) production that leads to membrane ionic pump failure and increases plasma membrane permeability. This results in bleb formation of the plasma membrane and ultimate rupture. There is an influx of neutrophils and macrophages in the surrounding tissues, leading to generalized inflammation.

1.3 Mechanism of cell death

Following an appropriate stimulus, the first stage or 'decision phase' of apoptosis is the genetic control point of cell death. This is followed by the second stage or 'execution phase', which is responsible for the morphological changes of apoptosis. There are four main groups of stimuli for apoptosis. The first group of stimuli causes DNA damage and includes ionizing radiation and alkylating anticancer drugs. The second group induces apoptosis via receptor mechanisms, either by receptor activation mediated by glucocorticoids (acting on the thymus), tumor necrosis factor-α (TNF-α), or by withdrawal of growth factors (nerve growth factor) and interleukin-3 (IL-3). The third group comprises biochemical agents that enhance the downstream components of the apoptotic pathway and includes phosphatases, and kinase inhibitors (e.g. calphostin C, staurosporine). The fourth group comprises agents that cause direct cell membrane damage and includes heat, ultraviolet light and oxidising agents (superoxide anion, hydrogen peroxide). Excessive production of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and the hydroxyl radicals, produces free radicals that damage lipid membranes, proteins, nucleic acids and extracellular matrix glycosaminoglycans. Many of these stimuli cause necrosis in larger doses.

In the decision phase, apoptosis is genetically controlled and two genes, Bcl-2 and p53 are involved. Bcl-2, is found on the mitochondrial membrane and endoplasmic reticulum. It may control calcium channels. It is now recognized that a family of mammalian proteins similar to Bcl-2, promotes or inhibits apoptosis. Proteins such as Bcl-2 and Bcl-x_L prevent apoptosis, whereas Bcl-2 associated x proteins (Bax) such as Bax, Bad, Bak and Bcl-x_S promote apoptosis. p53 is a 53-kDa

nuclear phosphoprotein that binds to DNA to act as a transcription factor, and controls cell proliferation and DNA repair. Activation of p53 blocks cell cycle in G_1 phase and this gives time to DNA repairing machinery to restore DNA before replication. On activation of p53 the cell could undergo either growth arrest or apoptosis. The ability of p53 to induce cell death or growth arrest resides in different parts of the peptide sequence. Mutations of p53 have been found in human cancers, e.g. colon carcinoma, and are associated with resistance to treatment . The gene *c-myc* is a proto-oncogene that encodes a sequence-specific DNA-binding protein that acts as a transcription factor and induces apoptosis in the presence of p53. The c-myc protein is elevated in many tumors.

In the execution phase, the central events in apoptosis are proteolysis and mitochondrial inactivation. Mitochondria involves cell death via at least three general mechanisms (37): (i) disruption of electron transport and ATP production, (ii) release of caspase-activating protein; and (iii) alteration of cellular reduction-oxidation potential. Disruption of electron transport has been recognized as early feature of cell death because ATP production is dropped and apoptosis is induced. During apoptosis, mitochondria releases caspase-activating proteins, such as cytochrome c, apoptotic-inducing factor (AIF). Once cytochrome c is released, the cell is committed to die by either a rapid apoptotic mechanism involving Apaf-1-mediated caspase activation or a slower necrotic process due to collapse of electron transport. The latter also occurs when cytochrome c is depleted from mitochondria, resulting in a variety of deleterious sequelae inducing generation of oxygen free radicals and decreased production of ATP. However, the actions of the caspases are varied; some are endonucleases that cleave DNA, some cleave cytoskeletal proteins and others cause a loss of cell adhesion. The integrity of the plasma membrane of the apoptotic cell is maintained initially, although budding of the cell membrane can occur later.

Alteration of cellular reduction-oxidation potential is a feature of apoptosis, which involves in mitochondria. Since mitochondria are the major source of superoxide anion (O_2^-) production in cells. An estimated 1 to 5% of electrons in the respiratory chain lose their way and participating mostly in formation of O_2^-. Anything that decreases the coupling efficiency of electron chain transport can increase production of superoxides. Superoxides and lipid peroxidation are increased during

apoptosis induced by myriad stimuli. However, generation of ROS may be a relatively late event, occurring after cells have entered a process of caspase activation (38). Table 1 lists the most important mammalian cellular systems that can generate ROI (39).

1.3.1 The death receptor pathway

The signal that initiates apoptosis may result from binding of a cell-surface death receptor or from damage to the genome. Death receptors that initiate apoptosis include the Fas receptor, the TNF receptor 1 (TNFR1), and death receptor 3 (DR3) (40).

The Fas receptor, initially known as CD95 or APO-1, is a transmembrane glycoprotein death receptor that is activated by binding of Fas ligand (FasL) to cell membranes. Ligation of Fas results in aggregation of the intracellular death domain, leading to the recruitment of signaling proteins and formation of the death-inducing signaling complex (DISC), which includes Fas, FasL, an adapter molecule, Fas-associated protein with death domain (FADD) and FLICE (FADD-like ICE). FADD binds to Fas via the death domain, and subsequently recruits procaspase-8 to the DISC. Following release of active caspase-8 from the DISC, a cascade of ICE-like proteases is activated. An anti-apoptotic factor, c-FLIP, has been shown to exist in two isoforms, c-FLIPS and c-FLIPL, which appears to be the predominant form expressed in mammalian cells. c-FLIP can be recruited to the DISC and can block further recruitment of procaspase-8 into the complex, thereby inhibiting the activation of caspase-8. In Fas-mediated apoptosis, two signaling pathways have been demonstrated (Figure 1). In type I cells, ligation of Fas leads to strong caspase-8 activation at the DISC thereby directly activating other caspases including caspase-3 in the absence of mitochondrial involvement. In type II Fas-mediated cell death, only a small amount of DISC is formed leading to the activation of a small amount caspase-8, which cleaves the cytosolic substrate Bid. The proteolytically modified Bid induces conformational changes in Bax that lead to pore formation in the mitochondrial membrane. Antiapoptotic members of the Bcl-2 family can block this process. Release of mitochondrial cytochrome c and ATP triggers the formation of the “apoptosome” with caspase-9 and the adaptor molecule Apaf-1. In the apoptosome caspase-9

undergoes autoactivation and further activates the effector caspases-3, -6, and -7. The active caspase-6 can activate additional caspase-8 molecules such that the apoptotic signal of Fas can be further amplified

The TNF receptor system mediates different biochemical pathways (41). TNF trimerizes TNFR1 upon binding, inducing association of receptors' death domains. Subsequently, a TNF receptor-associated death domain (TRADD) binds through its own death domain to the cluster receptor death domains. TRADD functions as a platform adapter that recruits several signaling molecule to the activated receptor: TNF receptor-associated factor 2 (TNFR2) and receptor-interacting protein (RIP) stimulating pathways leading to activation of NF-κB and JNK/AP-1. TNFR2 and RIP activate the NF-κB-inducing kinase (NIK), which in turn activates the inhibitor of κB (IκB) kinase complex (IKK). IKK phosphorylates IκB, leading to IκB degradation and allowing NF-κB to move to the nucleus to activate transcription. The pathway from TNFR2 and RIP to JNK involves a cascade that includes the mitogen-actvating protein (MAP) kinase MEKK1 (MAP/Erk kinase kinase 1), JNKK (JNK kinase), and JNK. MEKK1 is related to NIK, and it is implicated in the pathway (Figure 2).

1.3.2 Apoptotic signaling via mitochondria

Whereas death receptors respond to instructive signals from neighboring cells or soluble ligands, an alternative cell death pathway depends on mitochondrial dysfunction and is initiated in response to primarily cell-intrinsic cues (Figure 1) (40). Intracellular stimuli including DNA damage, glucocorticoids, perturbations in redox balance, ceramide generation, and loss of growth factor signals can trigger permeabilization of the outer mitochondrial membrane and leakage of mitochondrial pro-apoptotic effectors into the cytosol. One such effector is cytochrome c, which stimulates assembly of the mitochondrial 'apoptosome', that consists of cytochrome c, Apaf-1 and caspase-9. Cytosolic cytochrome c binds tightly to Apaf-1, and induces oligomerization of this protein, which subsequently recruits pro-caspase-9. In turn, caspase-9 is activated autoproteolytically and triggers activation of caspase-3.

A recently identified participant in an alternate cell death pathway is AIF, which translocates from mitochondria to nuclei during apoptotic conditions and

induces caspase-independent chromatin condensation and large-scale (50 kb) DNA cleavage (42).

1.4 Therapeutic possibilities and future directions

The widespread involvement of apoptosis in the pathophysiology of disease lends itself to therapeutic intervention. In diseases caused by increased cell loss, such as viral hepatitis and neurodegenerative disease, the aim will be to minimise apoptosis by modifying the signals which trigger the response (e.g. ROS) or interfering with the effectors (e.g. caspases and endonucleases). However, inhibition of apoptosis may be deleterious because new tumors may arise when damaged cells are prevented from committing suicide.

In diseases caused by deficient apoptosis, such as cancer, viral latency and autoimmunity, agents targeting receptors or regulatory molecules and agents targeting the final common pathway are attractive possibilities. The soluble form of Fas could prove useful for increasing apoptosis (e.g. in tumors). Antibodies to Fas or Fas-L may be useful in preventing apoptosis (e.g. in neurodegenerative disease). Preliminary successes in treating chronic inflammatory diseases, such as rheumatoid arthritis and ulcerative colitis, with TNF-α inhibitors have been reported, but these therapies have proved disappointing in sepsis. The regulatory molecule Bcl-2 may protect normal cells from death induced by cytotoxic agents. In contrast, decreasing Bcl-2 in cancer cells can reinstate chemo- and radio-sensitivity. This can be achieved by treatment with synthetic short DNA single strands called antisense oligonucleotides, which bind to specific messenger RNA sequences and prevent production of the offending protein. The caspases are of interest from a therapeutic point of view, as specific inhibitors exist. Tetrapeptide aldehydes are potent inhibitors of ICE but are toxic. They act by specifically binding to the protease and preventing cleavage of the target proteins. Inhibitors of ICE can inhibit apoptosis in a number of cell systems. They may have a role in sepsis and chronic inflammatory and neurodegenerative disease.

Table 1 Cellular ROI-forming system[a] (39).

Source or enzyme class	Enzyme	ROI	Localization	Cofactor
Mitochondrial electron transport proteins	NADH coenzyme Q reductase	$O_2^{-\cdot}$	Mitochondria	FMN, FeS
	Cytochrome b-c1 complex	$O_2^{-\cdot}$	Mitochondria	FeS, Fe-heme
	Cytochrome oxidase	$O_2^{-\cdot}$	Mitochondria	Fe/Cu, Fe-heme
Cytochrome P450 isoenzymes	Various isoenzymes and the associated NADPH cytochrome P450 reductase	$O_2^{-\cdot}$, H_2O_2	Endoplasmic reticulum, microsomes	Fe-heme, FAD, FMN
Oxidases	NAD(P)H oxidase (variable isoenzyne compositions in different cell types)	$O_2^{-\cdot}$	Plasma membrane	FAD, cytochrome b
	Xanthin oxidase	$O_2^{-\cdot}$	Cyoplasm	Fe
	Galactose oxidase	$O_2^{-\cdot}$	Cyoplasm	Cu
	Aldehyde oxidase	$O_2^{-\cdot}$	Cyoplasm	Mo
	Glycolate oxidase	H_2O_2	Peroxisomes	FMN
Dioxygenases	Indoleamine-2,3-dioxygenase	$O_2^{-\cdot}$	Cyoplasm	Fe-heme
	Tryptophane-2,3-dioxygenase	$O_2^{-\cdot}$	Cyoplasm	Fe-heme

Table 1 Cellular ROS-forming system[a] (continued)

Source or enzyme class	Enzyme	ROI	Localization	Cofactor
Cyclooxygenaes (prostagrandin syntheses)	COX-1	$O_2^{-\cdot}$	Cyoplasm	Fe-heme
	COX-2	$O_2^{-\cdot}$	Cyoplasm	Fe-heme
Lipoxygenase	5-LOX	$LOO^{\cdot}$, $ROO^{\cdot}$	Cyoplasm	Fe
	12-LOX	$LOO^{\cdot}$, $ROO^{\cdot}$	Cyoplasm	Fe
	15-LOX	$LOO^{\cdot}$, $ROO^{\cdot}$	Cyoplasm	Fe
Nitric oxide synthases	nNOS (NOS I)	$NO^{\cdot}$, $O_2^{-\cdot}$	Cyoplasm	FMN, FAD, Fe-heme, BH_4
	iNOS (NOS II)	$NO^{\cdot}$, $O_2^{-\cdot}$	Cyoplasm	FMN, FAD, Fe-heme, BH_4
	eNOS (NOS III)	$NO^{\cdot}$, $O_2^{-\cdot}$	Cyoplasm	FMN, FAD, Fe-heme, BH_4
Peroxidases	Myeloperoxidase	HOCl	Secreted from neutrophil	Fe-heme
	Lactoperoxidase	($OSCN^-$)	Milk, silva	Fe-heme

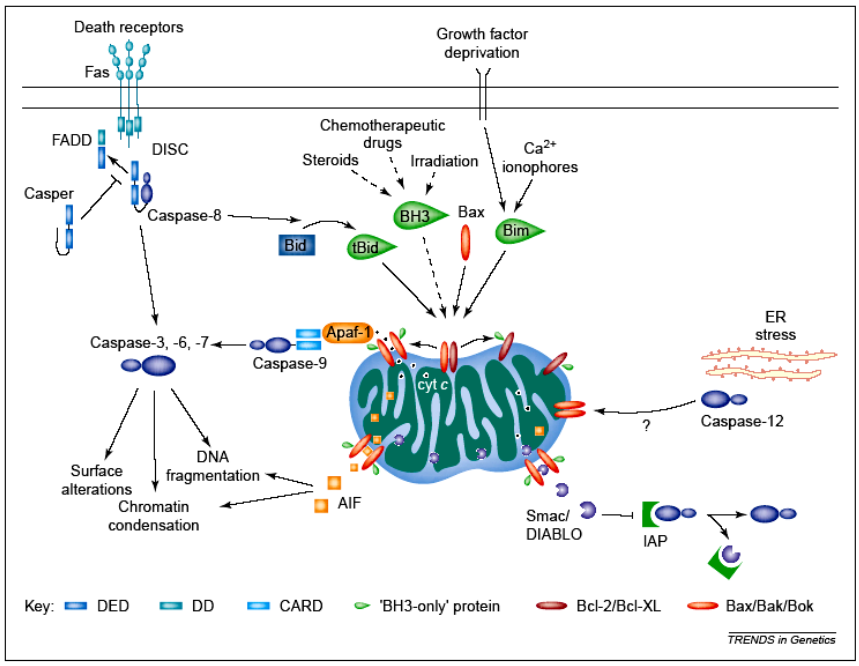


Figure 1 Overview of apoptotic signaling in mammalian cells.

Engagement of 'death receptors' (e.g. Fas) initiates recruitment of FADD and caspase-8 to the cytoplasmic regions of the receptor. Caspase-8 is autocatalytically activated and activates terminal caspases-3, -6 and -7. Once activated, these cleave select intracellular targets ultimately leading to the phenotypic manifestations of apoptosis. In response to specific death stimuli, distinct 'BH3-only' proteins, such as Bid and Bim, are activated. Bid involves proteolytic cleavage. Bim is disengagement from sequestering proteins. BH3-only proteins subsequently translocate to mitochondrial surfaces, where they associate with multidomain Bcl-2-related proteins. Anti-apoptotic Bcl-2 family members (e.g. Bcl-2, Bcl-x_L) are inactivated, and pro-apoptotic Bcl-2 members (e.g. Bax, Bak) are activated to potentially form mitochondrial membrane-spanning pores themselves or to interact with pre-existing membrane channels. Release of cytochrome c (cyt c) induces formation of the apoptosome in association with Apaf-1 and caspase-9. AIF induces hallmarks of apoptosis independent of caspases. Smac/DIABLO inactivates 'inhibitor of apoptosis proteins' (IAPs), thereby derepressing caspases. ER stress induces activation of caspase-12, perhaps cross-talking with the mitochondrial pathway. (Taken from Joza N, Kroemer G, Penninger JM. Genetic analysis of the mammalian cell death machinery. Trends Gen 2002;18:142-9.)

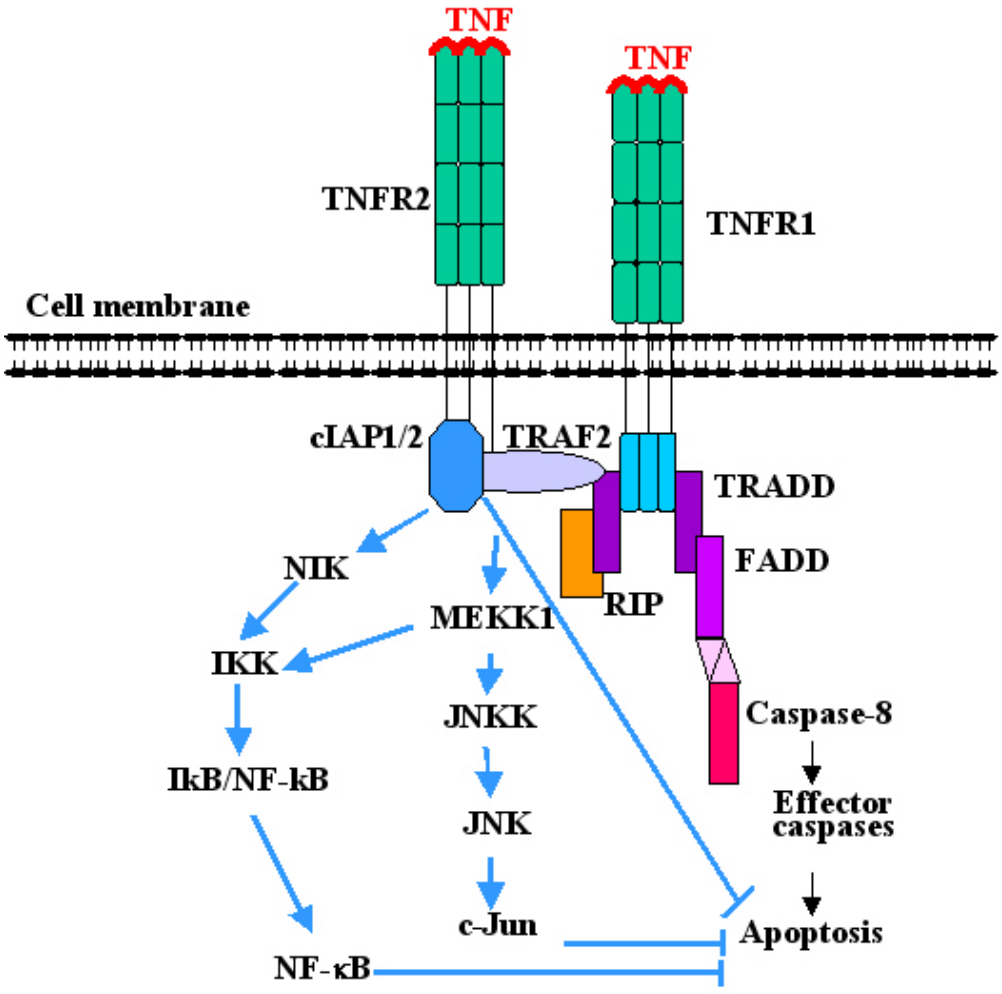


Figure 2 Signaling mechanism of TNF receptor.

Engagement of TNFR activates competing pathways: a proapoptotic pathway and an antiapoptotic pathway. Binding of TNF to TNFR1 induces trimerization of the receptor. The recruitment of FADD and caspase 8 to the clustered receptor complex leads to apoptosis. On the other hand, recruitment of TRADD, RIP and TRAF2 leads to activation of NF-κB, which initiates an anti-apoptotic pathway. (Modified from Ashkennazi A, Dixit VM. Death receptors: Signaling and modulation. Science 1998;281:1305-8)

1.5 Analysis of Apoptosis

1.5.1 Detection of changes in cell morphology

Dehydration that leads to cell shrinkage is characteristic and early feature of apoptosis. Condensation of chromatin and cytoplasm and nuclear fragmentation mediated by activation of caspases are other gross changes by initial swelling of the cell followed by rupture of its plasma membrane and lysis. All of these changes can be detected and measured by standard and scanning flow cytometries. Analysis of light scattered in the cell intersects the laser light beam reveals changes in cell structure: intensity of light scattered in the forward direction correlates with cell size, whereas intensity of light scattered at a right angle ("side" or 90^0 angle scatter) is affected by the cell's refractive and reflective properties and reveals optical in homogeneity, such as resulting from condensation of cytoplasm or nucleus and granularity. A decrease in forward light scatter is observed early during apoptosis, due to cell shrinkage (43); initially, there is little change in side scatter. When apoptosis is more advanced, cells become small and the intensity of forward scatter is decrease. Late apoptotic cells show markedly diminished intensity of both forward and side scatter signals. In contrast to apoptosis, cell swelling, which occurs early during necrosis, is detected by a transient increase in forward light scatter. Rupture of the plasma membrane and leakage of the cytosol during subsequent steps of necrosis correlate with a marked decrease in intensity of both forward and side scatter signals.

1.5.2 Analysis of mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta\Psi m$) results from the asymmetric distribution of protons and other ion on both sides of the inner mitochondrial membrane, giving rise to a chemical (pH) and electric gradient which is essential for mitochondrial function. The inner side of the inner mitochondrial membrane is negatively charged. When cells are induced to undergo apoptosis $\Delta\Psi m$ disruption occurs (44). Dissipation and collapse of $\Delta\Psi m$ has been observed to occur early during apoptosis (45). $\Delta\Psi m$ collapse results in releasing proteins: cytochrome c and AIF. Cytochrome c is essential for activation of pro-caspase-9 (46), and AIF is involves in proteolytic activation of apoptosis-associated endonuclease.

Flow cytometry is the instrumentation of choice for ΔΨm analysis in both whole cells and isolated mitochondria. Membrane-permeable lipophilic cationic fluorochromes such as rhodamine 123 (Rh 123) or 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$) can served as probe for ΔΨm (47-49), as they crosses plasma membrane and distributes itself inside cell. Its distribution reflects the electrical potential difference across the relevant membrane of mitochondria, which has a relatively high negative membrane potential. A decrease of $\Delta\Psi_m$ will affect accumulation of the probe and lead to detect low fluorescence by flow cytometry. Another probe of ΔΨm is the J-aggregate-forming lipophilic cationic fluorochrome 5,5',6,6' tetrechloro-1,1',3,3'-tetrarthylbenzimidzzolcabocyanine iodide (JC-1) (48); its uptake by charged mitochondria driven by the transmembrane potential is detected by the shift in color of fluorescence from green, characteristic of its monomeric form, to orange, which reflects its aggregation in mitochondria (50), collapse of ΔΨm is reflected by a reversed change of fluorescence from orange to green.

1.5.3 Detection of phosphatidylserine on the outer leaflet of the plasma membrane

In live cells, the phospholipids in the plasma membrane are asymmetrically distributed; phosphatidychlorine and sphingomyelin are exposed on external leaflet of the lipid bilayer while phosphatidylserine is on the inner surface. Early during apoptosis this asymmetry is disrupted, and phosphatidylserine (PS) is exposed on the outside surface. Binding of fluorochrome-conjugated annexin-V is widely used in flow cytometry studies of apoptosis. Annexin V can be conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Annexin V conjugated to FITC has been demonstrated to preferentially bind to PS in a Ca^{2+} dependent manner. Apoptotic cells will react with annexin V before the plasma membrane becomes permeable to cationic dyes such as propriodium iodide (PI). By staining cells with the combination of annexin V-FITC and PI, it is possible to identify unaffected, nonapoptotic cells (annexin V-negative/PI-negative), early apoptotic cells (annexin V-positive/PI-negative), and late apoptotic as well as necrotic cells (annexin V-positive/PI-positive) as shown in Figure 3.

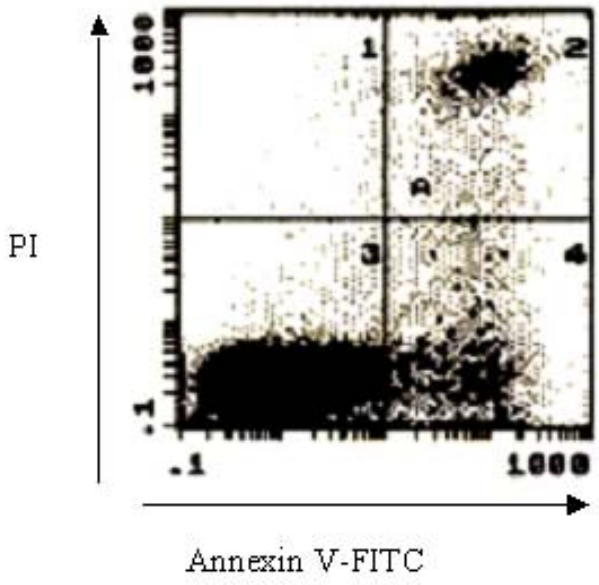


Figure 3 Quantitation of annexin V binding to apoptotic cells.

Annexin V-FITC labels PS, which flip to the outside of the plasma membrane during apoptosis. By staining cells with the combination of annexin V-FITC and PI, the stained cells in quadrant 3, 4 and 2 are nonapoptotic cells (annexin V-negative/PI-negative), early apoptotic cells (annexin V-positive/PI-negative), and late apoptotic as well as necrotic cells (annexin V-positive/PI-positive), respectively.

1.5.4 Detection of DNA fragmentation

The process of DNA fragmentation in apoptosis is mediated partly through endonuclease activity, which results in DNA cleavage and increase in number of 3' hydroxyl (3'OH) ends (51). Apoptotic cells can be identified by labeling the DNA strand breaks with fluorescently tagged deoxyuridine triphosphate nucleotides (F-dUTP) or with bromodeoxyuridine triphosphate (Br-UTP) and FITC labeled anti-BrdU antibody. Fluorescence intensity measured by flow cytometry will be proportional to the number of strand breaks. This technique is refered to TdT-mediated deoxyUridine triphosphate-biotin Nick-End Labeling (TUNEL) assay (52).

1.5.5 Analysis of caspase activation

Activation of caspases is the critical event of apoptosis, initiating the irreversible "execution" steps of cell death. Immunoprecipitation and immunoblotting techniques using caspase specific antibodies are the most common methods to measure caspase activation. One potential approach for cytometry employs fluorogenic or chromogenic substrates of caspases since the peptide substrates, on caspase-induced cleavage, can generate colored or fluorescing products.

The fluorochrome-labeled inhibitors of caspase assay (FLICA) measure activation of caspases in living cells (53). This technique involves binding of the specific inhibitor to active center of the enzyme. The ligands that specifically and covalently bind to caspase-active centers are fluorescein (*fam*)-labeled peptide-fluoromethyl ketone (*fmk*) inhibitors. These reagents penetrate into the plasma membrane and are relatively nontoxic to the cells for short-term incubation. As amino acid sequence of the peptide moiety provides specificity for the respective caspase, these reagents should be able to quantitate activation of each individual caspase. For example, *fam*-LEHD-*fmk* is expected to be a specific probe for the activation of caspase-9, while *fam*-DEVD-*fmk* is caspase-3-specific. Practically, some degrees of cross-reactivity does occur. The inhibitor that has only three-amino acid recognition peptide, *fam*-VAD-*fmk*, is generic to all caspases and can be used to detect activation of any of these enzymes.

1.5.6 Analysis of cleavage of poly(adenosine diphosphate-ribose) polymerase

In DNA repair poly(adenosine diphosphate-ribose) polymerase (PARP) is a nuclear enzyme, which is activated in response to DNA (54, 55). In many cells, early biochemical event in apoptosis is the proteolytic cleavage of PARP. During apoptosis PARP is cleaved by casepase-3 into distinct 89-kDa and 24-kDa fragments, which is considered to be another hallmark of programmed cell death. The development of antibodies that recognize the cleaved PARP products led to their use as immunocytochemical markers of apoptosis. The proteolytic PARP fragments can be detected by Western blotting or by cytometry (56).

1.5.7 Analysis of reactive oxygen species production

Reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) and superoxide anion, also involve in apoptotic mechanism. Peroxides and superoxide anion production are monitored by flow cytometry using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and dihydroethidium (HE), respectively (57-59). These dyes are stable non-polar compounds that readily diffuse into cells. Once inside the cells, the acetate groups of DCFH-DA are cleaved from the molecule by intracellular esterases to yield DCFH, which is trapped within the cells. Intracellular H_2O_2 or low-molecular weight peroxides, in the presence of peroxidases, oxidize DCFH to the highly fluorescent compound DCF. Thus, fluorescence intensity is proportional to the amount of peroxide produced by the cells.

Cytosolic HE exhibits blue fluorescence when excited by UV light. However, once this probe is oxidized by superoxide anion to ethidium, it intercalates in the cell's double-strand nucleic acid, staining its nucleus and cytoplasm with a bright red fluorescence, which is proportional to the intracellular superoxide anion level. The excitation and emission of DCFH is 488 nm and 525 nm, respectively. The excitation and emission of HE is 488 nm and 605 nm, respectively.

2. Erythropoiesis

2.1 Ontogeny of erythropoiesis

Ontogeny of erythropoiesis is shown in Figure 4. Erythropoiesis begins in the yolk sac, but site of erythropoiesis are shift during development (60). At about 5 weeks of gestation, the site of hematopoiesis changes from the yolk sac island to the liver. The liver becomes the prominent site of erythropoiesis in the fetus until about the twentieth week of gestation. Hematopoiesis subsequently occurs in the spleen and the bone marrow, which becomes the predominant and lifelong site of erythropoiesis.

The shifting sites of erythropoiesis coincide with changes in the hemoglobin composition of red blood cells and also with changes in other morphological and biochemical characteristics. The hemoglobin composition of the erythrocytes varies, depending on the gestation or postnatal development. These changes, known as hemoglobin switching, result from sequential activation and inactivation of gene within the α- and non α-globin gene cluster (61). Yolk sac erythroblasts produce three early embryonic hemoglobins, consisting of Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Gower 2 ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$). They are detectable only during the very earliest stages of embryogenesis. Erythrocytes produced in yolk sac are called primitive erythrocyte and are very large nucleated cells (average volume about 200 μL).

The major hemoglobin of intrauterine life is fetal hemoglobin (Hb F), which consists of α-chain and γ-chain ($\alpha_2\gamma_2$). γ-Globin gene expression begins early in embryogenesis, peaks during mid-gestation, and declines rapidly just prior to birth. Erythrocytes produced in fetal life cells are anucleated definitive lineages of erythrocytes. Fetal red cells tend to be larger and shorter lived than adult red cells. The volume of fetal red cells is approximately 125 μL, whereas an average volume of adult red cells is 80 μL. By 6 months of age in normal infant, the level of Hb F in blood is less than 1% of the total.

α-Globin gene expression starts early in the first trimester, peaks by about 10-12 weeks' gestation, and is sustained for life. β-Globin gene expression starts in early gestation, rises slowly, and reaches its peak within a few weeks of birth. The combination of α- with β-globin chains forms hemoglobin A (Hb A; $\alpha_2\beta_2$). Hb A level

is predominant (95-97%) after birth. The combination of α- with δ-globin chains forms hemoglobin A2 (Hb A_2, $\alpha_2\gamma_2$), which is also present in adult cells. Since δ-globin gene is very inefficiently expressed, only low level of Hb A_2 is present (1.5-3.5%).

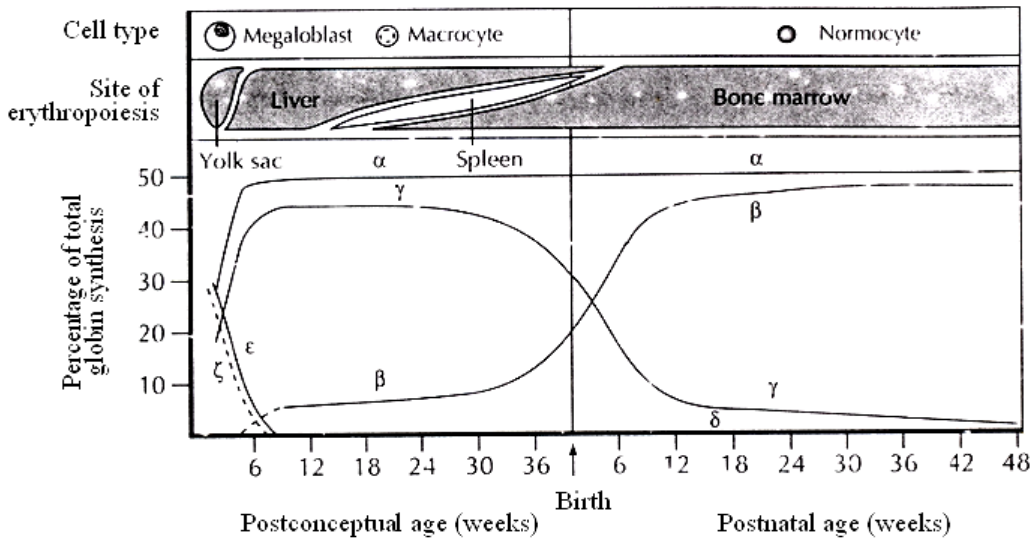


Figure 4 Ontogeny of erythropoiesis.

Erythriod cell types, their sites of production, and the different globins produced at each stage of development are shown. (Taken from Stamatoyannopoulos G, Grosveld F. Hemoglobin switching. In: The molecular basis of blood diseases. Stamatoyannopoulos G, Majerus PW, Perlmutter RM, Varmus H, editors. 3rd edition. Philadelphia: W.B. Sauders, 2001, p 135-82.)

2.2 Cellular compartment of erythropoiesis

Erythropoiesis is the formation of the erythrocyte or red blood cells, which is a highly regulated, multistep process. Erythropoiesis begins when a multipotent hematopoietic stem cell undergoes erythroid unilineage commitment and continues throughout the proliferation and terminal differentiation of erythroid progenitor cells (62). During this process, distinct cellular compartments are formed characterized by a progressive decline in proliferation and a parallel increase in differentiation potential. The erythroid committed progenitor compartment is composed of early erythroid progenitors (BFU-E) and late erythroid progenitors (CFU-E) that are not morphologically recognizable but can be defined functionally *in vitro* through their progeny (62). By contrast, the erythroid precursor cell compartment is composed of morphologically recognizable, maturing erythroid cells at different stages of maturation. The general properties and surface receptor of erythroid progenitor cells are summarized in Tables 3 and 4, respectively.

2.3 Characterization of erythroid lineage

The identification of erythroid lineage is facilitated by the use of lineage-restricted antigen, glycophorin A (63). This antigen appears only on the erythroid lineage and can be paired with other markers, which may have a wider distribution on other lineages (lineage associated antigens). By combination of cell morphology and cell surface antigen expression, it is possible to demonstrate the progressive changes of cell surface antigen expression during cell maturation. Figure 5 shows relative quantities of the particular antigens correlated with other changes of erythroid maturation. The earliest erythroblasts do not express glycophorin A but the cells are defined by cell morphology and expression of a large amount of transferrin receptor (or CD 71) on cell surface. Expressions of specific lymphoid and myeloid markers are also absence in these cells. Early erythroblasts express intermediate to high levels of glycophorin A and a high amount of transferrin receptor. Late erythroblasts express high levels of glycophorin A and decreasing level of transferrin receptor. The mature erythrocytes express high levels of glycophorin A and do not express transferrin receptor.

The expression of transferrin receptors on erythroid cells precedes the expression of glycophorin (Figure 5). Most erythroid colony-forming cells express low to intermediate amount of transferrin receptor. Expression of transferrin receptor is nearly maximal before glycophorin is expressed. Once erythroid cells express cell surface glycophorin, they are no longer able to form colonies (64). Erythroid lineage cells express much higher maximal level of cell surface transferrin receptor than do cells of other lineages (63). In addition, transferrin receptor persists to a very late stage of erythroid development (reticulocyte). These may be related to the unique requirement of erythroid cells for large quantities of iron needed for hemoglobin synthesis (63).

There is a parallel decrease in Hle-1 and transferrin receptor levels during erythroid maturation (Figure 5). The decreased expression of Hle-1, a common leukocyte antigen, and the increased expression of transferrin receptor are very early events following the commitment of cells to the erythroid lineage (63).

2.4 Steps in erythroid cell development

The conventional view of hematopoiesis invokes a hierarchy in which hematopoietic stem cells (HSCs) give rise to multipotential progenitors, which generate precursors committed to differentiation along selected lineages. HSCs are ultimately responsible for the production of all mature blood cells, including red cells, myeloid cells (neutrophils and macrophages), mast cells, megakaryocytes, and B- and T-lymphocytes. The survival and proliferation of cells at all the various stages require growth factors or cytokines that signal through cognate receptors. Progenitors first committed to erythroid development are not recognizable morphologically, but can be detected by *in vitro* colony assays. Such assays suggest that erythroid precursors often exhibit bipotential properties, and give rise to erythroid/mast and erythroid/megakaryocytic mixed colonies. Of interest, several cell-restricted transcription factors initially identified in erythroid cells are shared by the megakaryocytic and mast lineages. The first morphologically identifiable erythroid precursor cell is the proerythroblast. The proerythroblast requires the growth factor erythropoietin for its survival and proliferation, and expresses small quantities of globin mRNAs. Upon its differentiation, the proerythroblast matures progressively to

the late erythroblast stage. Staining a bone marrow sample with Wright's stain and observing by light microscopy allow the identification of six morphologic stages of erythrocyte as follow:

1. Proerythroblast

Cell size is variable, ranging from 12-25 μm. The nuclear:cytoplasmic ratio is high, with the nucleus occupying more than 80% of the cell. The cytoplasm is basophilic (intense dark blue) because of the high RNA content. The golgi apparatus may be visible as pale area next to the nucleus. The nucleus which is usually round to slightly oval, has dispersed fine clumps of chromatin and contained nucleoli.

2. Basophilic erythroblast

The cell is slightly smaller (12-17 μm) than the proerythroblast. The cytoplasm is basophilic, with the visible light area of golgi apparatus next to the nucleus. The nucleus is round and usually occupies about 75% of the cell. Its chromatin is dark violet and definitely coarser and more clumped than that of the pronormoblast. Parachromatin is slightly visible between the clumps of chromatin and nucleoli are no longer visible.

3. Polychromatic erythroblast

The cell is smaller (8-12 μm) than the proeythroblast. Throughout this stage of maturation, more cytoplasm becomes apparent as the nucleus becomes smaller and may be eccentric. The cytoplasm shows varies spectrum of blue color as hemoglobin synthesized. Blue RNA mixed with red hemoglobin gives the cytoplasm an opaque, blue-gray-violet color called polychromatophilia. Early polychromatophilic erythroblast is moderately polychromatophilic, differing from the intensely dark blue cytoplasm of the basophilic normoblast. Late polychromatophilic erythroblasts have a paler violet-blue-gray to slightly pinkish color. The nuclear chromatin is coarse and condensed. Distinct area of parachromatin is visible amid clumps of chromatin. Each polychromatophilic erythroblast usually gives rise to two orthochromatic erythroblasts. This is the last cell division during maturation.

4. Orthochromatic erythroblast

This cell is the last nucleated erythrocyte stage. It is the same size as or smaller than the polychromatophilic erythroblast. The cell is paler, violet-blue-gray cytoplasm, which is more pinkish than that of the polychromatophilic erythroblast

since hemoglobin is the main cytoplasmic constitutent. The nuclear chromatin is very dense, coarse and clumped. The nucleus is pyknotic. The nucleus is extruded at this stage and the cell becomes a reticulocyte.

5. Reticuclocyte

This cell is larger than the mature erythrocyte. It has irregular cytoplasm borders. The cytoplasm still contains small amount of RNA, producing varying amounts of polychromasia. After nuclear extrusion, reticulocytes are retained in the marrow for 2 to 3 days before releasing into the peripheral blood. The reticulocyte contains golgi apparatus remnant and residual mitochondria that permit continued aerobic metabolism and hemoglobin production. Reticulocyte also contains residual RNA, which may be stained supravitally. The staining causes the RNA to precipitate and aggregate into network of strand or clumps visible by light microscope.

6. Mature erythrocyte

This cell is approximately 7.2 μm in diameter. In the resting state it is biconcave disc. A Wright stain reveals a central paler area that fades gradually into reddish-pink cytoplasm. The central pallor corresponds to the indentation in the erythrocyte disc. The mature erythrocyte contains no mitochondria; therefore, neither protein nor hemoglobin is synthesized.

In vitro erythroid differentiation is also demonstrated by culturing of isolated stem cells with the appropriated cytokines such as IL-3, SCF, EPO, GM-CSF, depending on the modified system. Normal erythroid differentiation is quite the same as found in bone marrow. The stage of 14 days erythroid culture is shown in Figure 6.

2.5 Regulation of cell cycle during erythropoiesis

The basic cell cycle is divided into four phases: G_1, S, G_2 and M phases. Cell generates a copy of its genetic material during the synthetic or S phase and separates all the cellular components between two identical daughter cells during mitosis or M phase. G_1 and G_2 phases represent ‘gap’ periods, which cells prepare themselves for the successful completion of the S and M phases, respectively. G_0 is quiescent state, which cells cease proliferation, either due to specific anti-mitogenic signals or to the absence of proper mitogenic signaling. Cells in hematopoietic system has the capacity

to enter quiescence and all quiescent cells, except those that have reached a state of terminal differentiation, have the capacity to re-enter the cycle.

A family of cyclin dependent kinases (CDKs) and their regulatory subunits, the cyclins, governs progression around the cell cycle (Figure 7). As cell enter the G_0, cyclin D and E are synthesized sequentially and both are rate limiting for S phase entry. pRb is a negative regulator of progression that permit exit from an entry G_1 into S phase. Cyclin D binds to pRb directly. pRb is the critical substrate of CDK4 and CDK6. Cyclin E/CDK2 also phosphorylate pRb. Phosphorylation of pRb relieves its inhibitory effect on the transactivation function of E2F family transcription factors that are required for S phase. Contributing to the proliferative quiescence is a high level of p27, a cyclin-dependent kinase inhibitor (CIP) that regulates cell number and size by blocking initiation of G_1 buildup by binding G_1-specific CDKs. The disappearance of p27 clears the way for cyclins and the assembly of the stage-specific CDK2/4-cyclins that come and go to drive the cell through G_1 buildup into the S phase.

The cell cycle regulators of normal erythroid progenitor have been identified (65). BFU-Es are not actively proliferating (most are in the G_0/G_1 phase of cell cycle), whereas CFU-Es are actively proliferating (most are in the S phase of cell cycle). As CFU-Es differentiate to the late-stage erythroblast, they cease dividing and accumulate in G_0 phase before enucleation. In cord blood stem cells induced to differentiate along the erythroid lineage, the increased proliferation at CFU-E stage is accompanied by up-regulation of cyclin D3 and E and CDK2, whereas late stage erythroblasts down-regulate cyclin D3 and E (66). Murine erythroleukemia cells showed a sequential decline in CDK2 and CDK6 induced differentiation (67, 68). In murine erythroid cells in the erythropoietin-dependent stages of differentiation, $p21^{CIP}$ and $p27^{Kip1}$, two inhibitors of G_1-phase CDKs, were examined for their ability to bind CDK2, CDK4, and CDK6 (69). $p27^{Kip1}$ binds to CDK2 and CDK4 but mainly inhibits CDK2 activity. $p21^{CIP}$ does not bind these CDKs. These results suggest that inhibition of G_2-phase cyclins through their associated CDKs, specifically CDK2, plays a role in the cessation of cell division as CFU-Es mature to late erythroblasts.

Table 2 General properties of erythroid progenitor cells (70).

	CFU-GEMM	BFU-E	CFU-E
Self-renewal	+ +	+	0
Differential potential	Multipotent	Committed to erythropoiesis	Committed to erythropoiesis
Cycling status (% suicide with ^{3}Thymidine)	15-20	30-40	60-80
Cell density (g/dL)	<1.077	<1.077	<1.077
Incidence/10^{5} cells	2-5	40-120	200-600
Circulate in blood	+	+	+
Growth factor response			
EPO	+	+	+ +
TPO	+	+	+
Kit ligand	+	+	+
GM-CSF, IL-3*	+	+	+
FL	+	0	0
G-CSF, IL-6, IL-1	+	0	0
IGF, activin	0	0	+
TGF-β1	-	-	+ +

* Most of the cytokines listed exert synergistic effects.

Table 3 Surface antigens and receptors of erythroid progenitor cells (70).

Antigens/ Receptor	CFU-GEMM	BFU-E	CFU-E
CD34	++	++	-
CD33	+	+	0
c-kit	++	++	+
HLA-DR (-DP, -DQ)	++	++	+
EPOR	+	+	++
TNFR	+	+	++
EP-1	+	+	++
23.6	0	0	+
CD36	0	±	+
Glycophorin A	0	0	+
ABH, il	0	+	+
Adhesion molecule			
VLA-4 (CD49d/CD29)	++	++	++
VLA-5 (CD49e/CD29)	+	+	++
CD41	+	+	
CD11a/CD18	+	+	
CD44	+	+	
HCAM	+	+	

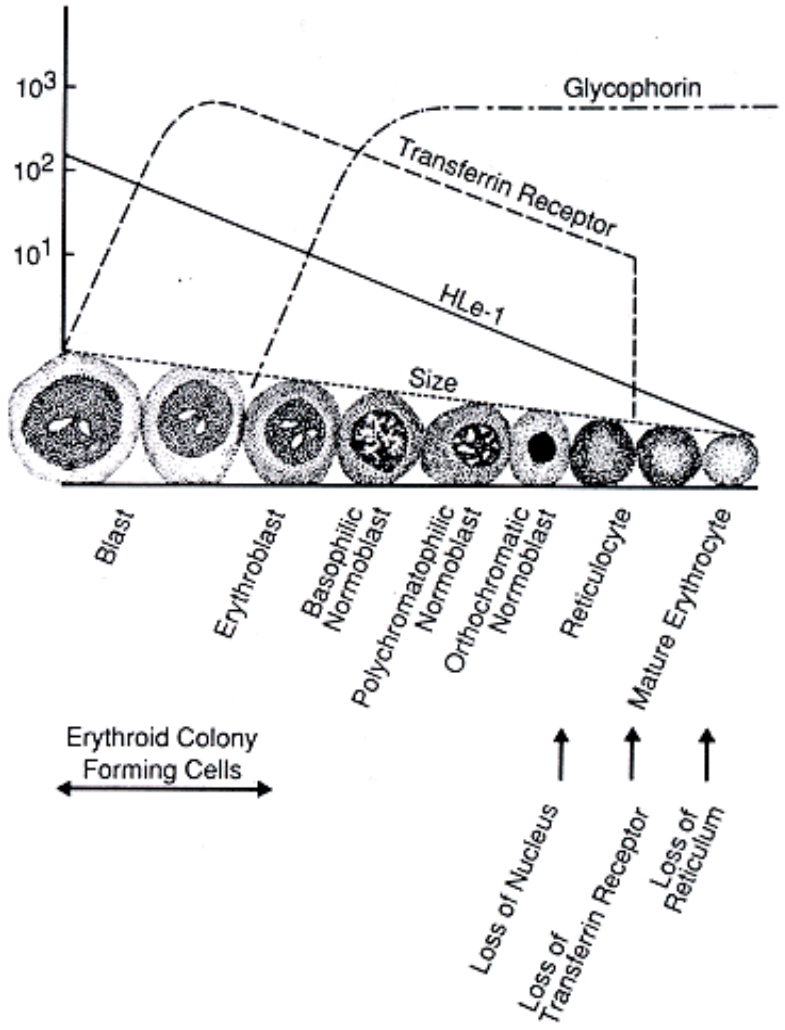


Figure 5 Characteristic of erythroid maturation.

The relative quantities of the cell surface antigens are depicted in the upper portion of the diagram. These antigenic characteristics can be correlated with cell size, morphology, nucleic acid content, and erythroid colony-forming cells. (Taken from Loken M.R, Shah VO, Dattilio KL, Civin CI. Flow cytometry analysis of human bone marrow: I. Normal erythroid development. Blood 1987;69:255-63.)

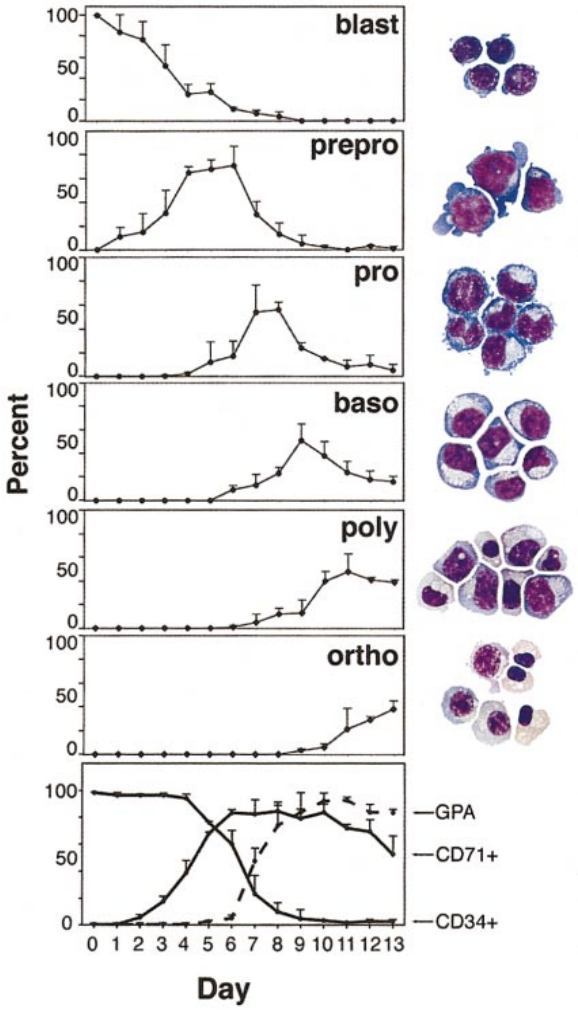


Figure 6 Erythroid maturation in vitro.

Cell types and expression of surface proteins are daily determination and quantitation . The 6 upper panels show daily percentage of a given cell type among all cells in culture calculated in a blinded fashion from cytospin preparations; blast indicates undifferentiated blast; prepro, preproerythroblasts; pro, proerythroblasts; baso, basophilic erythroblasts; poly, polychromatic erythrooblasts; ortho, orthochromatic erythroblasts. Stained cytospins performed on days 1, 5, 7, 9, 11, and 13 are shown on the right. The bottom panel demonstrates surface phenotype of cells in culture stained for glycophorin A (GPA), transferrin receptor (CD71), and CD34 (Taken from Wojda U, Noel P, Miller JL. Fetal and adult hemoglobin production during adult erythropoiesis: coordinate expression correlates with cell proliferation. Blood 2003;99:3005-13.)

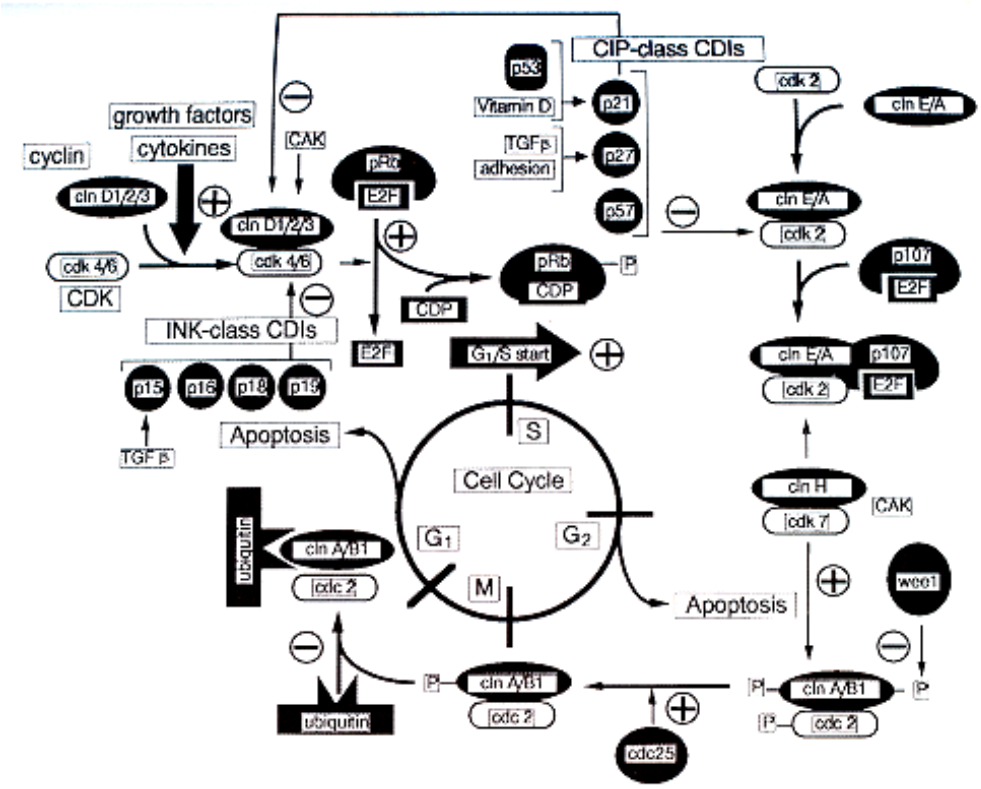


Figure 7 Regulation of cell cycle by cyclin-dependent kinase.

Competent for cell progression is determine by cyclin-dependent kinases (CDK) that monitor intracellular levels of cyclins and CDK inhibitory proteins (CDIs). CDKs mediate phosphorylation of pRb, which results in activation of E2F and CDP/cut-homeodomain transcription factors. These E2F-dependent and independent mechanism induce expression of gene required for the G_1/S phase transition. The activation of CDKs is also influenced by phosphorylation, e.g., weel or CDK-activating kinase (CAK), cdc25, ubiquitin dependent proteolysis, and induction of CDIs by p53. Growth factors and cytokines induce the activities of CDKs, which mediate the G_0/G_1 transition. Vitamin D- and TGF-β-dependent cell signaling pathway upregulate CDIs, e.g., p21 and p27, which block cell cycle progression and support s differentiation in the presence of tissue-specific regulatory factor. (Taken from Stein GS, Wijnen AJV, Frenkel B, Hushka D, Stein JL, Lian JB. Gene expression: the regulatory and regulated mechanisms. In: Stein GS, Baserga R, Giordano A, Denhardt DT, editors. The molecular basis of cell cycle and growth control. New York: Wiley-Liss, 1999, p 183-224.)

2.6 Growth factors and cytokines as regulators of erythropoiesis

Erythropoietin (EPO) and Stem cell factor (SCF) are essential cytokines for proliferation of erythroid progenitor cells (8). EPO is the pivotal factor that function to prevent apoptosis of most committed erythroid progenitor cells and their progeny (9). SCF including IFN-γ also have been reported to protect erythroid progenitor cells from apoptosis (8, 71, 72). These three factors will be reviewed as follows.

2.6.1 Erythropoietin

2.6.1.1 Erythropoietin

EPO is a 3.04 kDa glycoprotein, produced by peritubular cells in the kidneys of the adults and in the hepatocytes of the fetus. EPO is the lineage-specific regulator required for survival, proliferation and differentiation of committed erythroid progenitor cells. At low oxygen tension, the synthesis of EPO is stimulated, resulting in an increased erythroid cell production. Under normal steady-state condition, the concentration of circulating erythropoietin is equal to the amount necessary to maintain the red cell mass and to replace senescent and dying red cells. The normal level of EPO is in the range of 10-25 mU/mL (73). In anemia, a wide range of EPO level is found depending on the cause of anemia as shown in Table 4. With erythropoiesis the EPO level can be increased or paradoxically decreased such as in the idiopathic or primary cases. Some of the cases, in which EPO level is decreased, have been explained by disorder of erythropoietin receptor.

There are evidences that the physiologic relevant function of EPO is the regulation of apoptosis in late-stage progenitor cells, not regulation of cell cycling (8, 74, 75). EPO protects cells from apoptosis by upregulating the expression of anti-apoptotic protein Bcl-x_L (76, 77). Conversely, EPO deprivation results in caspase activation and apoptosis (78). The transcription factor GATA-1 is required for EPO-mediated upregulation of Bcl-x_L (34) and expression of erythroid gene involved in erythroid differentiation (79). In the absence of GATA-1, the differentiation process is arrested at the basophilic erythroblast stage, and cells die by apoptosis. A negative feedback loop was described in which the ligand of Fas expressed by mature erythroblasts activated Fas-mediated cell death of immature erythroblast (80). Fas ligation results in the activation of caspase-8, then caspase-3, and the cleavage of GATA-1, the inactivation of which leads to maturation arrest at

the basophilic erythroblast stage of differentiation (81). Normal erythroid differentiation requires transient activation of caspases (82). Effector caspases such as caspase-3 are transiently activated through the mitochondrial pathway during erythroblast differentiation and cleave proteins involved in nucleus integrity (lamin B) and chromatin condensation (acinus) without inducing cell death and cleavage of GATA-1. Cleavage of these proteins may account for the nuclear structural changes associated with the maturation of erythroblasts.

There is mixed evidence for a proliferative function of EPO in human BFU-E (83-86) and there is evidence that EPO is mitogenic for some erythroid cell lines (87, 88). *In vivo*, most CFU-E and proerythroblast are in DNA synthesis regardless of the EPO levels in the animals from which they are obtained. Several studies using CFU-E and proerythroblast of human show that they undergo apoptosis in the absence of EPO (8, 89). In a proerythroblast population, the fractions of the cells that undergo apoptosis depend on the concentration of EPO over the extensive range of concentrations (75). A model of erythropoiesis has been proposed based on EPO acting to prevent apoptosis in late-stage erythroid progenitor cells (74, 90). In the model (Figure 7), it is postulated that there is a period during the differentiation of erythroid progenitors during which EPO is required to prevent apoptosis. This period includes the CFU-E and subsequent proerythroblast stage and possibly the predecessors of the CFU-E, the mature BFU-E. The model also requires that individual progenitors within the EPO-dependent population exhibit a range of sensitivities to EPO such that there is an extended dose range of EPO over which individual progenitors may survive and continue proliferation and differentiation. Thus, the level of EPO ultimately controls erythrocyte production by regulating the number of dependent progenitors that survive or die.

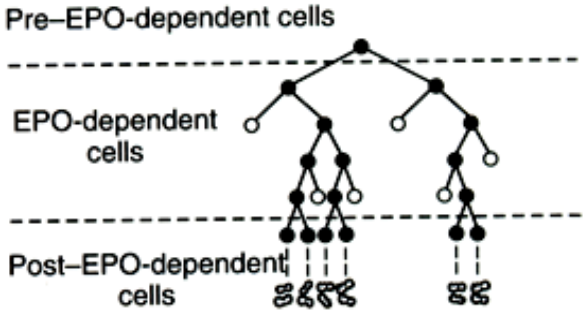
Table 4 Erythropoietin level in anemia (73).

Individual with normal renal function	Endogenous erythropoietin level (mU/mL)
Nonanemic individuals	10-25
Anemic individuals	
Mild anemia*	20-200
Severe anemia**	200-2,000
Transfusion-dependent anemia	2,000-20,000

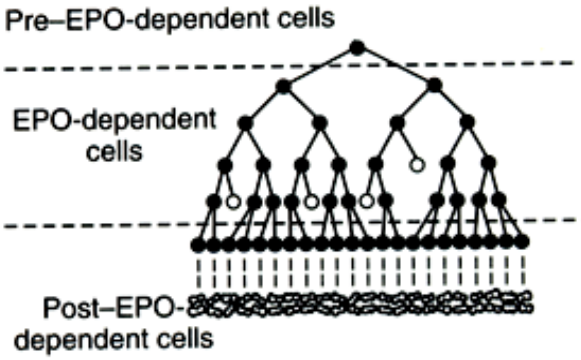
*Mild anemia caused by rheumatoid arthritis, chronic inflammatory and infectious diseases.

**Severe anemia caused by sickle cell anemia, acquired immunodeficiency syndrome (AIDS), refractory anemia.

A. Normal erythropoietin



B. Increased erythropoietin



C. Decreased erythropoietin

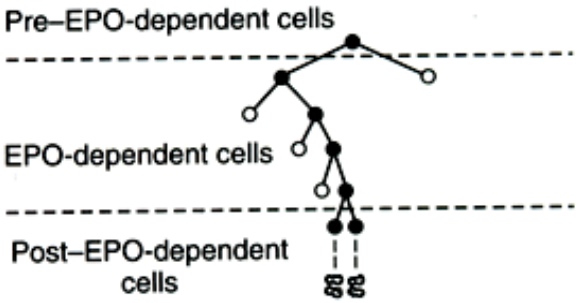


Figure 8 Model of erythropoiesis base on erythropoietin suppression of apoptosis.

Model proposed by Koury and Bondurant for expansion of erythropoiesis by prevention of apoptosis in erythroid progenitors. Surviving viable cells are indicated by solid dot; cells undergoing apoptosis caused by insufficient EPO are indicated by open circle. Before entering the EPO period, the progenitors can survive without EPO (pre-EPO-dependent cells). Cells surviving transit through the EPO-dependent period can complete maturation into reticulocytes without EPO and ultimately become red cells (post-EPO-dependent cells). Late erythroid progenitor cells, CFU-Es and proerythroblast are dependent on the continuous presence of EPO to suppress apoptosis and are heterogeneous with respect to their EPO sensitivity. Under normal condition (A), only a portion of late erythroid progenitor cells (those with lower EPO requirement) survive, generating erythroid precursors that produce normal amount of mature red cells. In conditions characterized by increased EPO production (B), nearly all erythroid progenitors, even those with high EPO requirements, survive, with maximum preamplification of erythropoiesis. In conditions characterized by decreased EPO production (C), the large majority of erythroid progenitor cells undergo apoptosis due to the low EPO level. Only a subpopulation of progenitors with very high EPO sensitivity and very low requirement survive; red cell production is inadequate and anemia develops. (Taken from Bondurant MC, Koury MJ. Origin and development of blood cells. In: Wintrobe's clinical hematology. Lee GR, et al. editors. 10th edition. Baltimore:Williams & Wilkins, 1999, p145-62.)

2.6.1.2 Erythropoietin receptor

Erythropoietin receptor (EPOR) is located on the surface of erythroid cells and undergoes phosphorylation in response to EPO. In human the receptor consists of 508 amino acids, including an extracellular domain, single hydrophobic transmembrane domain, and a cytoplasmic domain. EPOR belongs to the superfamily of cytokine receptor, which includes receptors for GM-CSF, IL-3 and IL-6. In common members, there are four conserved cystein residues and a WSXWS motif in the extracellular ligand-binding region. The single copy EPOR gene is located on chromosome 19 and contains eight exon, where exon 1-5 encode for extracellular region, exon 6 for the transmembrane, and exon 7-8 for the cytoplasmic domain. The latter two exons are thought to be the positive and negative regulatory regions of the receptor, respectively.

Within the erythroid lineage, the small numbers of EPOR are first expressed on BFU-E (about 300 receptors/cell). Their number increases and reaches about 1,000receptors/cell at CFU-E stage. Receptor expression then decreases with erythroid maturation. However, the highest number of EPOR is also found in the proerythroblast stage and is almost absent on reticulocytes (91).

The interaction between EPO and EPOR initiates the signal transduction pathways and ultimately EPO-induced cell proliferation leading to an increase in red cell mass. EPO induces homodimerization of the receptor molecule, which causes the associated protein kinase, Janus kinase 2 (JAK2), to autophosphorylate. Activated JAK2 phosphorylates EPOR and a number of other signal transduction proteins also become phosphorylated and signal transducer and activator of transcription (STAT) 5 are activated. When STAT5 is phosphorylated it can be translocated to the nucleus where it initiates gene transcription. In addition, other signaling pathway also activated such as Ras/MAPK cascade and PI3K (Figure 9). In the presence of EPO, the antiapoptotic effects largely mediated via activation of PI3K (92, 93). PI3K catalyses the formation of phosphatidylinositol–3,4-diphosphate, which acts in concert with phosphatidylinositol-dependent kinase to activate Akt/PKB. Akt phosphorylates many downstream kinases and transcription factors that mediate the antiapoptotic effect of PI3K-Akt pathway, such as NF-κB (94, 95). The

intracellular domain of the EPOR was also demonstrated to transmit signals leading to the activation of NF-κB and antiapoptotic signals were generated (22).

2.6.1.3 Erythropoietin and Bcl-x_L

The Bcl-2 protein family has been reported to participate in the regulation of apoptosis, cell growth and differentiation. It is divided into two main groups consisting of apoptotic repressors, such as Bcl-2, Bcl-x_L, and Mcl-1, and apoptotic promoters, such as Bax, Bcl-x_S, (the alternatively spliced *Bcl-x* gene product), Bad, Bak, and Bid (96). Among these Bcl-2 family members, Bcl-x_L, appears to be important for erythropoiesis. The expression of Bcl-x_L is dependent on EPO, which is essential for survival of late erythroid progenitor cells (33, 76, 97, 98). Bcl-x_L is greatly increased in erythroblasts during late stages of erythropoiesis (33). EPO-dependent activation of the factor STAT5 is probably involved in the induction of Bcl-x_L expression has been demonstrated (76, 98). EPO and GATA-1 signaling pathways also converge to promote erythroid cell survival through activation of Bcl-x_L expression (34). Survival activity of Bcl-x_L can be antagonized by heterodimerization with proteins containing a BH3 domain, which is the essential domain for the binding and proapoptotic activity of Bak, Bax, Bik, and Bid (99). Bcl-x_L is expressed in outer mitochondrial membranes where antagonization of their function is associated with rupture of the membrane, release of the proapoptotic factors cytochrome c, and activation of caspases (100).

2.6.1.4 Erythropoietin and anemia

Anemia defines commonly as a condition that the hemoglobin level less is than 12 g/dL. Anemia may occur from many causes related to the patient (hemoglobinopathies, thalassemia, gastrointestinal problems, etc.), to the disease (bone marrow infiltration, bowel resection, hypersplenism, diminished nutritional state, etc.) or to therapy (bone marrow and renal toxicity secondary to chemotherapy; drug-induced hemolysis, etc.). Several studies have shown that improving the hemoglobin level will increase quality of life. The most marked increment in the rate of improvement of quality of life takes place when hemoglobin levels are increased to between 11 and 12 g/dL. Anemia can be corrected by blood transfusion, which has the advantage of effecting a direct improvement if required, such as thalassemic diseases.

Bone marrow stem cells are self-renewing and able to support a normal hemoglobin level over a lifetime. Erythrocytes derive from committed stem cells that differentiate and multiply through the different erythroblastic stages. Erythropoietin is the most important in proliferation and differentiation of the erythroid pathway as previous mention. Hypoxia is sensed by the nephron, and the kidney responds with erythropoietin production. The erythropoietin binds to a specific receptor on the red blood cell progenitors, and its signaling induces proliferation and differentiation and has an anti-apoptotic effect.

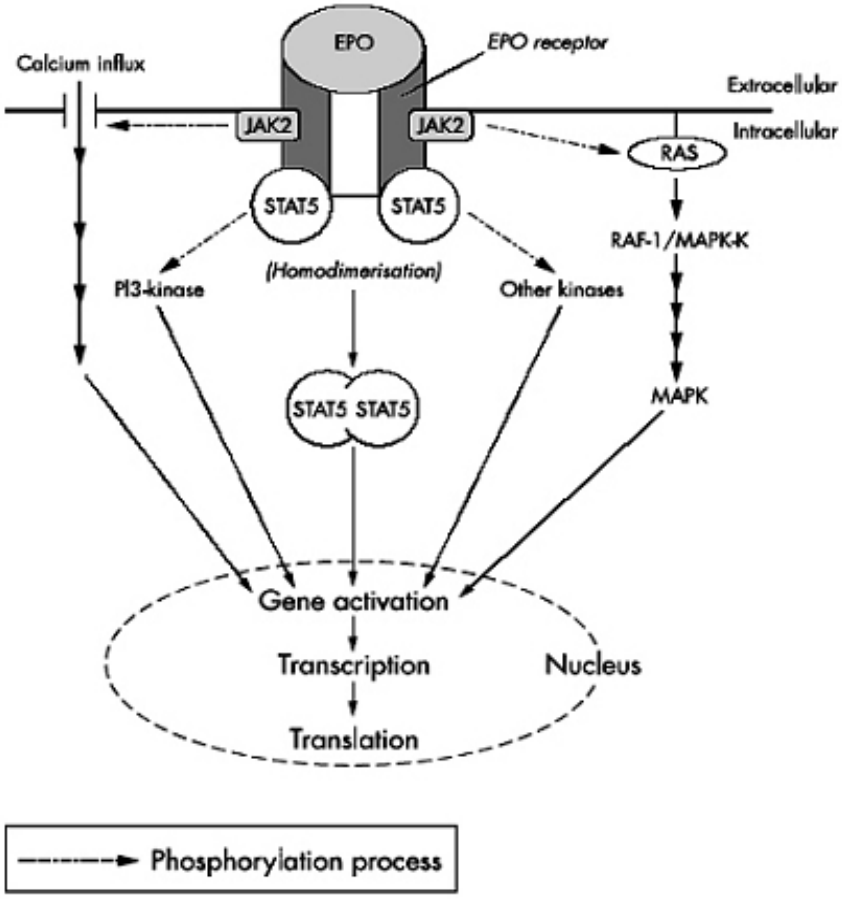


Figure 9 EPOR signaling pathway.

Simplistic view of the main signal transduction pathways activated by the EPOR. The binding of EPO to EPOR results in homodimerisation of EPOR followed by activation of several signal transduction pathways: JAK2/STAT5 system, G-protein (RAS), calcium channel, and kinases. (Taken from Ng T, Marx G, Littlewood T, Macdougall I. Recombinant erythropoietin in clinical practice. Postgrad Med J 2003;79:367-76.)

2.6.2 Stem cell factor

Stem cell factor (SCF) or c-kit ligand is a highly glycosylated 28-36 kDa protein encoded by a gene on the long arm of chromosome 12q22 (101). The widely expressed gene gives rise, via alternate splicing, to two mRNA species, one containing exon 6 (exon 6+) and the other is the splicing out exon (exon 6-) (102, 103). The transcript ratio of exon 6+ to exon 6- transcripts is approximately 3:1(104).

SCF promotes the proliferation and differentiation of the most primitive progenitor cells into committed progenitor cells. This factor is categorized as an early-acting hematopoietic growth factor. SCF has an essential role in the development of erythroid cells and affects intracellular signaling associated with proliferation, differentiation, and survival of erythroid progenitor cells (9, 105-107). It has been reported that SCF inhibits the activation of caspase-8 and caspase-3 without down-regulation of the surface expression of Fas and prevents Fas-mediated apoptosis of human ECFCs with Src-family kinase dependency (108). It is demonstrated that SCF prevented IFN-γ-induced apoptosis in human erythroid progenitor cells via increases the expression of FLIP (109).

The expression of SCF receptor, also known as c-kit or CD117, is studied among *in vitro* maturation of erythroblast (110). CD117 expression increased during the first week of erythroid culture and peaked on culture days 7 to 9. After culture day 9, the level of CD117 declined to lower levels. The rise in CD117 expression to high levels mirrored that of the transferrin receptor (CD71), and the subsequent reduction in CD117 was inversely related to increases in expression of glycophorin A. SCF has also regulated erythroid growth and Hb F production *in vivo* and primary cells (110-112). Base upon increase erythropoiesis and Hb F, SCF is thought to be involved in the stress response. SCF-related increases in Hb F are almost certainly related to signal transduction through its cellular receptor, CD 117 (113). After CD 117 dimerization, several signal transduction pathways are activated leading to gene transcription and cell cycle regulation (114). SCF- and EPO-mediated signals interact at various points along the Ras/Raf1/MAPK and PI3K pathways to affect specific biologic responses (115, 116). A role of EPO-activated STAT5 and PI3K in preventing apoptosis also has evidence SCF promotes the proliferation of erythroid

cells but does not completely protect them from apoptosis. SCF does not activate JAK2/STAT5, but it activates MAPK, JNK, and other pathways. STAT5 and PI3K contribute to erythroid cell survival, whereas MAPK activation drives proliferation (117). Erythropoietin-activated PI3K/Akt may also phosphorylate the Forkhead transcription factor in erythroid cells to promote survival (92, 118).

2.6.3 Interferon-γ

Interferons (IFNs) are a family of cytokines that elicit pleiotropic biological effects. IFNs consist of the multiple type I species (IFN-α, IFN-β, IFN-ω, and IFN-κ) and the one type II species (IFN-γ). Type I IFNs are clustered on chromosome 9 and consist of several α genes and pseudogenes and one β gene. These cytokines are induced in most cell types by viruses and dsRNA. In contrast IFN-γ, coded by a single gene on chromosome 12, is mainly secreted by activated T cells and natural killer cells. Following secretion from cells, IFNs mediate their effects by binding to cell surface receptors and thus activating members of the JAK kinase family. Activated JAK kinases phosphorylate STAT. STAT proteins homo-or heterodimerize and form complexes with other transcription factors to activate transcription of IFN-stimulated genes (ISGs). Gene products regulated by IFNs are the primary effectors of the IFN mediated biological responses (Figure 9). Both direct and indirect effects of IFNs result from regulation of gene expression. Alterations in gene expression have resulted in modulation of receptors for other cytokines, concentration of regulatory proteins on the surface of immune effector cells and activation of enzymes that modulate cellular growth and function.

The inhibitory effects of IFN-γ on human hematopoiesis *in vitro* have been demonstrated (119, 120). IFN-γ reduced erythroid colony formation, cell proliferation, and differentiation of highly purified human day-3 to day-6 BFU-Es in a dose-dependent manner and produced profound erythroblast apoptosis (120). This effect may be a result of enhanced specific binding, which is highest with the earliest BFU-E and declines progressively as maturation of erythroid progenitor cells (121). Exposure to high levels of EPO *in vitro* can overcome the inhibitory effect of IFN-γ on CFU-E colony formation by either marrow cells or cell enriched for CFU-E (122). Since EPO cannot overcome the inhibitory effect of IFN-α and IFN-β (122) and the

receptors of both cytokines act through the tyrosine kinase JAK1 and JAK2 kinase (123, 124), while IFN-γ receptor acts through the Jak1 and Jak2 kinase (123, 124). Jak2 also associated with EPO receptor and is tyrosine phosphorylated and activated following stimulation of responsive cells by EPO (125, 126). It is conceivable that, by its interaction with Jak2, IFN-γ created an environment to rescue of CFU-E by EPO.

It has been shown that IFN-γ markedly increased the percentage of cells expressing Fas on the surface of human erythroid colony-forming cells (ECFCs) as well as the intensity of Fas expression on these cells and induced the up-regulation and activation of caspase-8 and caspase-3 to produce apoptosis in human erythroid colony forming cells (ECFCs) (127, 128). Although SCF could prevent IFN-γ apoptotic induction in ECFCs, SCF did not decrease the surface expression of Fas on ECFCs (109). SCF protected ECFCs from IFN-γ-initiation apoptosis via increase in the expression of FLIP (109).

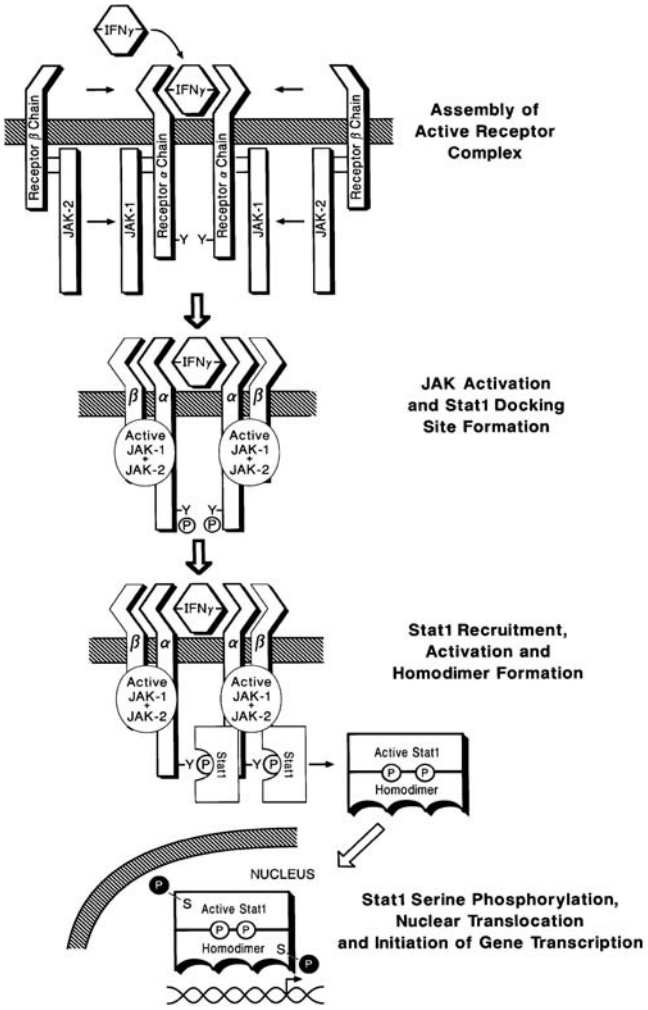


Figure 10 Signaling mechanism of the IFN-γ receptor.

IFN-γ induces the assembly of the complete receptor complex containing two α and two β subunits and JAK enzyme. JAK is activated and docking site for STAT1 is formed. STAT1 is recruited, activated and formed homodimer. Activated STAT1 translocates to the nucleus, and binds to a specific sequence in the promoter region of IFN-γ inducible genes and effects gene transcription. (Taken from Bach EA, Aguet M, Schreiber RD. The IFNγ receptor: a paradigm for cytokine receptor signaling. Ann Rev Immunol 1997; 15:563-91.)

3. Nuclear factor-κB

3.1 NF-κB activation

The Rel/NF-κB family transcription factors perform a vital role in mediating the cellular response to stress, inflammation, immune response, and apoptosis (129). Members of the Rel/NF-κB family transcription factors include Rel A (p65), NF-κB 1 (p50 and its precursor p105), NF-κB 2 (p52 and its precursor p100), RelB and c-Rel. These NF-κB family proteins share a conserved N-terminal ~300 amino acid region known as the Rel homology region (RHR) which is responsible for site-specific DNA binding, subunit dimerization, nuclear localization sequence (NLS). NF-κB family subunits associate in various combinations to form homodimers and heterodimers with distinct but overlapping functions. The functions of NF-κB dimers are generally restricted to specific cell types.

The most common form of NF-κB in a variety of cell types is the p50/p65 heterodimer. It exists in the cytoplasm as a complex with the inhibitor protein IκBα. IκBα inactivates the NF-κB complex by masking the NF-κB nuclear localization sequences. Upon activation of signaling pathways by extracellular signals, IκBα is rapidly phosphorylated and degraded, resulting in the release of the NF-κB complex, most commonly the p105/p65 heterodimer. The p105 subunit is cleaved into its active p50 form. This cleavage exposes the NLS on the p50 subunit. The p50/p65 heteroduplex then translocates to the nucleus where it activates gene transcription. NF-κB also induces the transcription of its own inhibitor, IκBα, causing an autoregulatory mechanism of NF-κB activity and generating the inactive form of NF-κB. The newly formed nuclear NF-κB-IκBα complexes are then exported out to the cytoplasm, thereby reestablishing the cytoplasmic pool of inactive NF-κB complexes primed for another round of activation to take place. The wide variety of genes regulated by NF-κB includes those encoding cytokines, chemokines, adhesion molecules, acute phase proteins, and inducible effector enzymes. Additionally, it is central to the overall immune response through its ability to activate genes encoding regulators of apoptosis and cell proliferation.

Activation of NF-κB occurs in response to extracellular stimuli (TNF-α, IL-1, LPS) or to chemical and physical stress and involves an apparently consecutive queue of signalling molecules linking the transcription factor to cell surface receptors. In the case of TNFα, ligand-induced trimerization of the TNFα receptors leads to recruitment of TRAFs as well as other signalling-molecules. TRAFs further transmit the signal by interaction with either NIK or MEKK1, type MAP3 kinases which resemble the most upstream point of integration of signals originating from diverse cellular stimuli such as TNF-α, IL-1 and LPS. NIK in turn activates IκB kinases (IKKs) that are part of a high-molecular weight signaling complex termed signalosome. Phosphorylation of IκBα by the IKKs triggers an ubiquitin ligase to ubiquitinylate IκBα and target it for degradation via the 26S proteasome. Thus, liberation of NF-κB from its inhibitory subunit exposes its previously masked NLS, leading to nuclear transport of the transcription factor where it can fulfil its biological function(s) (Figure 10).

3.2 NF-κB suppresses apoptosis

The antiapoptotic activity of NF-κB depends on gene induction (130). NF-κB induces the expression of the number of genes whose products can inhibit apoptosis, i.e. cellular inhibitor of apoptosis (c-IAPs), caspase 8-c-FLIP (FLICE inhibitor protein), A1 (also known as Bfl1), TRAF1 and TRAF2. As TRAF1 and TRAF2 are adaptor proteins required for optimal NF-κB and c-Jun kinase (Jnk) activation, their anti-apototic activity is most likely due to their ability to augment the activation of NF-κB (131). The other anti-apoptotic proteins may work in a coordinated manner to block apoptosis at multiple steps along the apoptotic signaling cascade (Figure 11).

3.3 Inhibition of NF-κB pathway

Activation of the NF-κB pathway can result from stimulation by a variety of different signal transduction pathways. Although IKK is a key regulator of the NF-κB pathway, ubiquitination of IκB and its subsequent degradation by the proteasome are also required for NF-κB activation. Furthermore, the differential nuclear translocation of members of the NF-κB family and the specific phosphorylation of these proteins

are also involved in the ability of the NF-κB proteins to activate gene expression. A number of different inhibitors can prevent NF-κB activation at different levels as shown in Figure 12. The information concerning the diverse inhibitors is summarized below (132-134).

3.3.1 Proteasome inhibitors

The last common step before NF-κB is freed from the cytoplasm is the ubiquitinated IκB by the 26S proteasome, thus, inhibitors of the ubiquitinated proteasome pathway suppress activation of NF-κB by stabilizing IκB. The best-characterized proteasome inhibitors are peptidyl aldehydes. They inhibit the chymotrypsin-like activity of the proteasome complex, but with distinct efficiencies. ALLnL (N-acetyl-leucinyl-leucynil-norleucynal or MG101) is a cysteine proteasome inhibitor, but less potent inhibitor of the proteasome than Z-LLnV (carbobenzoxyl-leucinyl-leucynil-norlvalinal or MG115) and Z-LLL (carbobenzoxyl-leucinyl-leucynil-leucynal or MG132) (135-137). Peptidyl aldehydes specifically block IκBα degradation but not IκBα hyperphosrylation, and do not cause chemical inactivation of NF-κB protein (138-141).

Lactacystin and its synthetic precursor, β-lactone, irreversibly block proteasome activity by acylating a threonine residue in the active site of the mammalian proteasome subunit X (135). For this reason, lactacystin is considered to be a more specific inhibitor of proteasome than the aldehyde peptides.

Boronic acid peptides (or dipeptide boronates), such as PS-262, PS-402, PS-341, PS-273, are originally used to act as proteasome inhibitor. They are more potent than their aldehyde analogs (135).

3.3.2 Protease inhibitors

The first protease inhibitors, which are shown to block NF-κB activation by blocking IκBα hyperphosphorylation and degradation, are alkylating agents including phenylalanine chloromethyl ketone (PCK), *N*-tosyl phenylalanine chloromethyl ketone (PTCK), 3,4-, dichloroisocoumarin (DCIC), and *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (142-144). These inhibitors are not specific since they also react with NF-κB proteins and abolish DNA-binding activity of other transcription factor, including AP-1, CREB and Oct-1 (144). The serine protease

inhibitors *N*-benzoyl-L-tyrosine ethyl ester (BTEE) and 4-(-2-aminoethyl)-benzensulfonyl fluoride (AEBSF) also block NF-κB activation by interfering with degradation and hyperphosphorylation of IκBα. However, these inhibitors do not abolish NF-κB binding activity by modifying NF-κB protein (143). Furthermore, there are artificial compounds, i.e. BAY-117821 and BAY-117083, which are pharmocologic small molecules that selectively an irreversibly block TNF-α induced phosphorylation of IκBα.

3.3.3 Glucocorticoids

Glucocorticoids, such as dexamethasone and prednisone, are widely used for their anti-inflammatory and immunosuppressive properties. These agents interact with the steroid receptor to downregulate the expression of specific genes that regulate the inflammatory process. There are several proposed mechanisms to explain the inhibitory effects of glucocorticoids on the NF-κB pathway. For example, dexamethosone induced expression of IκBαmRNA, resulting in the cytosolic retention of NF-κB (145, 146). A number cytokine genes regurated by NF-κB are repressed by glucocorticoids including GM-CSF, IL-2, IL-3, IL-6, IL-8, TNF-α (145, 146).

3.3.4 Nonsteroid-anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of chronic inflammatory states. NSAIDs inhibit COX activity to prevent prostaglandin synthesis. However, NF-κB inactivation involved in the actions of these agents has been reported (147). For example, aspirin and sodium salicylate are nonsteroidal anti-inflammatory drugs that are specific binding of ATP-binding to IKKβ (147). Thus, IKKβ-dependent phosphorylation of IκB is markedly reduced, preventing its degradation by the proteasome and activation of NF-κB pathway.

3.3.5 Immunosuppressive agents

CsA and tacrolimus (FK-506) are immunosuppressive agents used in organ transplantation to prevent graft-versus-host disease. These agents form a complex with the cellular protein cyclophilin, inhibit the activity of calcineurin, a calcium- and calmodulin-dependent serine/threonine phosphatase, to result in the inhibition of cell activation. Calcineurin is required for activation of the transcription factor NF-AT, which binds to the *IL2* promoter and is critical for regulating IL-2

expression in T lymphocytes. In addition, calcineurin can activate the NF-κB pathway (148).

FK-506 and CsA act by distinct mechanisms to inhibit the NF-κB pathway (148-151). It has been shown that FK-506 inhibited translocation of c-Rel, resulting in decreased expression of both IL-2 and its receptor. (151). CsA serves as a noncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome and thus prevents IκBα degradation and activation of the NF-κB pathway. CsA inhibits proteasome activity in vitro and suppresses LPS-induced IκB degradation in murine macrophages by stabilizing the ubiquitinated forms of IκBα (148). This finding suggests that a target of CsA in inhibiting the NF-κB pathway is the protease activity of the proteasome rather than kinases or ubiquitin ligases that regulate the signal-induced phosphorylation and ubiquitination of IκB, respectively. Similar results are seen in Jurkat cells, as well as human and mouse primary T lymphocytes, where CsA interferes with the degradation of IκBα following phorbol-ester and ionomycin stimulation without altering IκBα phosphorylation (149). The proteolytic processing of the NF-κB precursor p105, which also requires the 26S proteasome complex, is not affected by CsA treatment (150).

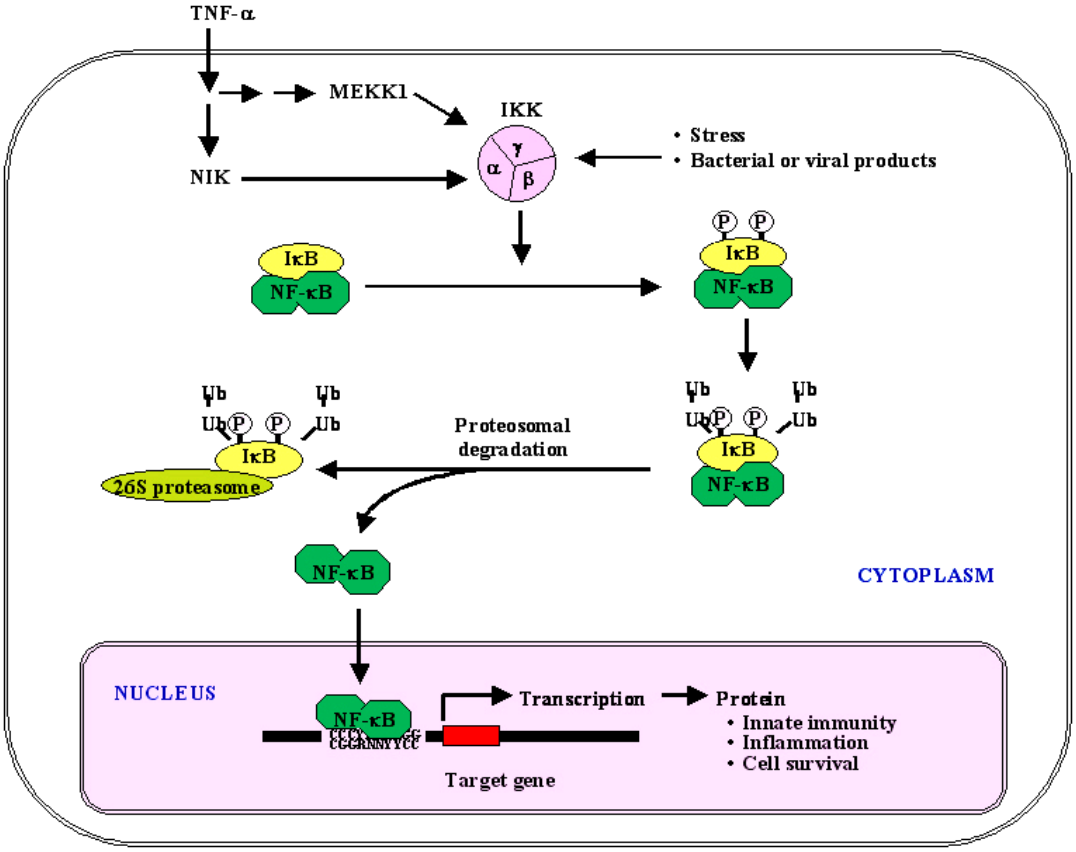


Figure 11 Activation of the NF-κB pathway by the IκB kinase complex.

Various signals can initiate a pathway that activates the IκB kinas complex (IKK), leading to the phosphorylation, ubiquitination and degradation of IκB. This allows NF-κB to enter the nucleus and regulate specific gene expression. (Modified from Karin M, Lin A. NF-κB at the crossroads of life and death. Nature Immunol 2002;3(3):221-7.)

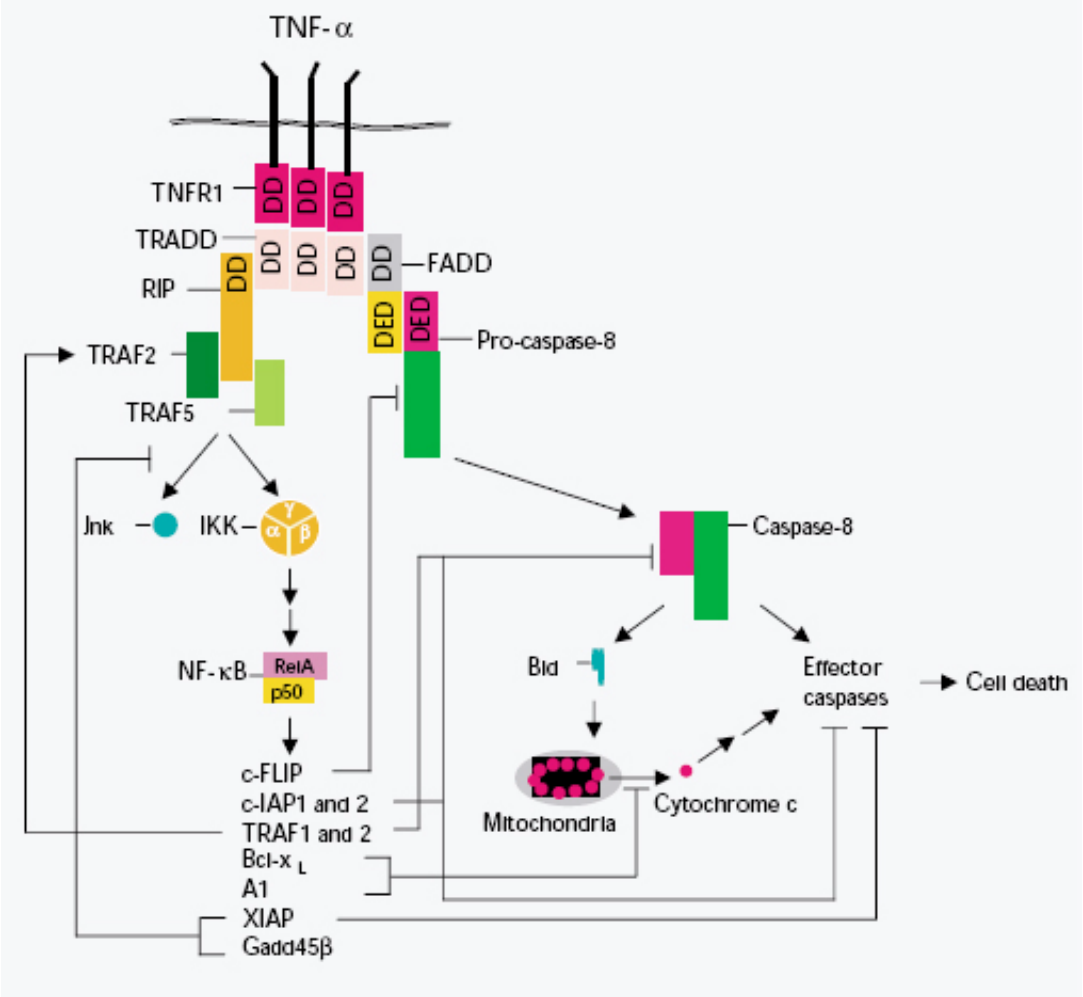


Figure 12 NF-κB induces a variety of antiapoptotic factors that can prevent TNF-α-induced apoptosis.

Induction of NF-κB by TNF-α leads to the transcriptional activation of genes that suppress apoptosis. The proteins encoded by the genes function at different levels in the caspase cascade to block apoptosis, or function at mitochondria to inhibit the cytochrome c releasing. (Taken from Karin M, Lin A. NF-κB at the crossroads of life and death. Nature Immunol 2002;3(3):221-7.)

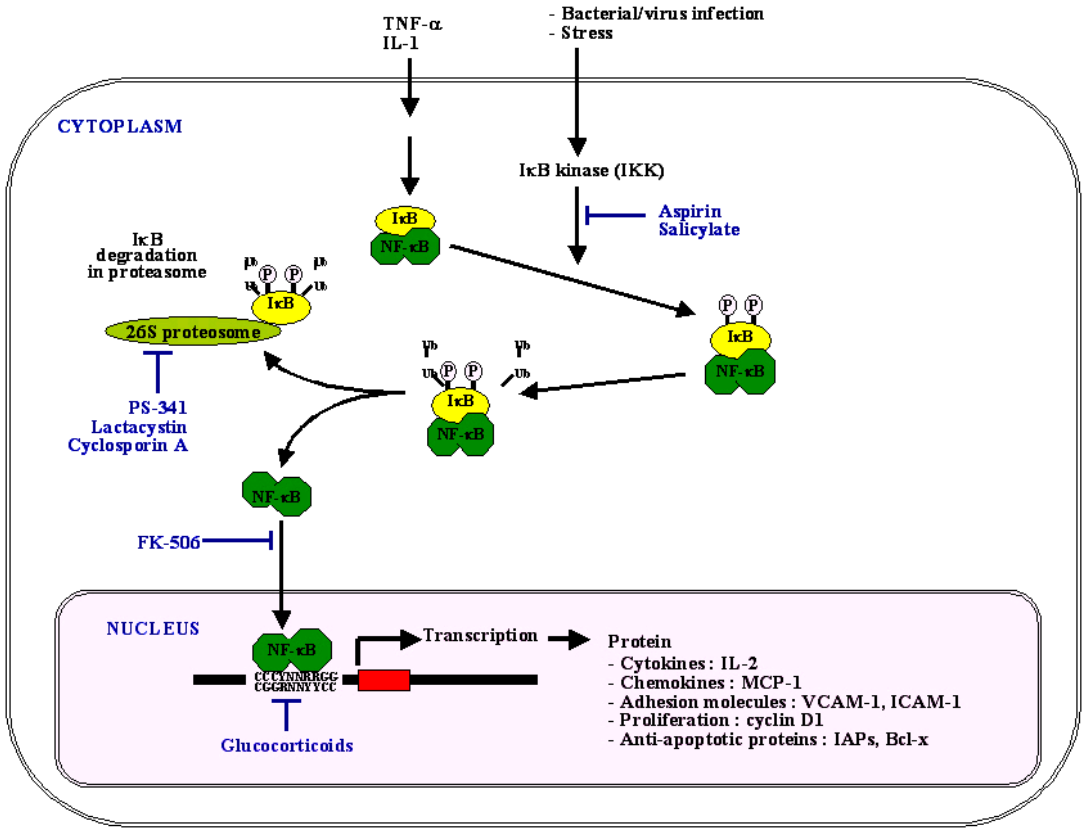


Figure 13 Inhibition of the NF-κB pathway.

A schematic illustrates the steps involved in the activation of the NF-κB pathway. Numerous drugs, natural products, and normal or recombinant proteins can act at several of these steps to interfere with NF-κB activation. (Modified from Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107:135-42.)

3.4 Cyclosporine A

Cyclosporin A (CsA) is an immunosuppressant, which was launched almost 20 years ago by Novartis. This drug is used to treat autoimmune diseases and prevent rejection in bone marrow and organ transplantation (152, 153). Originally identified by screening for antibiotics from microorganisms, these remarkable drugs suppress the immune system by altering activation of the genes that encode immune factors, such as IL-2, IL-3, IFN-γ. However, this suppression must be balanced with the need to maintain enough strength in the immune system to combat infection. So, side effects of cyclosporine treatment are not uncommon, and include an increased risk of cancer in patients who take these drugs long-term.

3.4.1 Structure and formulation

CsA is a highly lipophilic cyclic endecapeptide, which is isolated from *Tolypocladium inflatum* gams. The formula of CsA is $C_{62}H_{111}N_{111}O_{12}$, and its molecular weight is 1202.63 g. The structure of CsA is shown in Figure 13. The compound has several N-methylated amino acids. The peptide is neutral, rich in hydrophobic amino acids, insoluble in water and n-hexane, but soluble in many other organic solvents and in lipid.

The cyclosporine oral solution of Neoral® contains 100 mg/mL of cyclosporine dissolved in corn oil-mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil NF, DL-a-tocopherol USP, polylene glycol USP, and alcohol USP. The emulsifying form of the solution allows its distribution in the water.

3.4.2 Distribution and metabolism

The absorption and elimination of CsA were obtained under steady-state conditions after oral and intravascular administration of the drug. The drug is secreted mainly via the liver and bile and only to slight extent via the kidneys. Six percent of oral dose of CsA administered to human subjects was recover from the urine within 96 h. The proportion of unchanged drug in the urine was only 0.1% (Table 5). CsA is extensively metabolised by cytochrome P-450 III-A enzyme system in the liver, and to lesser degree in the gastrointestinal tract, and kidney. All metabolites contained the intact cyclic oligopeptide structure of parent compound. Observed modifications consisted of mono- and dihydroxylation and N-demethylation at various positions on the molecule. At least 25 metabolites have been identified from human bile, feces, blood, and urine. The major metabolites are M1, M9, and M4N,

which result from oxidation at the 1-beta, 9-gamma, and 4-N-demetylated positions, respectively.

Table 6 shows distribution of ^{3}H-CsA after 20 min addition of the drug to human blood, approximately 33%-35% in plasma, 4%-9% in lymphocytes, 5%-12% in granulocytes and 45%-58% in erythrocytes. At high concentrations, the binding capacity of leukocyte and erythrocytes becomes saturated. About 34% of the drug is present in the plasma, 5% as free drug, 24% bound to lipoproteins, and 5% bound to other plasma proteins.

3.4.3 Molecular mechanism of CsA action

The molecular mechanism of CsA action in T cell has been described (154). It is well established that CsA through formation of a complex with cyclophilin inhibits the phosphatase activity of calcineurin, which regulates nuclear translocation and subsequent activation of NF-AT transcription factors (Figure 14). In addition to the calcineurin/NF-AT pathway, recent studies indicate that CsA also blocks the activation of JNK and p38 signaling pathways triggered by antigen recognition, making CsA a highly specific inhibitor of T cell activation.

The effects of CsA on the human erythroid leukemic cell line K562 were recently reported (155). The proliferation of K562 cells were not inhibited by CsA but the differentiation of the cell line via activation of p38 and inactivation of ERK has been demonstrated (156). After K562 was treated with CsA for 4 days, the hemoglobinized cells were increase 3.3 times.

There is also evidence that CsA can inactivate NF-κB by non-competitive inhibiting proteasomal breakdown of IκB (149).

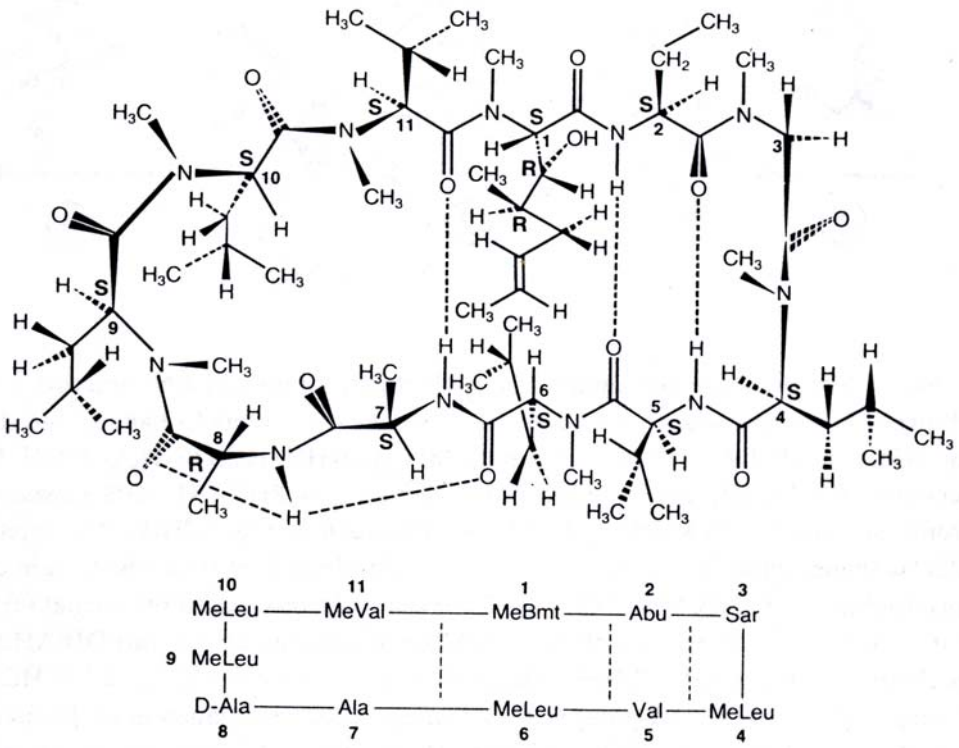


Figure 14 Structure and conformation of CsA.

Cyclosporin is a cyclic polypeptide containing 11 amino acid residues. The upper schematic represents the structure conformation of CsA in the solid state. The lower schematic represents the structure conformation of CsA in a polar solvent. (Taken from Wenger RM. Synthesis of ciclosporin and analogues: Structural and conformational requirements for immunosuppressive activity. Prog Allergy 1986;38:46-64.)

Table 5 Pharmacokinetic data of CsA in man (157, 158).

Absorption	
Absolute oral bioavialability	20-50% (mean 30%)
Plasma peak concentration time	2-4 hours
Distribution	
Binding to plasma proteins	90-95%
Apparent volume of distribution	1.5-7.0 /kg (mean 3.51 /kg)
Metabolism	
Extent of metabolism	99%
Number of components	24 known metabolites
Elimination	
Elimination half-life	1.2 hours (α-phase)
	27 hours (β-phase)

Table 6 Distribution of CsA in various concentrations among different blood (159).

Concentration in blood	Distribution (%)			
(ng/mL)	Plasma	Lymphocytes	Granulocytes	Erythrocytes
500	33	4	5	58
100	35	7	6	53
25	33	9	12	45

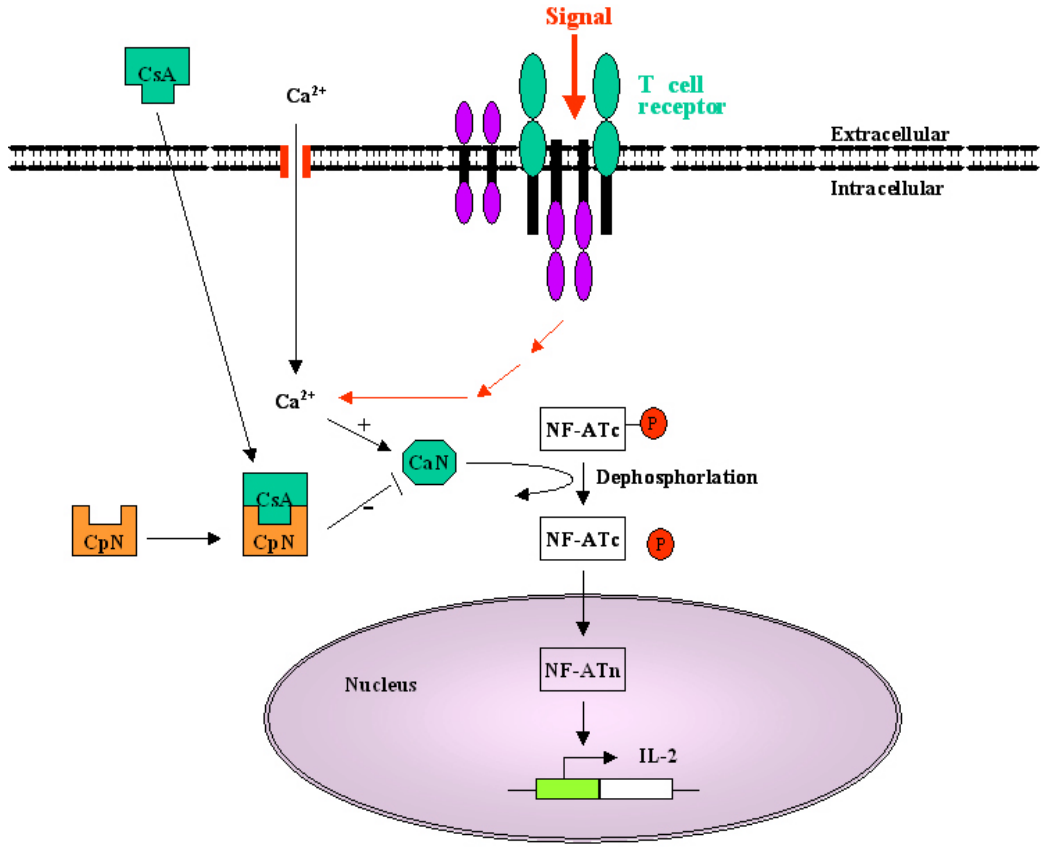


Figure 15 Mechanism of action of CsA.

In the cytoplasm, CsA binds to its immunophilin, cyclophylin (CpN), forming a complex between cyclosporine and CpN. The cyclosporine–CpN complex binds and blocks the function of the enzyme calcineurin (CaN), which has a serine/threonine phosphatase activity. As a result, CaN fails to dephosphorylate the cytoplasmic component of the nuclear factor of activated T cells (NF-ATc), and thereby the transport of NF-ATc to the nucleus and the binding of NF-ATc to the nuclear component of the nuclear factor of activated T cells (NF-ATn). The NF-ATc–NF-ATn complex binds to the promoter of the interleukin 2 (IL-2) gene and initiates IL-2 production. (Modified from Stepkowski SM. Molecular targets for existing and novel immunosuppressive drugs. Expert Reviews in Molecular Medicine. Cambridge University Press, 2000.)

4. The thalassemias

The genetic disorders of hemoglobin can be classified into two groups: (i) the thalassemias, in which there is impaired synthesis of normal globins; (ii) hemoglobin variants, in which there is a structural alteration in one of the globin chains.

4.1 Genetic control of hemoglobin

The amounts and types of hemoglobin produced at any age are determined primarily by the selective expression of the individual genes encodeing each globin chain. Human globin genes are located in two clusters: α-like genes in about 30 kb of DNA on the short arm of chromosome 16, and β-like gene in about 70 kb of DNA on the short arm of chromosome 11. The α-like globin genes arrange in the order 5'-ζ-ψζ-ψα_2-ψα_1-α_2-α_1-θ_1- 3'. The β-like globin genes arrange in the order 5' ψβ_2-ε-$^{G}\gamma$-$^{A}\gamma$-ψα_1-δ-β- 3'. Each gene shares certain basic organizational features. Each contains three coding regions (exons) separated by two non-coding regions (introns). When a globin gene is transcribed, messenger RNA is copied from one of the strands of DNA of the particular gene. While it is still in the nucleus of the red cell precursor, the intron sequences are removed and the exon sequences spliced together in the correct order to form the template for the production of a globin chain. This processed molecule moves into the cytoplasm, where it acts as the blueprint whereby appropriate amino acids are strung together to form a definitive globin chain. In adult red cells α- and β-globin chains synthesised in this way combine with heme to form definitive hemoglobin molecules. There are also critical regulatory regions of DNA that are involved in ensuring that globin chains are produced in appropriate amounts in the correct tissues at the right time of development. The thalassemias result from mutations or gene deletions that involve one or other of these complex steps.

4.2 Definition and classification

The thalassemias are a heterogeneous group of inherited disorders of hemoglobin synthesis; all characterized by the absence or reduced output of one or more of globin chains of hemoglobin. They can be classified at three levels: clinical classification, genetic classification and molecular classification (160).

4.2.1 Clinical classification

For practical clinical approach the thalassemias are classified as descriptive symptoms with no strict genetic basis. They are thalassemia major, thalassemia intermedia and thalassemia minor. Thalassemia major is severe anemia and transfusion dependent, such as β-thalassemia major. Hb levels of the patients are usually 6 g/dL or lower. Thalassemia major consists mainly of two categories: homozygous α^0-thalassemia and numerous of β-thalassemia diseases. Thalassemia minor is asymptomatic. It is represent the carrier state, such as β-thalassemia trait and α-thalassemia trait. Thalassemia intermedia describes conditions that are associated with a more severe degree of anemia than the trait but they are not as severe as the major forms, such as Hb H disease. Hb levels of the patients are 7 g/dL or higher in the steady state. Generally, patients have very mild symptoms or are free symptoms, not requiring blood transfusion. However, complications do occur in these patients. They have moderately shortened life span, but a few can attain old age.

4.2.2 Genetic classification

The thalassemias are classified according to their genetic basis by describing the globin chain that synthesized at a reduced rate. There are two globin-gene clusters that are involved in controlling of globin synthesis. The α-like gene cluster is on chromosome 16 and the genes are arranged in the order 5'-ζ-ψζ-ψα2-ψα1-α2-α1-θ1-3'. The β-like gene cluster is on chromosome 11 and the genes are arranged in the order 5'-$\psi\beta_2$-ε-$^{G}\gamma$-$^{A}\gamma$-$\psi\alpha_1$-δ-β-3'. As shown in Table 7, the thalassemias are classified into α, β, γ, δβ, δ, εγδβ varities, depending on which chain or chains are synthesized at reduced rate.

4.2.3 Molecular classification

The molecular classification is classified according to their molecular pathology. It is developed a more accurate approach to the designation of different type of α and β thalassemias. For example, the genotype of patient with the clinical picture of β-thalassemia disease according to $\beta^{39C\rightarrow T}$ mutation, a common Mediteranian nonsense mutation, at the homozygous pairs of β globin chain loci would have the genotypes αα/ $\beta^{39C\rightarrow T}$ $\beta^{39C\rightarrow T}$. Compound heterozygotes for this

mutation and another common RNA processing mutation would have the genotypes $\alpha\alpha/\ \beta^{39C\rightarrow T}\ \beta^{IVSI,\ 1G\rightarrow T}$.

4.3 Common thalassemia in Thailand

Distribution of α-thalassemia in throughout Southeast Asia is heterogeneous. In Thailand, the overall frequency of a thalassemia is 20-30%. The frequency of α^0-thalassemia is higher in the Northern than in the Southern part; 10% in Chiengmai and 3.5% in Bangkok whereas α^0-thalassemia is between 16-20%. The frequency of β-thalassemia is 3-9%. The frequency of Hb Constant Spring is found at least 4%. The overall frequency of Hb E is about 13%, with the highest frequency of about 50% observed in the northeast of the country.

4.3.1 α-Thalassemia

α-Thalassemia is characterized by reduced rate of globin production. The gene deletion is the major cause of α-thalassemia and that non-deletion is very rare. The two α-globin genes, α_1 and α_2, have a high degree of structural similarity and produce identical product of α-globin chain. In α^0-thalassemia (α-thalassemia 1), there is a deletion that removes about 17.5 kb of DNA including the α_1 and α_2 globin genes from the α-globin gene cluster (--). In α^+-thalassemia (α-thalassemia 2), only one α−globin gene in the chromosome is functional (-α). There are two types of α^+-thalassemia: one involves a deletion of 4.2 kb of DNA including the α_2-globin gene ($-\alpha^{4.2}$) and another involves a deletion of 3.7 kb of DNA between the linked α_1- and α_2-globin genes and results in a hybrid α gene ($-\alpha^{3.7}$).

α-Thalassemia can be defined depending on the number of α-globin gene deletion in the diploid genome. Deletions of 1, 2, 3, and 4 globin genes result in α^+-thalassemia (-α/αα), α^0-thalassemia (--/αα), Hb H disease (--/-α) and Hb Bart's hydrops fetalis (--/--), respectively. α^+-Thalassemia and α^0-thalassemia are carriers and asymptomatic. Hb H disease is intermediate form. Hb Bart's hydrops fetalis is severe form.

4.3.2 β-Thalassemia

The β-thalassemias are a heterogeneous group of disorder characterized by decreased or absent β globin chain synthesis. Point mutations and

small deletions or insertions in the nucleotide sequences are mainly responsible for the molecular defects of β-thalassemia around the world and in Thailand. Two types of β-thalassemias are known: β^+-thalassemia, in which the production of β-chains is reduced, and β^0-thalassemia, in which the production of β-globin chain is entirely eliminated. β^+-thalassemia generally involves defects at RNA processing or promoter region of the gene. In some cases, it results from a mutation within the introns of the β globin gene. In β^0-thalassemia, the mutation may result in a complete block at transcription or RNA processing, leading to lacking of the β-globin mRNA production and the absence of β-globin chain synthesis. In some cases, point mutation in the DNA sequence lead to a nonsense mutation providing a production of an incomplete β-globin chain.

Reduction of β-globin chain synthesis leads to an imbalanced α-/non-α-globin synthesis. This is the major factor in determining the severity of disease in the β-thalassemia syndromes. The mutations causing β-thalassemia result in a deficit of β-globin production that ranges from minimal (mild β^+-thalassemia alleles) to a complete absence (β^0-thalassemia alleles).

4.3.3 Hb Constant Spring

Hb Constant Spring (Hb CS, $\alpha_2^{142\ Ter\rightarrow Gln}\beta_2$) is a single base mutation of the termination codon (TAA→CAA) of the α_2-globin gene. When mutation changes the stop codon to one that encodes an amino acid it allows mRNA translation to continue to the next inphase termination codon (UAA) located within the polyadenylation signal (AAUAAA). Thirty-one amino acids are addded to the end of the peptide chain. Although α^{CS}-mRNA can be detected in bone marrow of the patients, it is absent from their peripheral blood suggesting its instability. Because of the remarkable reduction in α^{CS}-globin chain production, Hb CS behaves similar to α^+-thalassemia.

Hb CS heterozygotes resemble α^+-thalassemia but MCV is higher than normally seen in this condition. Homozygotes have symptom with mild anemia, jaundice and splenomegaly. Hb CS is often inherited in double heterozygous condition (Hb H with Hb CS) and has unusually severe form of Hb H disease.

4.3.4 Hb E

Hb E ($\alpha_2\beta_2^{\ 26\ Glu\rightarrow Lys}$) is a point mutation of β-globin chain variant. The β-globin chain codon 26 Glu→Lys mutation (GAG→AAG) partially activates cryptic splice site towards the 3' end of exon 1, resulting in a proportion of abnormally spliced mRNA (161). Thus, β^E globin is synthesized in a reduced amount resulting in a mild thalassemia phenotype. HbE is also mildly unstable and may be susceptible to oxidant damage.

Hb E heterozygotes are clinically normal and have only minor hematologic changes. Hb E homozygotes exhibit more microcytes but are still asymptomatic. Compound heterozygote for Hb E and β-thalassemia gives rise to serious clinical disease with a phenotype ranging from mild anemia to the most severe form of β-thalassemia major.

4.4 Pathophysiology of thalassemia

4.4.1 The β-thalassemias

4.4.1.1 Clinical feature of the β-thalassemias

The β-thalassaemias result from over 150 different mutations of the β globin genes that result either in the absence of the β-globin chains (β^0-thalassaemia) or a reduction in their output (β^+-thalassaemia). This results in imbalanced globin chain synthesis and production of an excess of α chains, which precipitate in the red cell precursors, leading to their destruction in the bone marrow or peripheral blood. This process causes severe anaemia, which in turn leads to increased erythropoietin production and expansion of the ineffective bone marrow as shown in Figure 15, Table 7. The severe ineffective erythropoiesis results in erythroid marrow expansion to as much as 30 times the normal level. Both an increase in plasma volume as a result of shunting through expanded marrow and progressive splenomegaly exacerbate anemia. Increased erythropoietin synthesis may stimulate the formation of extramedullary erythropoietic tissue, primarily in the thorax and paraspinal region. Marrow expansion also results in characteristic deformities of the skull and face, as well as osteopenia and focal defects in bone mineralization, Marrow hyperplasia leads ultimately to increased iron absorption and progressive deposition of iron in tissues.

4.4.1.2 Ineffective erythropoiesis and apoptosis in β-thalassemia

Using ferrokinetic analysis, in normal, 75 percent to 90 percent of Fe^{59} entered marrow erythropoiesis appeared in circulating red blood cells in 7 to 10 days. In contrast, in some cases of Cooley's anemia, 15 percent of Fe^{59} was incorporated in circulating erythrocytes indicated that the extent of ineffective erythropoiesis or intramedullary hemolysis could account for as much as 60 percent to 75 percent of total erythropoiesis (162).

The evidence of ineffective erythropoiesis in thalassemia was first reported as an electron microscopic study of marrow fragments from patients with homozygous β-thalassaemia. (3). There were 3% of early polychromatic erythroblast and 20% of late polychromatic erythroblast contain intracytoplasmic α-globin chain precipitates. Various nuclear abnormalities were found including the loss of parts of the nuclear membrane and the presence of intranuclear α-globin chain precipitates, and these abnormalities were virtually confined to the non-dividing, late polychromatic erythroblasts. The cytoplasm of the bone marrow reticulum cells contained early and late polychromatic erythroblasts at various stages of degradation, providing direct evidence of ineffective erythropoiesis.

Ultrastructural studies revealed several abnormalities in orthochromatic erythroblast in patients with homozygous β-thalassemia. Theses included glycogen accumulation, plasma membrane enfolding, vacuole formation, hyaline figures, and Heinz body (163). Polychromatic and orthochromatic erythroblast contained cytoplasmic aggregates of electron dense material (3). β-Thalassemia erythroid precursors containing apparent α-globin chain precipitates had decreased protein synthetic capacities (164).

The erythropoietic cells of β-thalassemia/Hb E showed marked ultrastructural abnormalities that were qualitatively and quantitatively similar to those seen in homozygous β-thalassemia (165). The most striking abnormality in β-thalassemia/Hb E was the presence in high proportion of the erythroblasts and marrow reticulocytes of multiple intracellular inclusions. These inclusions appeared as mass varied in size and shape, had indistinct edges, tended to fuse together and were not

attached to the cell membrane. The inclusions were indistinguishable from α-globin chain precipitates present in homozygous β-thalassemia. In β-thalassemia/Hb E precipitates were first found in the early polychromatic erythroblasts and both the quantity of precipitate per affected cell profile and the proportion cell profiles containing precipitates increased at the late polychromatic erythroblast stage. Precipitates were virtually confined to cytoplasm in the early polychromatic erythroblasts but were found in both the nucleus and the cytoplasm in the late polychromatic erythroblasts. Other abnormalities encountered in the nuclei of the late polychromatic erythroblasts were: (1) intranuclear clefts, (2) loss or duplication of part of nuclear membrane, (3) separation of the nuclear membrane from the nucleus, and (4) marked irregularities in nuclear shape. In some cell profiles the duplication, or loss of the nuclear membrane was found adjacent to mass of precipitate. In addition, phagocytosed erythroblasts, mainly late polychromatic erythroblasts, at various stages of degradation were found within some of the bone marrow macrophages.

In severe untreated β-thalassemia, erythropoiesis may be increased by a factor of up to 10, more than 95 percent of which may be ineffective. Ineffective erythropoiesis is a result of the myriad deleterious effects of a relative excess of α-globin chains. This relative excess interferes with most stages of normal erythroid maturation: both intramedullary death of red-cell precursors through arrest in the G1 phase of the cell cycle and accelerated intramedullary apoptosis of late erythroblasts have been demonstrated. Studies of the consequences of the accumulation of excess α-globin chains and their degradation products within the red cell membrane and its skeleton have also demonstrated abnormalities in the ratio of spectrin to band 3 and in the function of band 4.1. The observation that the presence of excess membrane iron may aggravate membrane changes has led to interest in the red-cell membrane as a potential therapeutic target in β-thalassemia. In a mouse model, increased cellular rigidity and decreased stability in connection with membrane-associated α-globin chains have reportedly been ameliorated during exposure to agents that bind membrane iron. Mechanisms of damage to red cells and precursor in β-thalassemia are summarized in Table 8.

Normally, the number of progenitor cells in blood is high prior to birth and decreases rapidly after birth. Only small numbers of progenitor cells have been in adult blood (166). However, circulating BFU-E and CFU-GM progenitor cells are increase in thalassemic patients, especially those who were splenectomized. The number of progenitor cells also positively correlated with the number of nucleated red cell in the blood. This reflects either increased marrow hematopoiesis or an evidence of extramedullary hematopoiesis. Recent studies show that the extent of apoptosis is variable in each type of thalassemias and apoptosis is increased with increasing erythroid maturity (4-6). Thalassemic erythroid precursors underwent apoptosis at a rate that was 3 to 4 times normal. Because thalassemic marrow has between 5- to 6-fold more erythroid precursors than healthy marrow, thus an absolute increase in erythroid precursor apoptosis of about 15-fold above healthy (4). The extreme rates of erythroid hyperplasia and apoptosis might be characteristic of more severely affected patients.

4.4.2 The α-thalassemias

4.4.2.1 Clinical feature of the α-thalassemias

The α-thalassemias show several important differences from β-thalassemia (Table 9). Because α-globin chains are shared by fetal and adult hemoglobin the disease is manifested in both fetal and adult life. Furthermore, excess γ- and β-globin chains do not precipitate immediately in the bone marrow as α-globin chains, but produce the physiologically useless and unstable tetramers: γ_4, (Hb Bart's) and β_4 (Hb H). The pathophysiology of the α-thalassemias differs from that of the β-thalassemias. The homozygous state for α^0-thalassemia results in the intrauterine death with a profoundly anemic and hydropic fetus: the Hb Bart's hydrops fetalis syndrome. Mothers carrying babies of this type commonly have toxemia of pregnancy and postpartum bleeding. Compound heterozygotes for α^0 and α^+-thalassemia (--/-α) have a milder illness characterised by anemia and splenomegaly which is called hemoglobin H disease. Carriers for α^0-thalassemia (--/αα) and homozygotes for α-thalassemia (-α/-α) have a mild hypochromic anemia, while carriers for α^+-thalassemia have no hematological abnormalities. The pathophysiology of Hb Bart's hydrops fetalis in fetus and Hb H disease in adult are shown in Figure 16.

4.4.2.2 Ineffective erythropoiesis and apoptosis in α-thalassemia

The primary kinetic lesion in Hb H disease is hemolysis (167-171). Ferrokinetic study showed that red cell ^{59}Fe red cell utilization in Hb H at 14 days was approximately 62% while normal erythropoiesis was 85% (171). This rate signified moderate to mild ineffective erythropoiesis, explaining in part the inability of apparently increased marrow erythropoiesis to compensate for moderate degree of hemolysis. Studies assessing plasma levels of soluble transferrin receptor also suggested that there is a component of ineffective erythropoiesis in Hb H disease (172, 173). Furthermore, morphologic data indicate that excess β-globin chains precipitated in marrow erythroid precursors (1, 174), where they might cause some intramedullary cell death.

It has shown that excess β-globins precipitate in marrow erythroid progenitor cells (2). Such deposition of insoluble β-globin could be provided the basis of oxidant or other sorts of cellular injury, leading to intramedullary erythroid progenitor death. Compared with healthy controls, marrow erythroid precursor from patients with Hb H and Hb H/CS diseases showed the extent of apoptosis is approximately twice of the normal whereas that with Hb CS/CS had about 4 times of the normal (5). The increased apoptosis in Hb CS/CS suggests that the α^{CS}-globin chain accumulation may begin at the erythroid precursor stage and cause increased apoptosis.

A consequence and hallmark of apoptosis is the movement of phosphatidylserine from the inner to the outer leaflet of the membrane bilayer. Macrophages recognize cell-bearing phosphatidylserine via CD36 receptor on their outer surface, and remove such cells efficiently as part of apoptotic process. An additional mechanism for the enhance removal of α-thalassemic erythroid precursor cells from the marrow maybe the excessive surface Ig G. In Hb H disease, these marked cells may be attacked and removed by marrow macrophage with Fc receptor.

Table 7 The thalassemias and related disorders (160).

α-Thalassemia
α^0
α^+
Deletion ($-\alpha$)
Non-deletion (α^T)
β-Thalassemia
β^0
β^+
δβ Thalassemia
$(\delta\beta)^0$
$(\delta\beta)^+$
$(^A\gamma\ \delta\beta)^0$
εγδβ Thalassemia
Hereditary persistence of fetal hemoglobin
Deletion
$(\delta\beta)^0$
$(\delta\beta)^+$
$^G\gamma\ (\gamma\beta)^+$ (Hb Kenya)
Non-deletion
Linked to β globin genes
$^G\gamma\ \beta^+$
$^A\gamma\ \beta^+$
Unlinked to β globin genes

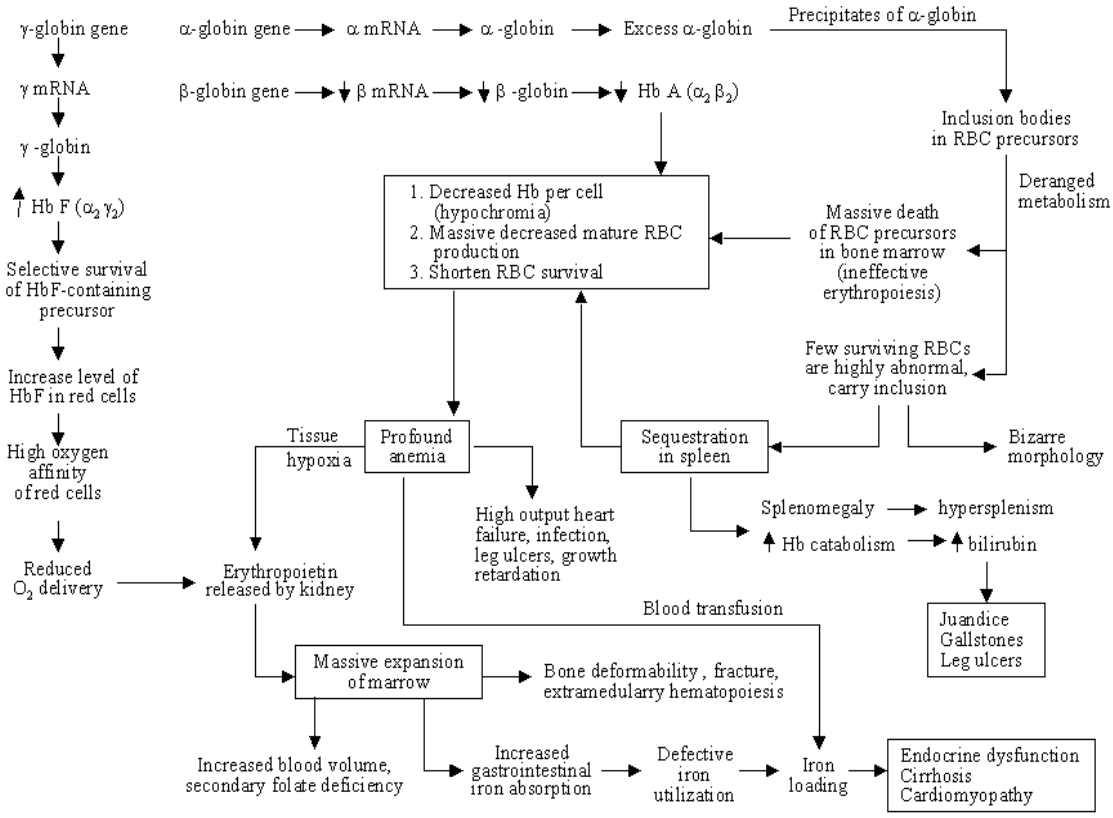


Figure 16 The pathophysiology of β-thalassemia.

The diagram outlines the pathogenesis of clinical abnormalities resulting from the primary defect in β-globin chain synthesis. (Modified from Schwartz E, Benz, Jr. EJ, Forget BG. Thalassimia syndromes. In: Hoffman R, Benz EJ, Jr., Shattil SJ, Furie B, Cohen HJ, Silberstein LE, editors. Hematology: basic principles and practice. 2nd edition. New York: Cherchill Livingstone, 1995, p 586-610.)

Table 8 Summary of mechanisms of damage to red cells and precursor in β-thalassemia (160).

Red cell precursors; ineffective erythropoiesis
Imbalance globin synthesis, α-globin chain precipitation
Inclusion body formation
Hemichrome formation
Oxidative damage to membrane components by heme, hemin and iron
Defective maturation of erythroid precursors
Accelerated apoptosis
Enhanced phagocytic activity
Red cell; hemolysis
α-globin chain precipitation
Inclusion body formation-mechanical trauma
Hemichrome formation, Band 3 clusters, Ig G binding
Oxidative damage to membrane components by heme, hemin and iron, band 4.1 oxidation
Low ATP and potassium; increased calcium
Metabolic consequences of hypersplenism

Table 9 Some important differences between α- and β-thalassemia (160).

	α-Thalassemia	β-Thalassemia
Mutation	Gene deletion common	Gene deletion rare
Properties of excess globin	Soluble γ_4 and β_4 tetramers	Insoluble α chain aggregates
	Slow rate of hemichrome formation	Rapid rate of hemichrome formation
	Band 4.1 not oxidized	Oxidation of band 4.1
	Binds to band 3	Less interaction with band 3
Red cells	Overhydrated	Dehydrated
	Rigid	Rigid
	Membrane hyperstable	Membrane unstable
	Decreased p50	Decreased p50
Anemia	Mainly hemolysis	Mainly dyserythropoietic
Bone changes	Rare	Common
Iron loading	Rare	Common

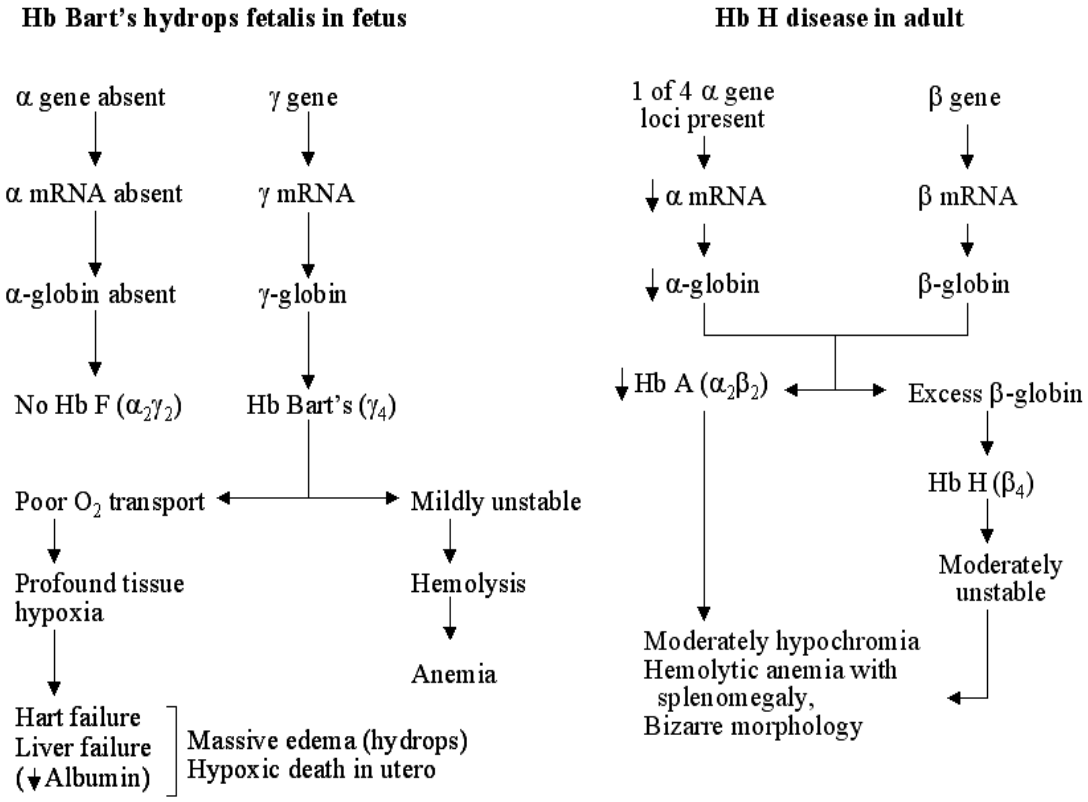


Figure 17 The pathophysiology of Hb Bart's hydrops fetalis and HbH disease.

The diagram illustrates the outline of pathogenesis of Hb Bart's hydrops fetalis and Hb H disease. (Modified from Schwartz E, Benz, Benz EJ, Jr., Forget BG. Thalassimia syndromes. In: Hoffman R, Benz EJ, Jr., Shattil SJ, Furie B, Cohen HJ, Silberstein LE, editors. Hematology: Basic principles and practice. 2nd edition. New York: Cherchill Livingstone, 1995, p 586-610.)

CHAPTER 4
MATERIALS AND MATHODS

Materials

1. Subjects

This study was performed by using blood samples from healthy normal volunteers and β-thalassemia/Hb E patients. All diagnoses were based on standard hematologic criteria, hemoglobin analyses, and DNA analyses. All patients were in stable condition, without complication, and none had received blood transfusion within 3 months before the study. 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.

2. Samples

Forty mL of heparinized peripheral blood of Japanese normal volunteers or 30 ml of heparinized blood of β-thalassemia/Hb E patients were collected. The blood samples were kept at 4^{o}C. Purification of erythroid colony forming cells was processed within 24 hours after collection.

3. Antibodies

3.1 Immunomagnetic beads: anti-CD3, anti-CD11b, anti-CD15 and anti-CD45RA antibodies (Miltenyi Biotech, Auburn, CA)

3.2 Hematopoietic progenitor enrichment cocktail containing anti-CD2, -CD3, -CD14, -CD16, -CD19, -CD24, -CD56, CD66b, anti-glycophorin A, and magnetic colloid (Stem Cell Technologies, Inc, Vancouver, BC)

3.3 Anti-NF-κB p65 (c-20)(Santa Cruz Biotechnology, CA)

3.4 Anti-actin clone AC-74 antibody (Sigma, Chemical Co, St. Louis, MO)

3.5 Anti-Bcl-x antibody (B22630, Transduction, Laboratories, Lexington, KY)

3.6 Goat-anti mouse Ig2a conjugated with horse reddish peroxidase (Sigma Chemical Co, St Louis, MO)

3.7 Rhodamine-conjugated with donkey anti-rabbit Ig G antibody (AP182R, Chemicon International, CA)

4. Cytokines

4.1 rhEPO (Chugai Phamaceitical, Tokyo, Japan)

4.2 SCF (Kirin-Brewery Co. Ltd, Tokyo, Japan)

4.3 rhIL-3 (Kirin-Brewery Co. Ltd, Tokyo, Japan)

4.4 rhIFN-γ (Shionogi Co. Ltd, Osaka, Japan)

5. Instruments

5.1 Vario-Macs stand (Miltenyi Biotech, Auburn, CA)

5.2 StemSep stand (Stem Cell Technologies Inc., Vancouver, BC)

5.3 Epics Elite ESP flow cytometer (Coulter Co., Miami, FL)

5.4 FACScan flow cytometer (Beckton-Dickinson, Franklin Lakes, NJ)

5.5 Leica DM Irbe confocal microscope

5.6 Bio-Rad confocal microscope, model MRC 600

5.7 Analytical balance

5.8 Autoclave

5.9 Autopipette

5.10 CO_2 incubator

5.11 Cytocentrifuge

5.12 Hand tally counter

5.13 Hemocytometer

5.14 Inverted microscope

5.15 Light microscope

5.16 Minigel vertical electrophoresis

5.17 Pipette aid

5.18 Refrigerated centrifuge

5.19 Suction pump

5.20 Transfer machine

5.21 UV spectrophotometer

5.22 Water bath

6. Glass ware, plastic ware and supplies

6.1 Vario-Macs columns (Miltenyi Biotech, Auburn, CA)

6.2 StemSep^{TM} column (Stem Cell Technologies Inc., Vancouver, BC)

6.3 Alumium foil

6.4 Beakers, glass, 50, 100, 250, 500 and 1,000 mL

6.5 Bottle, glass, 250, 500 and 1,000 mL

6.6 Color printing Pack (Sony, UPC-1010)

6.7 Cover glass 22x22 mm

6.8 Culture flask T25 and T75 (Felcon)

6.9 Culture dishes, plastic, 35 mm

6.10 Cylinder 50, 100, 250, 1,000 and 2,000 mL

6.11 Eppendorf tube 1.5 mL

6.12 EDTA tube 5 ml, vacutainer

6.13 Filter paper, diameter 70 mm circle

6.14 Glass slide

6.15 Heparinized coated tube 10 ml, vacutainer

6.16 Millipore filter 0.22, 0.45 and 0.8 μm

6.17 Parafilm

6.18 Pasture pipette, glass

6.19 Pipette tips 30, 200 and 1,000 μL

6.20 Plastic tube 12x75 mm, 10x100 mL

6.21 Seropipette, glass, 1, 5 and 10 mL

6.22 Sterile conical centrifuge tube, plastic, 15 and 50 mL

6.23 Suction volumetric flask 1,000 mL

6.24 Syringe, disposable, 5, 10, 25 and 50 mL

6.25 X-ray film

7. Reagents

7.1 Reagent for cell culture

7.1.1 Lymphocyte separation medium (LSM; density 1.0770-1.0800 g/mL; ICN Biomedicals, Aurora, OH)

7.1.2 Histopaque (density 1.0770, Sigma Chemical Co., St. Louis, MO)

7.1.3 Red cell lysis buffer containing 0.16 mol/L NH_3Cl, 10 mmol/L $KHCO_3$, 5 mmol/L EDTA

7.1.4 Dulbecco phosphate buffer saline (DPB-S; GIBCO BRL, Grand Island, NY)

7.1.5 DPBS-citrate containing 4 g of trisodium citrate in 1 L of DPB-S

7.1.6 25% Human serum albumin (HSA)

7.1.7 Human serum albumin containing 40 mL of 25% HSA and 60 mL of D-PBS-citrate

7.1.8 Sample buffer containing 1 mL of 25% HSA and 49 ml of 5 mM EDTA-PBS

7.1.9 Priming buffer containing 12 mL of 25% HSA and 38 mL of PBS

7.1.10 Iscoves modified Dulbecco medium (IMDM; GIBCO BRL, Grand Island, NY)

7.1.11 Heat-inactivated fetal calf serum (FCS; Commonwealth Serum Laboratories, Melbourne, Australia)

7.1.12 Human AB serum

7.1.13 1,000 U/mL of penicillin and streptomycin solution (GIBCO BRL, Grand Island, NY)

7.1.14 F-12 medium (Sigma Chemical Co., St. Louis, MO)

7.1.15 Detoxified bovine serum albumin (BSA; Stem Cell Technologies Inc., Vancouver, BC)

7.1.16 Iron-saturated transferrin (Boehringer Mannheim, Germany)

7.1.17 Lipid suspension containing 2.8 µg/mL oleic acid; 4.0 µg/mL L-α-phosphatidylcholine; 3.9 µg/mL cholesterol (Sigma Chemical Co., St. Louis, MO)

7.1.18 Wright's Giemsa satin

7.2 Reagent for cell viability assay

7.2.1 Trypan blue solution containing 0.4% trypan blue, 0.8%NaCl and 0.06%KPO_4

7.3 Reagent for apoptosis assay

7.3.1 Annexin V-fluorescein-5-isothiocyanate apoptosis detection kit (Immunotech, Marseille, France)

7.3.2 Glycophorin A-PE (BD Bioscience, Pharmingen, San Diego, CA.)

7.3.3 CD 71-FITC (BD Bioscience, Pharmingen, San Diego, CA.)

7.4 Reagent for erythroid colony-forming assay by plasma clot

7.4.1 10% Citrated human AB plasma

7.4.2 Benzidine solution containing 2 g of 3,3' dimethoxybenzidine (Sigma) in 100 absolute methanol

7.4.3 H_2O_2 solution containing 1 mL of 30% H_2O_2, 22.5 mL of absolute methanol and 40 mL of distilled water

7.5 Reagent for determination of NF-κB expression by Western blotting

7.5.1 Cell lysis buffer containing 10 mmol/L HEPES, pH 7.9; 10 mmol/L KCl; 0.1mmol/L EDTA, pH 8; 0.1mmol/L EGTA; 1 mmol/L DTT; 0.5 mmol/L PMSF; 2 µg/mL leupeptin; 2 µg/mL aprotinin

7.5.2 Nuclear extraction buffer containing 20 mmol/L Hepes, pH 7.9; 0.4 mol/L NaCl; 1 mmol/L EDTA, pH 8; 1 mmol/L EGTA; 1 mmol/L DTT; 1 mmol/L PMSF; 2 µg/mL leupeptin; 2 µg/mL aprotinin

7.5.3 Whole cell lysis buffer containing 50 mmol/L Tris-HCl, pH 8, 150 mmol/L NaCl; 0.5% NP-40, 100 mmol/L NaF; 1 mmol/L EDTA, pH 8;1 mmol/L EGTA; 0.08 mmol/L PMSF; 0.01 mg/ml leupeptin, 0.01 mg/ml aprotinin

7.5.4 Bio-Rad protein assay kit (Bio-Rad, Hercules, CA)

7.5.5 10% Sodium dodecyl sulfate-polyacrylamide gel

7.5.6 PVDF membrane (Immobilon, IPVH00010)

7.5.7 PBS containing 0.1% Tween 20 (PBST)

7.5.8 PBST with 5% skim milk

7.5.9 Chemiluminescence detection system (ECL, PRN2106; Amersham)

7.5.10 0.0625 mol/L Tris-HCl, pH 6.8, containing 2% SDS

7.6 Reagent for NF-κB assay by confocal microscopy

7.6.1 PBS containing 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$

7.6.2 Formaldehydye

7.6.3 0.2% Triton-X

7.6.4 10% Fetal calf serum (FCS)

7.7 Reagent for determination of Bcl-x expression by Western blotting

7.7.1 Cell lysis buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DDT, 2% SDS, and 10% Glycerol

7.7.2 Bio-Rad protein assay kit (Bio-Rad, Hercules, CA)

7.7.3 12.5% Sodium dodecyl sulfate-polyacrylamide gel

7.7.4 PVDF membrane (Immobilon, IPVH00010)

7.7.5 PBS containing 0.1% Tween 20 (PBST)

7.7.6 PBST with 5% skim milk

7.7.7 Chemiluminescence detection system (ECL, PRN2106; Amersham)

7.8 Reagent for analysis of mitochondrial transmembrane potential and ROS production of mitochondria by flow cytometry

7.8.1 100 mM of $DiOC_6$ (Aldrich Chemical, Milwaukee, WI) as stock solution

7.8.2 400 nM of $DiOC_6$ as working solution. To prepare working solution, 1 μL of 100 mM was diluted with 1 mL of PBS to get 100 μM of $DiOC_6$ solution. Then, 5 μL of 100 μM was diluted with 1.25 mL of PBS to get 400 nM of $DiOC_6$ solution.

7.8.3 22 mM of HE (Polysciences Inc., Warrington, PA.) as stock solution

7.8.4 200 mM of as working solution. To prepare working solution, 1 μL of 22 mM was diluted with 109 μL of PBS.

Methods

1. Purification of erythroid colony forming cells (ECFCs)

ECFCs from normal and thalassemic subjects were prepared as described (71, 175, 176). Light-density mononuclear cells were separated from 40 mL of heparinized peripheral blood buffy coat by density centrifugation using lymphocyte separation medium. Red blood cells were lysed by suspending the mononuclear cell pellet in red cell lysis. Platelets were removed by cell centrifugation through phosphate-buffered saline (PBS) containing 10% human serum albumin. Adherent cells were depleted by a 1 hour incubation in a polystyrene tissue-culture flask at 4°C. Nonadherent cells were collected and negative selection were then performed using anti-CD3, anti-CD11b, anti-CD15 and anti-CD45RA antibodies and immunomagnetic beads with Vario-Macs columns or using hematopoietic progenitor enrichment cocktail, which contained anti-CD2, -CD3, -CD14, -CD16, -CD19, -CD24, -CD56, CD66b, anti-glycophorin A, and magnetic colloid with StemSep column. The remaining cells were cultured in Iscoves modified Dulbecco medium containing 15% heat-inactivated FCS, 15% pooled human AB serum, 2 U/mL rhEPO, 20 ng/mL SCF, 10 ng/mL rhIL-3, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a high-humidity, 5% CO_2, 95% air incubator (day 0). On day 3 of the culture, the cells were centrifuged over LSM, then collected and incubated under the same conditions, but without rhIL-3. On day 7 of the culture,

the cells were collected and assessed for the purity of the day 7 ECFCs with proerythroblast-like features. This was 95%±3% and these cells were used in the following experiments.

2. Serum-free liquid cultures of ECFCs

Day 7 ECFCs ($1x10^5$-$1x10^6$ cells/mL) were suspended in serum-free liquid medium containing 50% IMDM, 50% F-12 medium, 1% detoxified bovine serum albumin, 300 µg/mL iron-saturated transferrin, lipid suspension (176), 100 U/mL penicillin, and 100 mg/mL streptomycin. The cell suspension was then incubated at 37°C for 30 minutes in a high-humidity, 5% CO_2, 95% air incubator with or without CsA as indicated. Then, rhEPO or rhIFN-γ, was added and the cells were incubated for the indicated times.

3. Erythropoiesis assessment

Erythropoiesis was assessed by counting the cells in serum culture and then the fold expansion of the cells was calculated from total erythroid cell numbers/starting cell number at day 0, 3, 7, 10, 13 and 16. Cell count was performed by using hemocytometer and light microscope.

4. Cell viability assay

To determine cell survival, viability of the cells was assessed by trypan blue exclusion test. Cell suspension was mixed with 0.4% trypan blue solution for 5 minutes. Then, the cells were counted by using hemocytometer and light microscope. The viable cells were unstained whereas the dead cells were stained blue. Percentage of viability was calculated from the number of unstained cells/(the number of unstained cells+the number of blue stained cells).

5. Apoptosis assay

To investigate cell-undergone apoptosis, measuring membrane redistribution of phosphatidylserine was assessed using an annexin V-fluorescein-5-isothiocyanate (FITC) apoptosis detection kit according to the manufacturer's protocol. Cell suspension ($1x10^5$ cells) was washed once with cold PBS, and the cell pellets were

suspended in 400 μL of diluted binding buffer, then, treated with 2 μL of annexin V-FITC and 2 μL of propriodium iodide (PI) for 10 minutes on ice in the dark. After that, the cells were analyzed with the Epics Elite ESP flow cytometer or FACScan flow cytometer. Annexin V-positive cells was apoptotic cells.

6. Erythroid colony-forming assay by plasma clot

To determine the colony-forming capacity, the day 7 ECFCs were cultured in serum-free liquid cultures as indicated, and plasma clot assays were performed as described (8, 71). One mL of cultured medium containing IMDM, 20% FCS, 1% BSA, 10 ng/mL SCF, 2 U/ml rhEPO, 10% citrated human AB plasma and 600 ECFCs were seeded on 3 plates of 35-mm culture dishes and incubated at 37°C in a high-humidity, 5% CO_2, 95% air incubator for 7 days. After fixation, the colonies were stained with 3,3' dimethoxybenzidine. The colonies of 8 or more hemoglobinized cells represented original CFU-E, and aggregated consisting of 2 to 7 hemoglobinized cells were defined as small erythroid clusters. Colonies consisting of 8 to 19 and 20 to 49 of hemoglobinized cells were referred as medium and large erythroid colonies, respectively (175).

7. Determination of NF-κB expression by Western blotting

To examine NF-κB protein translocation from the cytoplasm to the nucleus in ECFCs, nuclear and cytoplasmic fractions were prepared as described (177, 178), and analyzed by Western blotting. Day 7 ECFCs ($1.0\text{-}2.0\text{x}10^6$) were washed once with PBS, and the cell pellets were suspended in 400 μL of cold cell lysis, on ice for 15 minutes, and then 25 μL of 10% NP-40 was added. Homogenates were spun at 3,000 rpm, 4^0C for 1 minute and supernatants were collected as the lysed cytoplasm fractions. The pellets were suspended in 25-50 μL of cold nuclear extraction buffer, and were then incubated on ice for 15 minutes with intermittent mixing. The supernatants were collected as nuclear lysates. For whole cell lysate preparation, Day 7 ECFCs ($1.0\text{-}2.0\text{x}10^6$) were washed once with PBS, and the cell pellets were suspended in 400 μL of cold whole cell lysis buffer, vortex for 15 seconds, then spun at 10,000 rpm, 4^0C for 10 minutes and supernatants were collected as the whole cell lysate.

Three volume of each lysate and one volume of 3x sample buffer were well mixed, then stored at -20°C until analysis.

Whole cell lysate, cytoplasmic and nuclear lysates were boiled for 5 minutes, cooled down and spun at 10,000 rpm at 4^0C for 5 minutes. The protein concentration of each sample was quantitated using the Bio-Rad protein assay kit. Samples containing 40 μg of protein were loaded on each lane of a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in PBS containing 0.1% Tween 20 (PBST) with 5% skim milk at 4^0C overnight, and followed by three washes with PBST. The membranes were then incubated in PBST containing 5% skim milk with anti-p65 (C-20) antibody (1:1,000), as the primary antibody, at room temperature for 2 hours, followed by three washes with PBST. The membranes were incubated in PBST containing 5% skim milk with horseradish peroxidase (HRP) conjugated anti-rabbit Ig G antibody (1:2,000) as a secondary antibody at room temperature for 40 minutes, followed by three washes with PBST. Specific signals were detected on x-ray films using an enhanced chemiluminescence detection system. To remove the antibodies, the membranes were incubated in 0.0625 mol/L Tris-HCl (pH 6.8) containing 2% SDS at 50°C for 1 hour and reblotted first with mouse anti-actin clone AC-74 antibody (1:2,000) as the primary antibody, and then with HRP conjugated with goat-anti mouse Ig2a (1:4,000) as the secondary antibody. Specific signals were detected as described above. K562 cell lysate was used as a positive control.

8. NF-κB assay using confocal microscopy

To confirm NF-κB protein translocation from cytoplasm to nucleus in ECFCs, cells were processed and analyzed by immunocytochemistry as described (179, 180). Day 7 ECFCs ($1x10^4$- $1x10^5$) were washed with PBS containing 0.9 mM $CaCl_2$ and 0.33 mM $MgCl_2$, and deposited on slides by cytocentrifugation. The cells were fixed with the vapour of formaldehydye for 30 minutes, permeabilised with 0.2% triton-X, and blocked with PBS containing 10% FCS at room temperature for 2 hours. The samples were then incubated with PBS containing anti-NF-κB (p65) antibody (1:50) at 4^0C overnight, incubated with PBS containing 0.1% BSA with rhodamine-

conjugated with donkey anti-rabbit Ig G antibody (1:50) for 1 hour at room temperature in the dark, and then the cells were analyzed by Leica DM Irbe confocal microscope or Bio-Rad confocal microscope.

9. Determination of Bcl-x expression by Western blotting

Expressions of Bcl-x were analyzed by Western blotting as described (71). Briefly, Day 7 ECFCs ($1x10^6$) were lysed in cell lysis buffer. Protein concentrations were determined using the Bio-Rad protein detection kit. Twenty µg of cellular protein was separated on 12.5% SDS-PAGE and blotted to a PVDF membrane. The Bcl-x and actin proteins were detected on the same membrane by incubation in PBST containing 5% skim milk with anti-Bcl-x antibody (1:1,000) and anti-actin clone AC-74 antibody (1:2,000) as a primary antibody at 26°C for 1 hour. The membranes were then incubated with HRP conjugated rabbit Ig G (1:2,000) and HRP conjugated with goat-anti mouse Ig2a (1:4,000) as a secondary antibody, respectively, at 26°C for 1 hour. Specific signals were detected by the ECL system and K562 cell lysate was used as a positive control.

10. Analysis of $\Delta\Psi_m$ and ROS production of mitochondria by flow cytometry

$\Delta\Psi_m$ and ROS production of mitochondria were monitored by flow cytometry using 3,3'-dihexyloxacarbocyanine ($DiOC_6$) and hydroethidine (HE), respectively, as described (45, 48, 59). Day 7 ECFCs (5.5×10^5) were incubated with 40 nM of $DiOC_6$ and 2 µM of HE at 37°C for 15 minutes in a high-humidity, 5% CO_2, 95% air incubator. Cells were centrifuged at 3,000 rpm for 5 minutes, and then resuspended in 500 µL of PBS, followed by analysis on the flow cytometer. The cells with low ΔΨm and high ROS, which were defined to apoptotic cells, were investigated.

11. Statistical analysis

Arithmetic means and standard deviations were calculated. The t-test was used to determine significant differences between the groups. The differences were considered significant if $p<0.05$.

CHAPTER 5
RESULTS

1. Erythropoiesis assessment

Erythroid colony forming cells were purified from peripheral blood of a healthy normal and 3 β-thalassemia/Hb E patients by negative selection and cultured in serum medium. The cell number was counted at the indicated time points (Table 10) and the increasing number of the cells was calculated from total erythroid cell numbers/starting cell number at day 0, 3, 7, 10, 13 and 16. Figure 18 showed the fold increase in cell numbers during *in vitro* erythroid culture in normal and β-thalassemia/Hb E. In normal, about 40-fold increase in cell numbers was seen at day 7 and the peak was reached, over 150-fold increase by day 10. Thereafter, cell numbers were relatively constant. The total increase in the numbers of erythroid cells was correlated with the patient's severity. The one who was milder with higher Hb level (8.8 g/dL) had more increased in the number of erythroid cells 10-fold expansion at day 7 to the peak of 140 fold increase by day 13. While two more severe cases with less Hb level (6 and 6.2 g/dL) had only 40 and 20 fold increased of erythroid cells at day 10 from the 20 and 10 fold increased at day 7, respectively. In addition, some day 7 thalassemic erythroid precursor cells in serum culture showed pyknotic nucleus, a small nuclear fragment, or vacuole in the cytoplasm (Figure 19).

2. Cyclosporine A induced apoptosis of normal ECFCs

To study the effect of CsA on apoptosis of normal erythroid progenitor cells in the presence of EPO or IFN-γ, the effect of EPO or IFN-γ on apoptosis of ECFCs in serum-free medium was first verified (Table 11 and Figure 20). Dose-dependent effects of EPO (0.1 0.5, 1, 10 U/mL) (Figure 20A) and IFN-γ (0.1, 1, 10, 100, 1,000 U/mL) (Figure 20B) on reduction of ECFC apoptosis were evident. The results showed that 10 U/ml of EPO and 1,000 U/mL of IFN-γ retarded apoptosis to the same

level, 29.7±9.5% and 29.3±4.6%, respectively (mean ± SD of four replicates of two independent experiments). Thus, dose effect of CsA for induction of apoptosis of ECFCs was evaluated in the presence of 10 U/mL EPO or 1,000 U/mL IFN-γ. In the absence of EPO or IFN-γ, addition of CsA did not increase the percentage of annexin V-positive cells (Figure 21A). A dose-dependent effect of CsA (0, 5, 10, 50 and 100 μM) on apoptosis of ECFCs in the presence of EPO was demonstrated (Table 12 and Figure 21B). However, a less effect of CsA was observed in the presence of IFN-γ (Figure 21C).

ECFCs were also treated with or without 10 μM CsA at 37^0C for 30 minutes, and cultured in the absence of cytokine or in the presence of 10 U/mL EPO or 1,000 U/mL IFN-γ for 16 hours before plasma clot assays were performed. A marked reduction of number and size of erythroid colonies was induced in the CsA-treated cells cultured in the presence of EPO, whereas the CsA-treated cells cultured in the presence of IFN-γ showed less effect (Table 13 and Figure 22B).

3. Cyclosporine A induced apoptosis of thalassemic ECFCs

Whether the optimum condition that CsA produced apoptosis of thalassemic erythroid progenitor cells in the presence of EPO or IFN-γ, the effects at various concentrations of EPO (0.1 0.5, 1, 10 U/mL) or IFN-γ (500, 1,000 and 2,500 U/mL) on survival of ECFCs in serum-fee medium was first verified at various times (16, 24, 48 and 72 hours) (Tab 14 and Figure 23). The viability of ECFCs was maintained by EPO as dose- and time-dependent manners (Figure 23A). IFN-γ maintained cell viability as dose-independent and time-dependent manners (Figure 23B). Although the survival of ECFCs was reduced with increasing times, 10 U/mL and 20 U/mL were the maximum dose of EPO to maintained survival of ECFCs. In contrast, 500 U/mL to 2,500 U/ml of IFN-γ could maintain survival of ECFCs at the same level at any indicated times. The surviaval of ECFCs was maintained about 90% in the presence of EPO 10 U/mL and about 85% in the presence of IFN-γ 1,000 U/mL, and both conditions showed clear different effect when compared with no cytokine at 24 hours incubation. Thus, EPO 10 U/mL and IFN-γ 1,000 U/mL were selected to study effect of CsA (5, 10, 50 and 100 μM) at varying times (16, 24, 48 72 hours) (Table 15 and

Figure 24). This study demonstrated that both cytokines had a dose- and time-dependent effect on survival of thalassemic ECFCs. Ten μM of CsA treatment for 24 hours showed that EPO and IFN-γ could maintain survival of ECFCs at the same level (about 65% for EPO and 61% for IFN-γ). However, after 24 hours, without CsA treatment, EPO protected cell death more than IFN-γ about 10%.

To confirm that CsA reduced survival of thalassemic ECFCs by apoptotic induction, ECFCs were treated with various concentration of CsA (5, 10, 50 and 100 μM), and cultured in the presence of EPO 10 U/ml or IFN-γ 1,000 U/ml for 24 hours before apoptotic assessment. The result showed that CsA induced apoptosis of thalassemic ECFCs as dose-dependent manner in the presence of EPO (Table 16 and Figure 25A), not in the presence of IFN-γ (Table 16 Figure 25B). Apoptosis of normal and thalassemic ECFCs, which treated with or without CsA10 μM and cultured with or without EPO 10 U/mL or IFN-γ 1,000 U/mL, were studied in more samples. Table 17 and Figure 26 show the percentage of control of annexin V-positive cells in normal and thalassemia. The percentage of control of annexin V-positive cells in each experiment were calculated from 100% control, which referred to the percentage of annexin V-positive cells of CsA-untreated cells cultured with EPO. The result showed that apoptosis of thalassemic ECFCs was significant higher than that of normal ECFCs, 274.0$\pm$29.0 %control for thalassemia and 210.3$\pm$10.0 %control (Figure 26 and Table 17). However, the significant difference of apoptosis between CsA-treated and CsA-untreated cells, cultured in the presence of EPO or IFN-γ, was only found in normal but not in thalassemia (Figure 26).

4. Effect of CsA on expression and the localization of NF-κB in normal and thalassemic ECFCs

Apoptosis induction by CsA was found in normal and thalassemia. An involvement of NF-κB in the apoptosis of erythroid progenitor cells was first investigated in normal peripheral blood. Day 7 ECFCs were treated with 10 μM of CsA at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL for 24 hours. Western blot analysis showed that, in all conditions, the amount of NF-κB (p65) in whole cell lysate at 16 hours was more than that

determined at 24 hours culture. The detected amount of NF-κB might result from the remaining viable cells because the amount of NF-κB was much decreased in the condition without cytokine at 24 hours (Figure 27A, lanes 2 and 5). However, in the presence of EPO or IFN-γ, the amount of NF-κB was still prominent.

Whether NF-κB activation involved in apoptotic retardation of EPO or IFN-γ, the amount of NF-κB in the cytoplasmic and the nuclear fractions were analysed. Without any cytokine, NF-κB was detected only in the cytoplasmic extract (Figure 27B, lane 1 compared with lane 4). In the presence of EPO, NF-κB was found both in the cytoplasmic and the nuclear lysates of ECFC (Figure 27B, lanes 2 and 5), and the amount in the nuclear lysate was reduced by CsA treatment (Figure 27B, lane 3 compared with lane 6). In contrast, when ECFCs were cultured with IFN-γ, NF-κB was found only in the cytoplasmic lysate (Figure 27B, lanes 7 and 8). In addition, the intensity of NF-κB in the cytoplasmic and the nuclear fractions of cell cultured with EPO was more than that in IFN-γ condition. This suggested that the expression of NF-κB in ECFCs cultured with EPO should be greater than that in ECFCs cultured with IFN-γ. The expression of NF-κB was further analysed by confocal microscopy. As shown in Figure 28B, a high intensity of rhodamine dye was present in ECFCs cultured with EPO. This indicated that large amounts of NF-κB were present in the cytoplasm and nucleus. CsA-treatment resulted in a decreased intensity of the dye both in the cytoplasm and the nucleus (Figure 28E). ECFCs cultured without cytokine (Figures 28A and 26D) or with IFN-γ (Figures 28C and 28F) also showed less intensity of rhodamine stain in the cytoplasm and nucleus as compared with cells cultured with EPO.

Whether EPO retarded apoptosis of thalassemic erythroid progenitor cells via NF-κB involvement, day 7 ECFCs were treated with 10 and 50 μM of CsA and cultured without cytokine or with EPO 10 U/mL for 24 hours before investigation by confocal microscopy (Figure 29). A high intensity of rhodamine dye was present in ECFCs cultured with EPO (Figures 29A and 29B) indicating that the large amounts of NF-κB were present in the cytoplasm and the nucleus. Treatment with CsA 50 μM resulted in a marked decrease in intensity of the dye, both in the cytoplasm and the nucleus (Figures 29E and 29F). This indicated that CsA reduced the expression of NF-

κB in thalassemic erythroid progenitor cells in the presence of EPO. ECFCs cultured with IFN-γ showed less intensity of rhodamine stain in the cytoplasm and nucleus as compared with cells cultured with EPO (Figure 29G). ECFCs treated with CsA 50 μM before culturing cells with IFN-γ showed rhodamine stain only in the cytoplasm. This indicated that NF-κB was present in the cytoplasm, not in the nucleus.

5. Effect of CsA on Bcl-x expression in normal ECFCs

To evaluate an involvement of Bcl-x in the regulation of apoptosis of normal ECFCs via NF-κB, day 7 ECFCs were treated with 10 μM of CsA and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 u/mL for 24 hours. Then, the expression of Bcl-x was investigated by Western blotting. To standardise the amount of Bcl-x expression, Bcl-x/actin ratio was further determined by NIH image software. The level of Bcl-x expression was increased in the cells cultured with EPO (0.56) (Figure 30, lane 2), not in the cells cultured with IFN-γ (0.32) (Figure 30, lane 4), as compared with that in the cells cultured in the absence cytokine (0.33) (Figure 30, lane 1). The level of Bcl-x expression of CsA-treated cells were decreased in cells cultured with EPO (0.34) (Figure 30, lane 6), and without cytokine (0.17) (Figure 30, lane 5) but not in the cells cultured with IFN-γ (0.33) (Figure 30, lane 8). Two independent experiments showed that Bcl-x expression was only increased in cells cultured with EPO, not in IFN-γ (Table 18). This means that EPO and IFN-γ induced the expression of Bcl-x via different mechanism.

6. CsA disturbed ΔΨm and ROS production in normal ECFCs

Mitochondrial function also plays role in apoptosis of the cells. ΔΨm and ROS production was then investigated. Day 7 ECFCs were treated with 10 μM of CsA and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL for 24 hours, and ΔΨm plus ROS were then analysed by using $DiOC_6$ and HE, respectively. Figure 29A represents a typical histogram from three independent experiments. Figure 31B shows mean $\pm$ SD of the percentages of the cells with low ΔΨm and high ROS, which defined as apoptotic cells, in cells cultured with EPO (45.7$\pm$9.5%) and IFN-γ (37.6$\pm$10.1%) were significantly decreased as compared with no cytokine

(85.4±6.7%). CsA-treated cells also showed the same pattern: 60.3±6.4% for EPO, 52.4±19.1% for IFN-γ, and 83.6±3.9% for no cytokine. The significant difference of the cells with low ΔΨm and high ROS was found between cells cultured with cytokine and without cytokine, but not found between CsA-treated and CsA-untreated cells (Figure 31B).

Table 10 The number of normal and thalassemic erythroid-derived cells *in vitro* during 16 days culture.

Days of erythroid culture	Normal *	Thalassemia		
		Subject 1*	Subject 2*	Subject 3*
0	$1.55x10^5$	$1.75x10^5$	$7.0x10^5$	$1.99x10^5$
3	$1.75x10^5$	$1.0x10^5$	$5.33x10^5$	$1.75x10^5$
7	$59.23x10^5$	$21.49x10^5$	$168x10^5$	$23.32x10^5$
10	$239.43x10^5$	$135.68x10^5$	$254.52x10^5$	$7.28x10^5$
13	$240.75x10^5$	$241.0x10^5$	$217.42x10^5$	ND
16	$223.5x10^5$	$204.0x10^5$	ND	ND

ND = not done

*Hb levels were 13.6 g/dL for normal, 8.8 g/dL for subject 1, 6.0 g/dL for subject 2, 6.2 g/dL for subject 3.

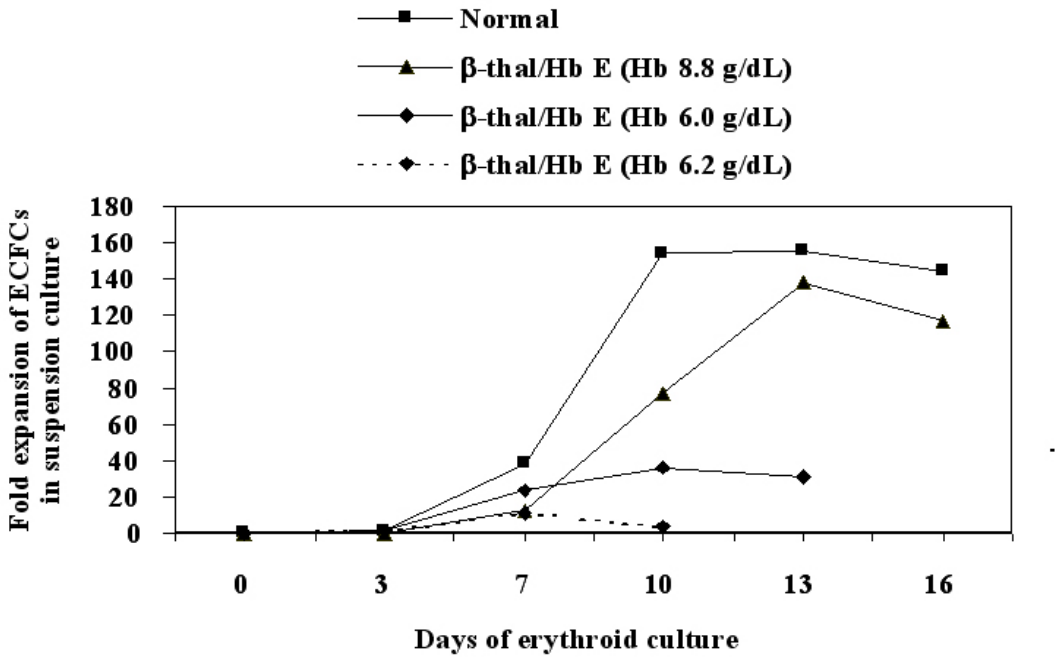


Figure 18 ***In vitro*** ineffective erythropoiesis of thalassemic erythroid progenitor cells from peripheral blood.

ECFCs of normal and thalassemic peripheral blood were cultured in the serum medium. The cells were counted every four days. Fold expansion was calculated from total cell number/starting cell number at serial time points. The graph shows maturation arrest of thalassemic erythroid cells indicating an ineffective erythropoiesis in thalassemia.

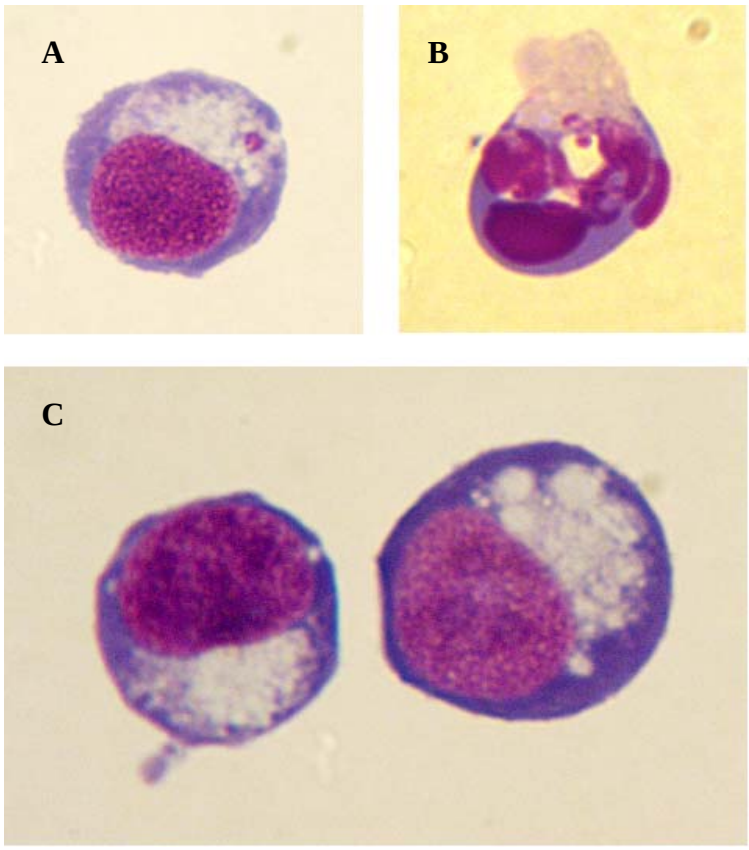


Figure 19 Abnormal morphology of day 7 thalassemic erythroblast in serum culture.

ECFCs from peripheral blood of β-thalassemia/Hb E were cultured in the medium containing containing 15% FCS; 15% pooled human AB serum, 2 U/mL rhEPO, 20 ng/mL SCF, 10 ng/mL rhIL-3, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37^0C in a high-humidity, 5% CO_2, 95% air incubator. The cells at day 7 were prepared by cytospin and stained with Wright's Giemsa. A) cell with nuclear fragment, B) cell with pyknotic nucleus, and C) cell with vacuole in cytoplasm.

Table 11 Anti-apoptotic effect of both EPO and IFN-γ on normal erythroid progenitor cells. Data represent mean ± SD of annexin V-positive cells (%) of four replicates of two independent experiments.

Cytokine	Concentration (U/mL)	Mean ± SD of annexin V-positive cell (%)
No cytokine	-	79.5 ± 6.2
EPO	0.1	53.7 ± 10.3
	0.5	43.9 ± 7.6
	1	35.0 ± 11.6
	10	29.7 ± 9.5
IFN-γ	0.1	72.7 ± 4.1
	1	66.8 ± 5.7
	10	38.5 ± 6.1
	100	28.0 ± 7.4
	1,000	29.3 ± 4.6

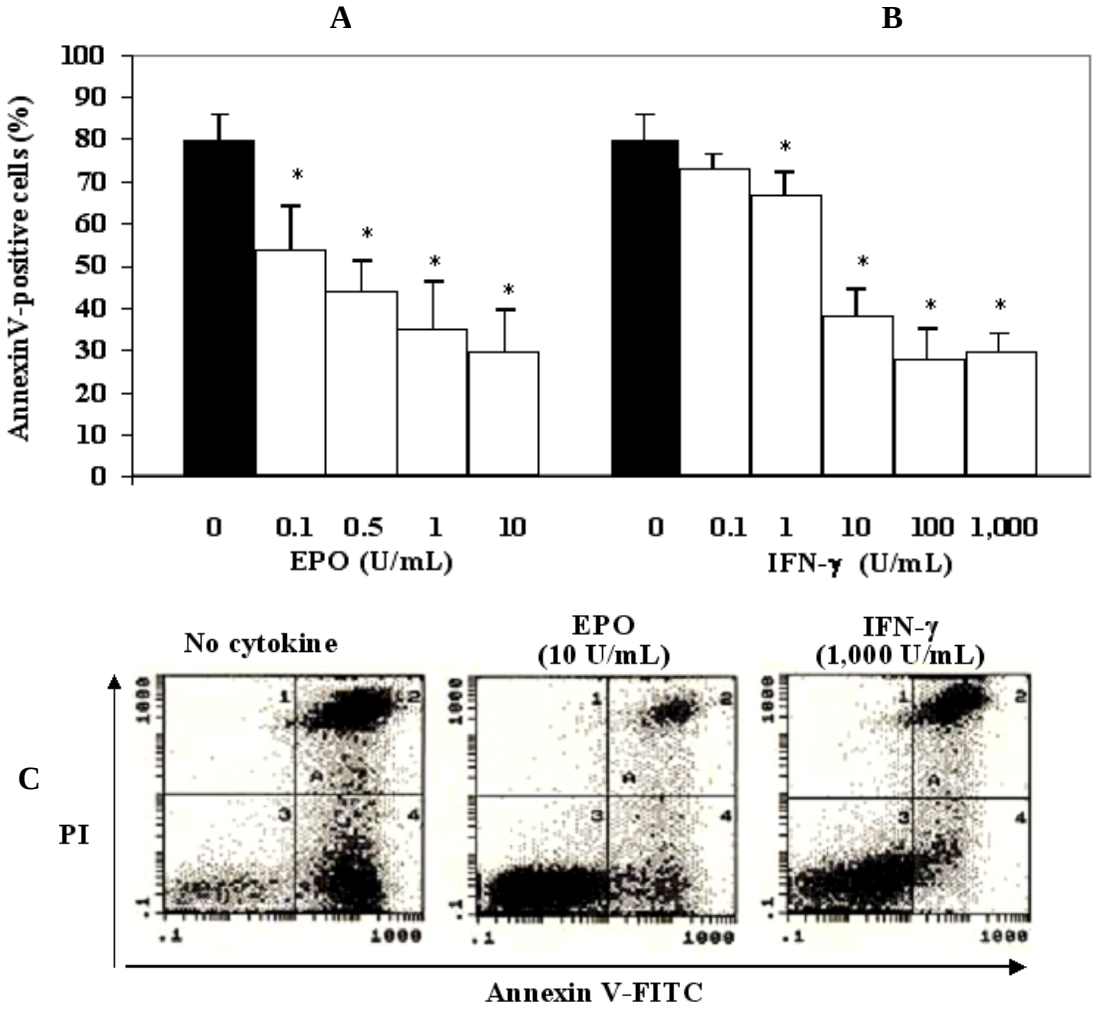


Figure 20 Anti-apoptotic effect of both EPO and IFN-γ on normal erythroid ECFCs.

Normal day 7 ECFCs in serum-free medium were cultured without cytokine or with various concentrations of EPO (0.1, 0.5, 1 and 10 U/mL) or IFN-γ (0.1, 1, 10, 100, and 1,000 U/mL) at 37^0C for 24 hours. Both EPO (Figure 20A) and IFN-γ (Figure 20B) resulted in apoptotic retardation as dose-dependent manner. Figure 20A show mean $\pm$ SD of two independent experiments. Figure 20C represents some histograms of apoptotic cells, which were detected by flow cytometer using annexinV-FITC and PI. Asterisk indicated the significant difference (p<0.05) between cells cultured with and without cytokine.

Table 12 Effect of CsA on apoptotic induction of normal ECFCs. Data represent mean ± SD of annexin V-positive cells (%) of four replicates of two independent experiments.

Cytokine	CsA concentration (μM)	Mean ± SD of annexin V-positive cell (%)
No cytokine	0	82.5 ± 4.0
	5	79.8 ± 5.1
	10	80.3 ± 9.6
	50	77.1 ± 1.1
	100	75.9 ± 12.4
EPO 10 U/mL	0	29.6 ± 2.4
	5	56.2 ± 4.1
	10	51.0 ± 11.7
	50	58.4 ± 9.1
	100	74.7 ± 3.4
IFN-γ 1,0 00 U/mL	0	41.9 ± 8.8
	5	45.7 ± 4.2
	10	46.2 ± 5.7
	50	49.3 ± 6.3
	100	56.0 ± 4.1

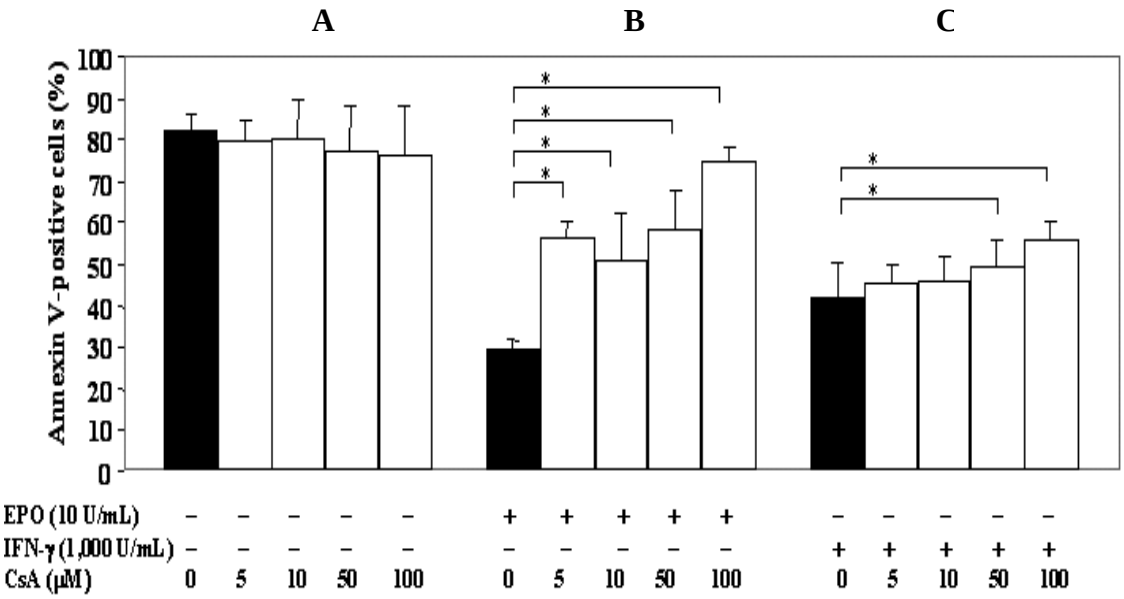


Figure 21 Effect of CsA on apoptotic induction of normal ECFCs.

Normal day 7 ECFCs were treated with various concentrations of CsA (0, 5, 10, 50 and 100 μM) at 37^0C for 30 minutes, then cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 24 hours. Dose-dependent effect of CsA on apoptosis of ECFCs in the presence of EPO was clearly demonstrated (Figure 21B), whereas there was a lower effect in the presence of IFN-γ (Figure 21C), and no effect in the absence of cytokine (Figure 21A). Mean $\pm$ SD of each bar represents data from four replicated of two independent experiments. Asterisk indicated the significant difference (p<0.05) between CsA-untreated and CsA-treated cells in the presence of EPO or IFN-γ.

Table 13 CsA reduced colony formation capacity of normal ECFCs. Data represent mean ± SD of a triplicate experiment.

Cytokine	CsA 10 μM	Small cluster	Medium colony	Large colony
No cytokine	-	13.7 ± 1.5	3.0 ± 1.0	2.7 ± 2.1
	+	1.0 ± 0	1.7 ± 0.6	0.3 ± 0.6
EPO 10 U/mL	-	43.7 ± 2.1	31.3 ± 2.1	7.7 ± 2.5
	+	1.7 ± 2.1	1.7 ± 0.6	0 ± 0
IFN-γ 1,000 U/mL	-	56.3 ± 5.5	18.0 ± 5.3	2.3 ± 1.5
	+	34.3 ± 6.7	12.0 ± 3.0	2.7 ± 2.1

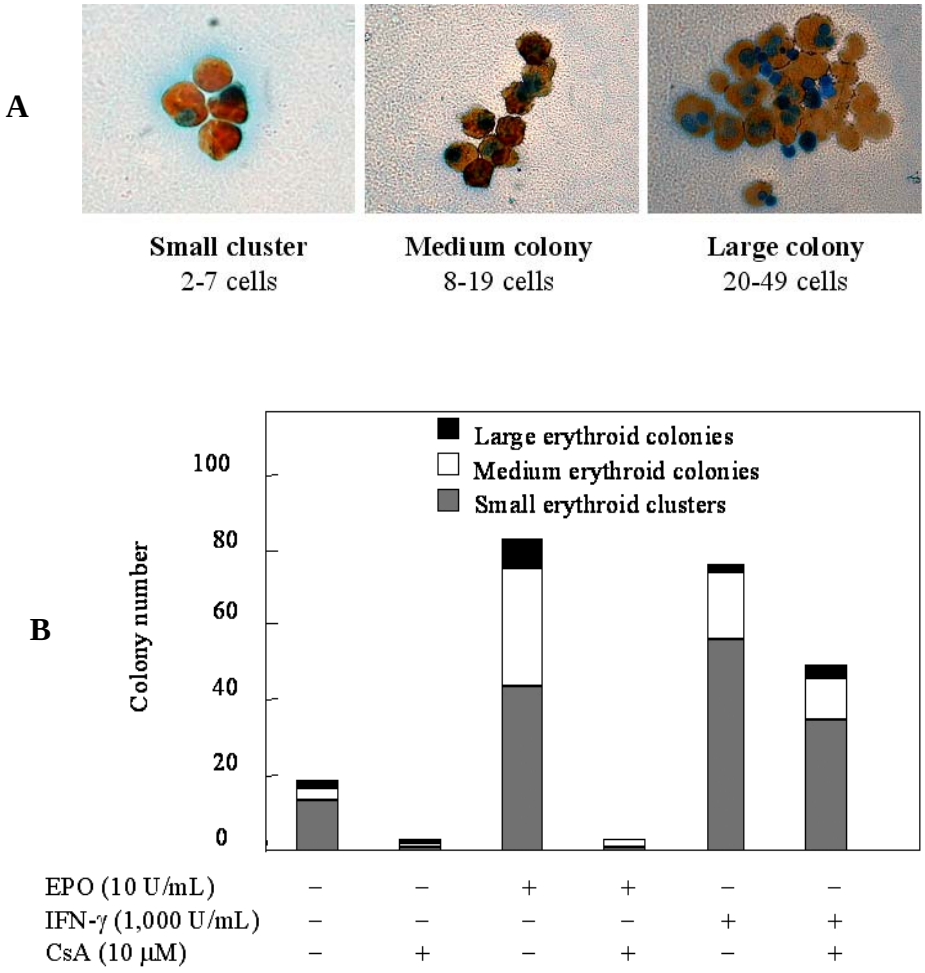


Figure 22 CsA reduced colony formation capacity of normal ECFCs.

Plasma clot assay was performed after normal day 7 ECFCs were treated without or with CsA 10 μM at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 16 hours. The cells were seeded in triplicate and incubated at 37°C for 7 days. Hemoglobin-positive colonies were examined by staining with 3,3' dimethoxybenzidine and hematoxylin, and then colony-forming unit-erythroid (CFU-E) were observed and defined as in Figure 22A. CsA reduced the colony formation capacity of ECFCs in the present of EPO more than that in the presence of IFN-γ as shown in Figure 22B. Figure 22B shows a representative result of two independent experiments.

Table 14 Effect of EPO and IFN-γ on viability of thalassemic ECFCs during 72 hours culture. Data represent mean ± SD of cell viability (%) of a duplicate experiment.

Cytokine	Concentration (U/mL)	Time (hours)			
		16	24	48	72
No cytokine	-	86.5 ± 3.3	63.4 ± 8.8	31.7 ± 5.9	28.2 ± 3.0
EPO	0.5	84.2 ± 0.6	82.9 ± 1.2	64.5 ± 6.9	55.7 ± 5.8
	1	91.8 ± 0.6	90.9 ± 2.6	67.3 ± 2.6	55.0 ± 2.9
	10	90.8 ± 0.6	95.1 ± 0.7	79.1 ± 8.2	71.7 ± 3.5
	20	91.1 ± 2.5	91.9 ± 1.5	83.1 ± 1.0	68.3 ± 2.7
IFN-γ	500	91.4 ± 1.7	86.0 ± 4.0	68.8 ± 1.6	52.8 ± 0.4
	1,000	92.9 ± 4.8	89.0 ± 1.8	76.0 ± 3.5	56.7 ± 6.6
	2,500	89.9 ± 0.5	85.5 ± 8.9	68.7 ± 5.8	56.4 ± 6.8

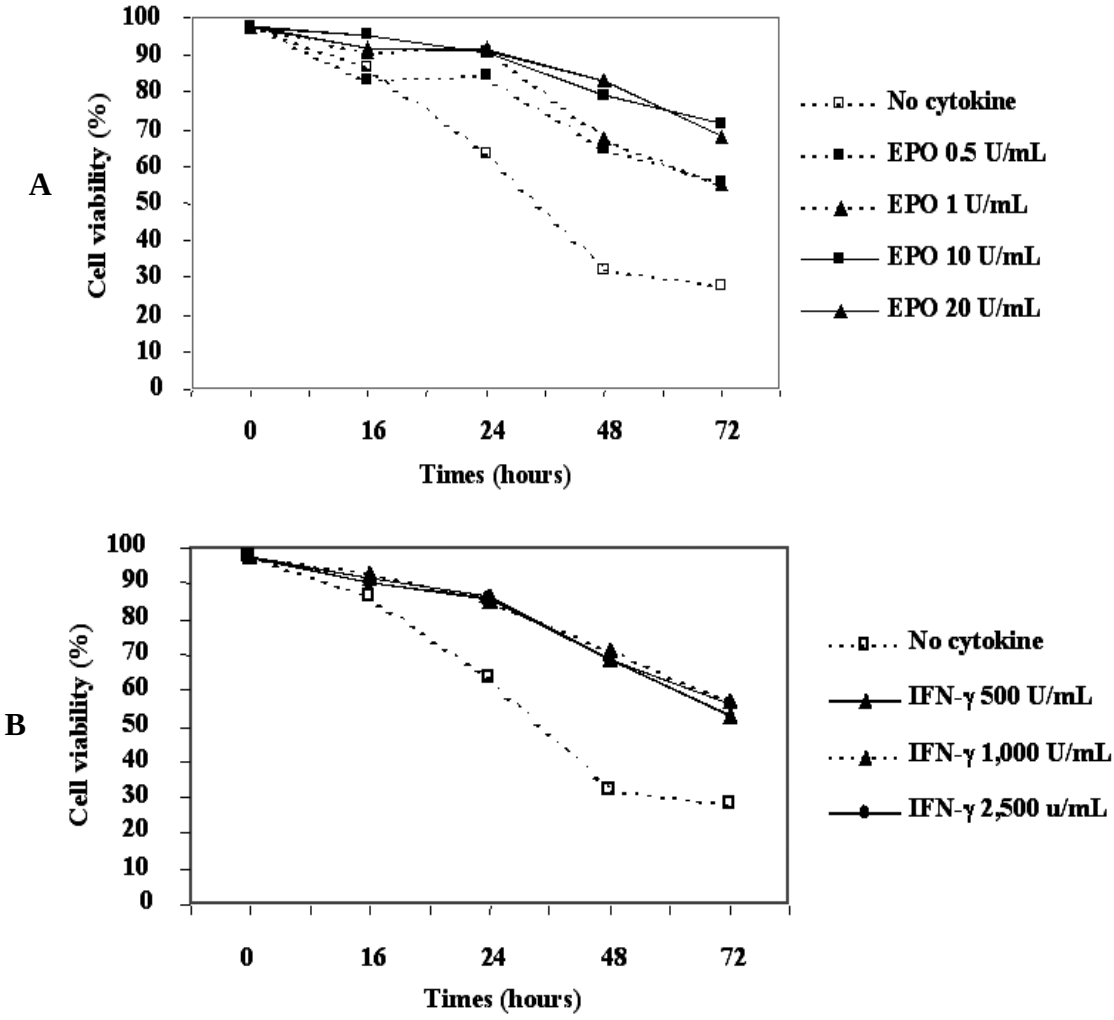


Figure 23 Effect of EPO and IFN-γ on viability of thalassemic ECFCs during 72 hours culture.

Thalassemic day 7 ECFCs in serum-free medium were cultured without cytokine or with various concentrations of EPO (0.1, 0.5, 1 and 10 U/mL) or IFN-γ (0.1, 1, 10, 100, and 1,000 U/mL) at 37^0C for 16, 24, 48 and 72 hours. Cell viability was determined at each time point. Both EPO and IFN-γ protected cell death as dose- and time-dependent manner as shown in Figures 23A and 23B, respectively. Figures 23A and 23B showed mean of duplicated data of the same sample.

Table 15 Effect of CsA on cell viability of thalassemic ECFCs during 72 hour culture. Data represent mean ± SD of cell viability (%) of a duplicates experiment.

Cytokine	CsA concentration (μM)	Time (hours)			
		16	24	48	72
EPO	0	95.1 ± 2.5	90.8 ± 0.7	79.1 ± 8.2	71.5 ± 3.1
	5	82.6 ± 3.7	63.9 ± 9.6	40.7 ± 2.1	26.2 ± 2.6
	10	68.8 ± 2.9	64.4 ± 1.5	23.6 ± 5.9	19.8 ± 4.4
	50	53.4 ± 23	50.8 ± 1.1	9.9 ± 6.9	1.7 ± 2.4
	100	40.6 ± 4.4	32.9 ± 6.1	0 ± 0	0 ± 0
IFN-γ	0	89.5 ± 2.8	88.0 ± 4.4	66.8 ± 5.7	37.6 ± 2.7
	5	85.0 ± 7.1	71.4 ± 5.1	46.4 ± 6.4	19.4 ± 6.1
	10	68.1 ± 4.6	61.3 ± 7.5	32.4 ± 3.4	18.1 ± 7
	50	71.4 ± 6.7	60.5 ± 2.2	12.4 ± 6.1	0 ± 0
	100	46.0 ± 5.7	35.1 ± 4.2	4.5 ± 1.1	0 ± 0

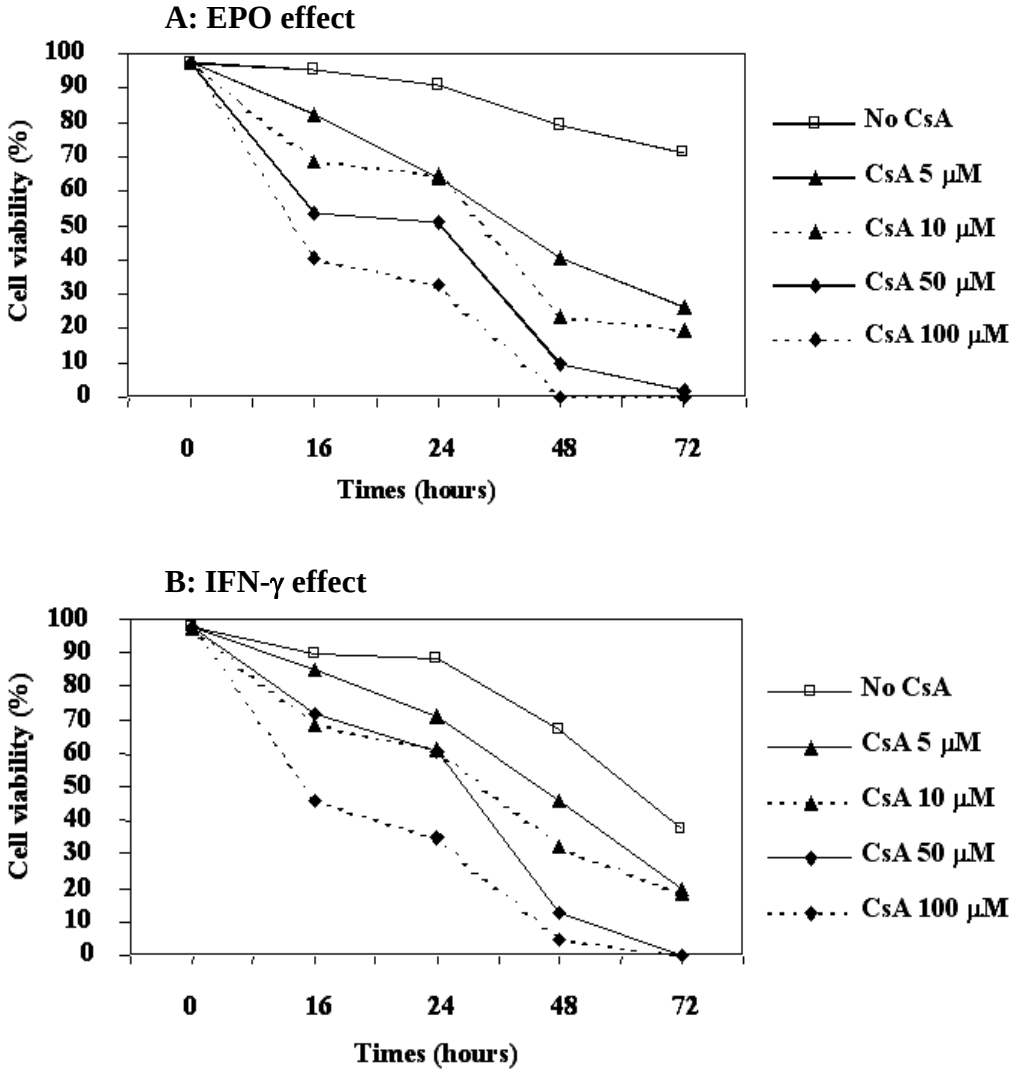


Figure 24 Effect of CsA on viability of thalassemic ECFCs during 72 hours culture.

Thalassemic day 7 ECFCs were treated with various concentrations of CsA at 37^0C for 30 minutes, then cultured with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 16, 24, 28 and 72 hours. CsA induced cell death as dose- and time-dependent manner in the presence of both EPO and IFN-γ as shown in Figures 24A and 24B, respectively. Figures 24A and 24B showed mean of duplicated data of the same sample.

Table 16 Effect of CsA on apoptotic induction of thalassemic ECFCs. Data represent mean ± SD of annexin V-positive cells (%) of a duplicates experiment.

Cytokine	CsA concentration (μM)	Mean ± SD of annexin V-positive cell (%)
No cytokine	0	52.1 ± 2.9
EPO 10 U/mL	0	16.1 ± 1.4
	5	30.9 ± 3.6
	10	38.3 ± 14.2
	50	47.9 ± 8.9
	100	52.4 ± 2.7
IFN-γ 1,0 00 U/mL	0	22.0 ± 6.1
	5	33.7 ± 9.1
	10	38.4 ± 5.0
	50	45.7 ± 6.8
	100	55.1 ± 8.5

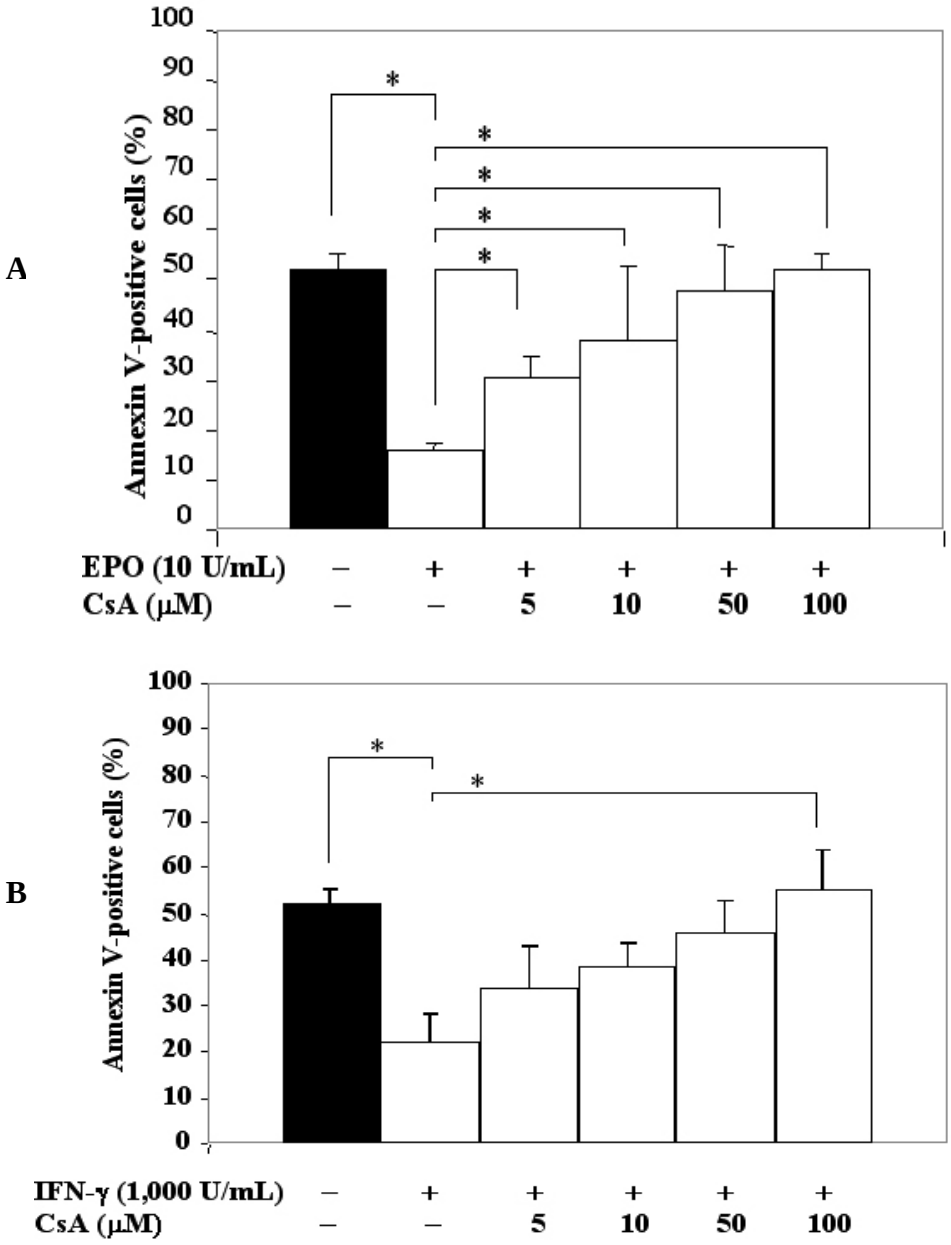


Figure 25 Effect of CsA on apoptotic induction of thalassemic ECFCs.

Thalassemic day 7 ECFCs were treated without or with various concentrations of CsA at 37^0C for 30 minutes, then cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 24 hours. Figures 25A and 25B show dose-dependent effect of CsA on apoptosis of ECFCs. Mean $\pm$ SD of each bar represents data from three independent experiments. Asterisk indicated the significant difference (p<0.05) between CsA-untreated and CsA-treated cells (*) and between cells cultured with and without cytokine (**).

Table 17 The relative apoptotic induction by CsA in normal and thalassemia. Data represent mean ± SD of annexin V-positive cells (%control) of 5 and 6 independent experiments in normal and thalassemia, respectively. The percentage of control of annexin V-positive cells in each experiment were calculated from 100% control, which referred to the percentage of annexin V-positive cells of CsA-untreated cells cultured with EPO.

Cytokine	CsA 10 μM	Normal	Thalassemia
No cytokine	-	210.3 ± 10.0	274.0 ± 29.0
	+	205.4 ± 32.4	240.9 ± 39.3
EPO 10 U/mL	-	96.0 ± 29.1	100.0 ± 22.0
	+	169.9 ± 24.0	123.1 ± 47.7
IFN-γ 1,000 U/mL	-	116.9 ± 27.1	129.1 ± 25.0
	+	157.9 ± 12.9	160.1 ± 34.5

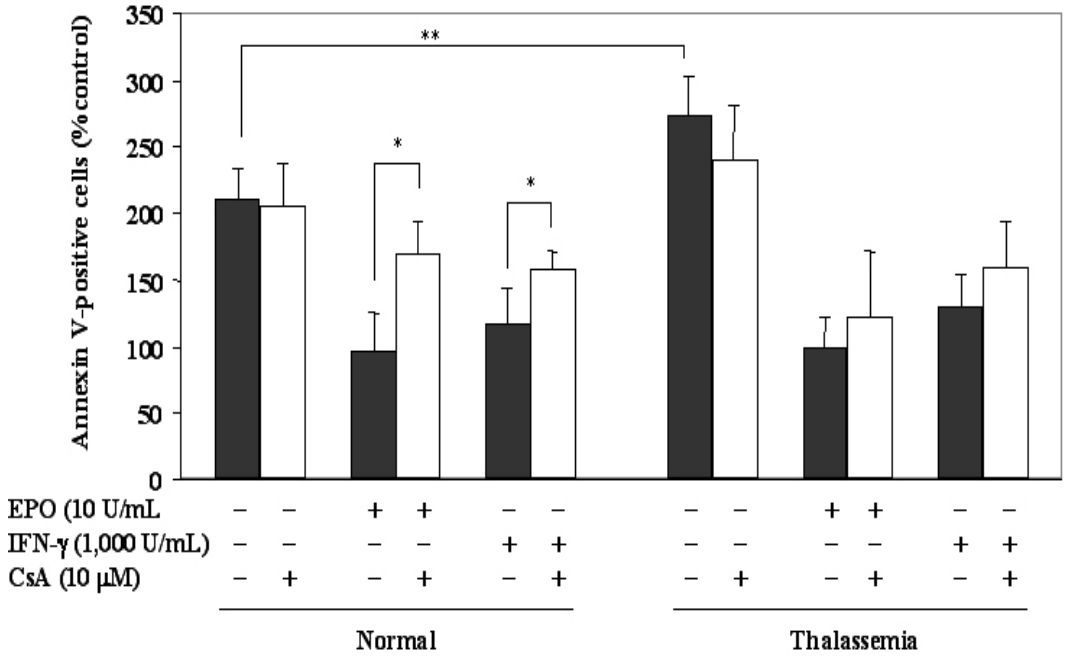
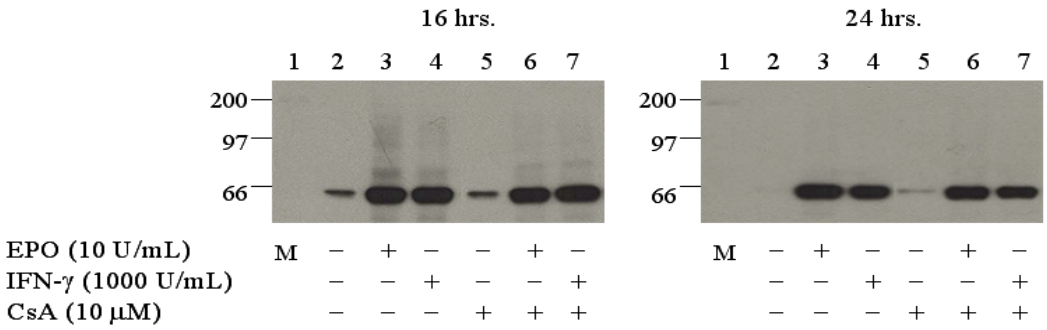


Figure 26 The relative apoptotic induction by CsA in normal and thalassemia.

Normal and thalassemic day 7 ECFCs were treated without or with CsA 10 μM at 37^0C for 30 minutes, then cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 24 hours. Annexin V-positive cells, which were detected from CsA-untreated cells cultured with EPO was defined to 100% control. Each value of annexin V-positive cells in each experiment was calculated from 100%control, that is, annexin V-positive cells (%control) = (%annexin V-positive cells in test condition x100)/ %annexin V-positive cells of CsA-untreated cell culture with EPO. Black bar represents without CsA treatment. White bar represents CsA treatment. Mean $\pm$ SD of %control of each bar represents data from independent experiments, 5 for normal and 6 for thalassemia. Asterisk indicated the significant difference (p<0.05) between CsA-untreated cells and CsA-treated cells (*) and between normal and thalassemic cells in the absence cytokine (**). This result showed significant apoptotic induction by CsA in the presence of EPO and IFN-γ in normal, not in thalassemia. In addition, thalassemia showed increased significant difference of apoptotic cells in the absence of cytokine, as compared with normal.

A: Whole cell lysate of ECFCs at 16 and 24 hours



B: Cytoplasmic and nuclear lysates of ECFCs at 24 hours

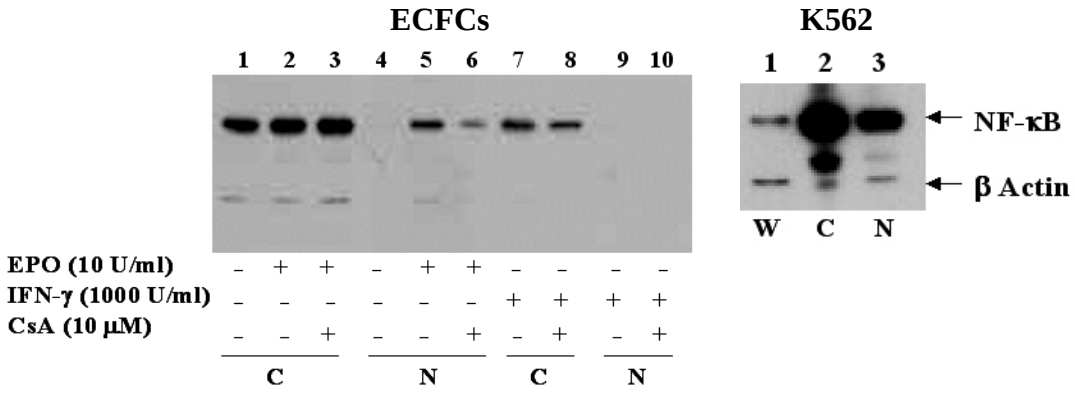


Figure 27 Effect of CsA on the expression of NF-κB in normal ECFCs.

Normal day 7 ECFCs were treated without or with CsA 10 μM at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 16 and/or 24 hours as indicated. Whole cell lysate or cytoplasmic and nuclear lysates were prepared, and NF-κB (p65) was determined by Western blot. Photographs illustrate the band intensity of NF-κB (p65) in whole cell lysate (W), cytoplasmic (C) and nuclear (N) lysates in each condition. Figure 27A shows NF-κB (p65) in whole cell lysate of ECFCs at 16 hours, which is more than that at 24 hours in all conditions. Figure 27B shows that CsA treatment reduced amount of NF-κB (p65) in nuclear lysate of ECFCs in the presence of EPO (lane 6). In contrast, in the presence of IFN-γ, NF-κB (p65) was detected only in cytoplasmic lysate of cells with or without CsA treatment (lane 7, 8). NF-κB (p65) in K562, a positive control, is shown in Figure 27B. The representative photographs show a result of two independent experiments.

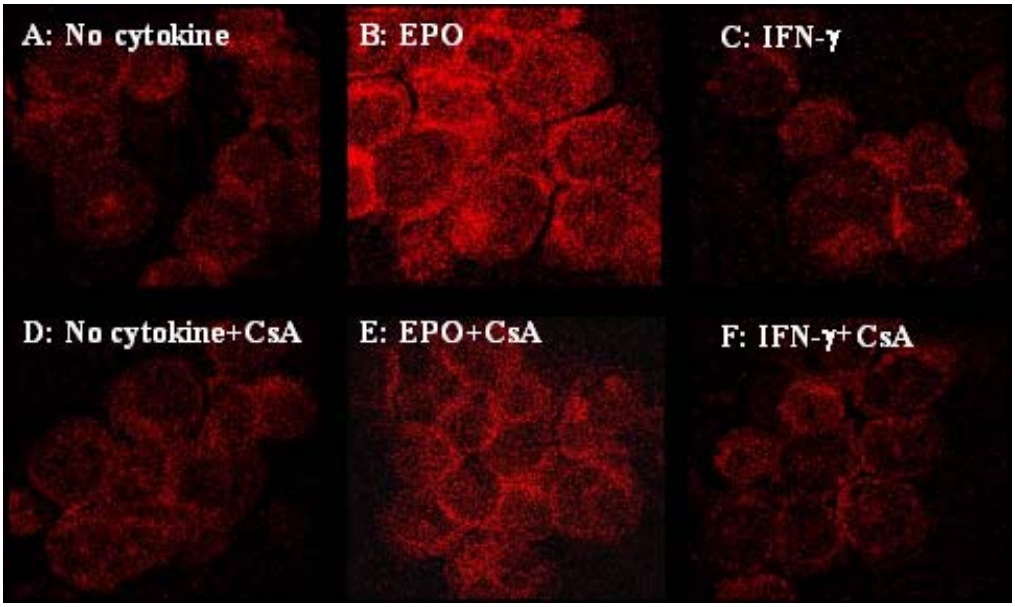


Figure 28 NF-κB expression in normal ECFCs.

Normal day 7 ECFCs were treated without or with CsA 10 μM at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 24 hours. Then, the cells were processed with anti-rabbit NF-κB (p65) polyclonal antibody and subsequently with donkey anti-rabbit IgG rhodamine conjugated for confocal microscope analysis. Photograph illustrates the intensity of NF-κB (p65) in cytoplasm and nucleus of the cells in each condition. CsA treatment reduced amount of NF-κB (p65) in ECFCs in the presence of EPO. The representative photograph shows a typical result of two independent experiments.

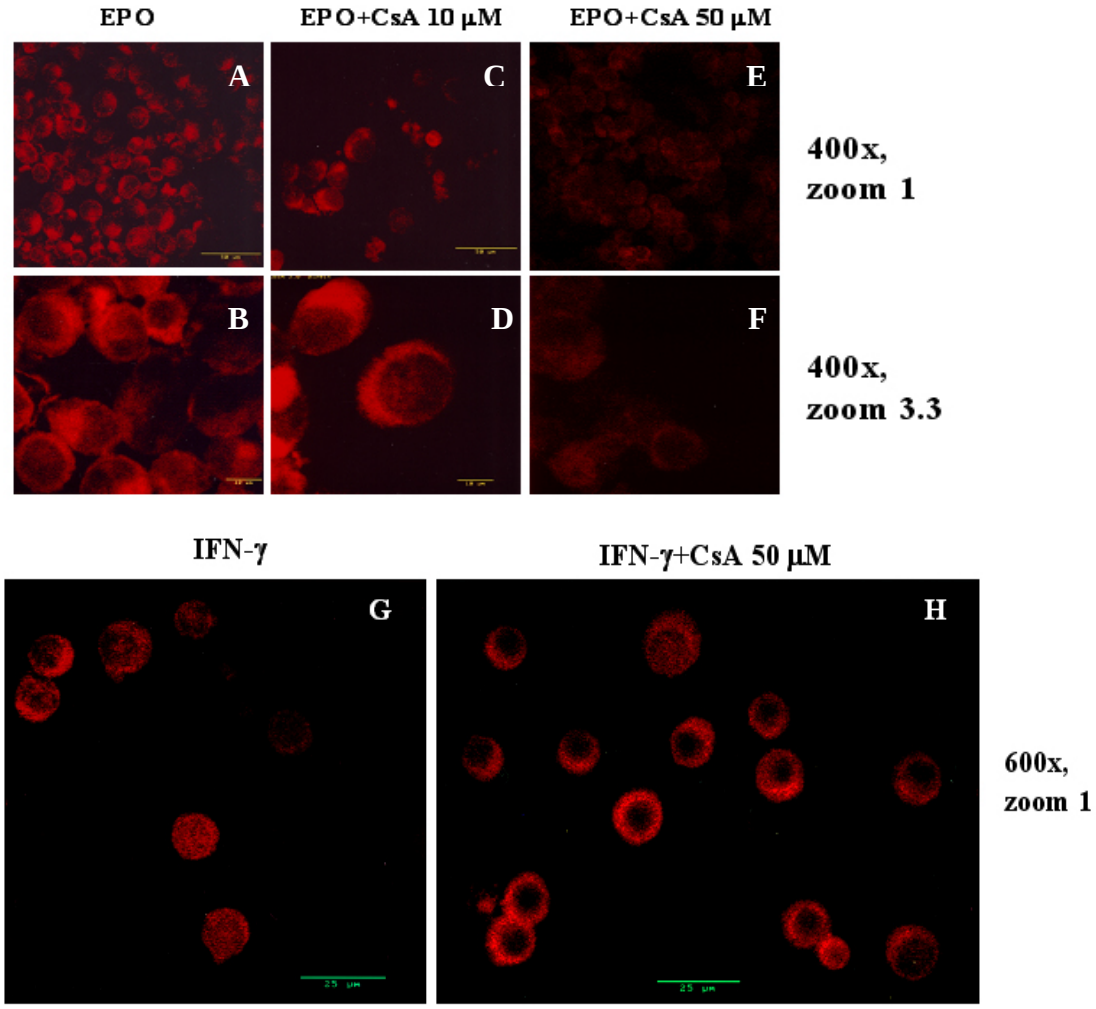


Figure 29 NF-κB expression in thalassemic ECFCs.

Thalassemic day 7 ECFCs were treated without or with CsA 10 and 50 µM at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL at 37^0C for 24 hours. Then, the cells were processed with immunocytochemistry staining and observed by confocal microscope. Photographs illustrate the intensity of NF-κB (p65) in cytoplasm and nucleus of the cells. In the presence of EPO, CsA reduced amount of NF-κB (p65) in the cells as dose-dependent manner. From the left to the right of the upper and middle panels show cells cultured with EPO (A, B), with EPO plus CsA 10 µM (C, D), and with EPO plus CsA 50 µM (E, F), respectively. From the left to the right of the lower panels show cells cultured with IFN-γ (G), with IFN-γ plus CsA 50µM (H), respectively. The representative photographs show a typical result of two independent experiments.

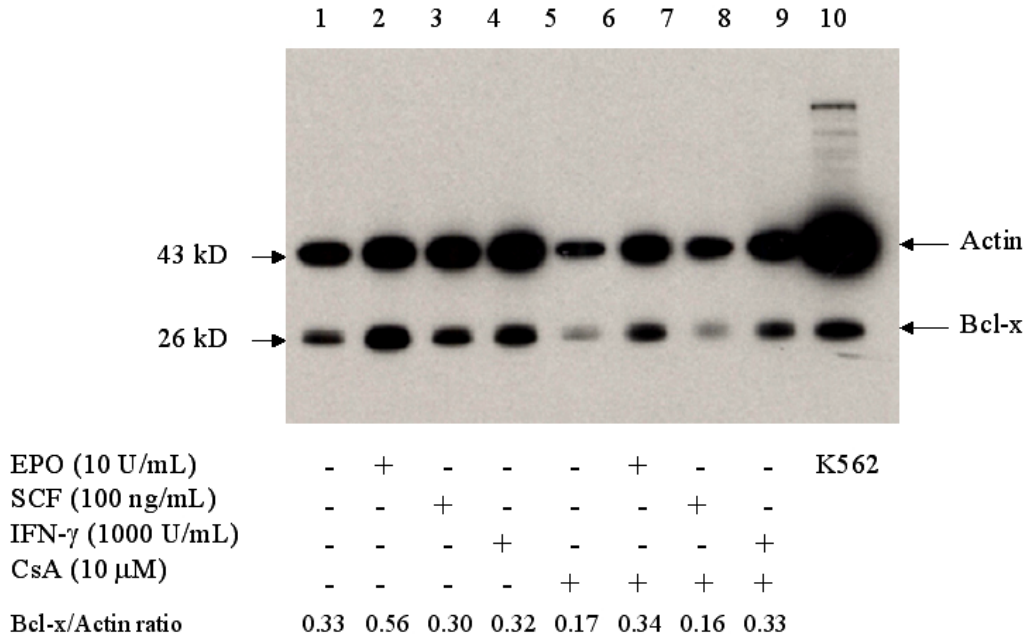


Figure 30 Effect of CsA on expression of Bcl-x in normal ECFCs.

Normal day 7 ECFCs were treated without or with CsA 10 μM at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL or SCF 100 ng/mL at 37^0C for 24 hours. Then, Bcl-x were analysed from whole cell lysates by Western blotting. Photograph illustrates the band intensity of Bcl-x. CsA treatment reduced amount of Bcl-x in the cells cultured without cytokine and with EPO, SCF, but not with IFN-γ as shown in band intensity and Bcl-x/actin ratio. The representative photograph shows a typical result of three independent experiments. K562 cell line was used as a positive control.

Table 18 Effect of CsA on expression of Bcl-x in normal ECFCs. Data represent as Bcl-x/actin ratio of two independent experiments.

Cytokine	CsA 10 μM	Sample 1	Sample 2	Mean
No cytokine	-	0.33	0.34	0.34
	+	0.17	0.16	0.17
EPO 10 U/mL	-	0.56	0.42	0.49
	+	0.34	0.34	0.34
IFN-γ 1,000 U/mL	-	0.32	0.26	0.29
	+	0.33	0.21	0.27

Table 19 Disturbance of mitochondrial function of normal ECFCs by CsA. Data represent mean ± SD of the low MPT and high ROS cells (%) of three independent experiments.

Cytokine	CsA 10 μM	Mean ± SD of the low MPT and high ROS cells (%)
No cytokine	-	85.4 ± 6.7
	+	83.6 ± 3.9
EPO 10 U/mL	-	45.7 ± 9.5
	+	60.3 ± 6.4
IFN-γ 1,000 U/mL	-	37.6 ± 10.1
	+	52.4 ± 19.1

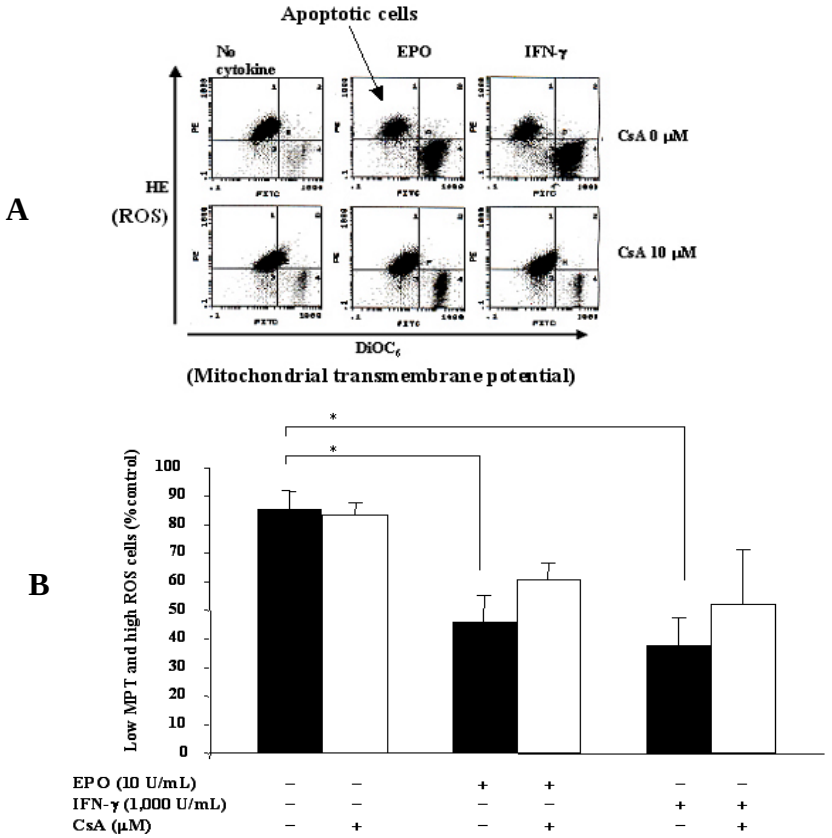


Figure 31 Disturbance of mitochondrial function of normal ECFCs by CsA.

Normal day 7 ECFCs were treated without or with CsA 10 μM at 37^0C for 30 minutes and cultured without cytokine or with EPO 10 U/mL or IFN-γ 1,000 U/mL at 37^0C for 24 hours. Then, cells were incubated with $DiOC_6$ and HE at 37°C for 15 minutes, and analysed by flow cytometry. Figure 31A shows the representative histograms of the typical results. Fluorescent intensity of $DiOC_6$ refers to ΔΨm. Fluorescent intensity of HE refers to ROS. The low ΔΨm and high ROS cells were defined to apoptotic cells (quadrant 1). From the left to the right of the upper panel shows mitochondrial function of CsA-untreated cells cultured with no cytokine, EPO and IFNγ, respectively. From the left to the right of the lower panel shows mitochondrial function of CsA-treated cells with no cytokine, EPO and IFNγ, respectively. Figure 31B shows mean $\pm$ SD of three independent experiments. In the presence of EPO and IFN-γ, CsA disturbed mitochondrial function at the same level. Asterisk indicated the significant difference (p<0.05) between cells cultured with and without cytokine.

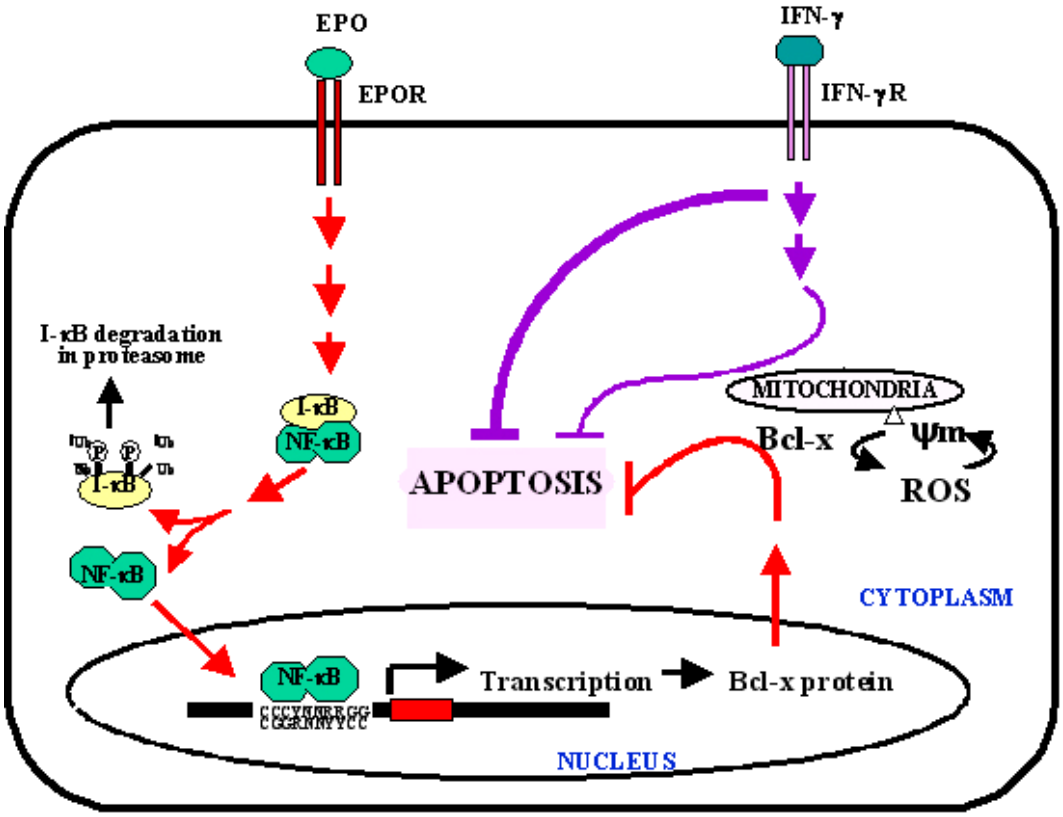


Figure 32 The proposed mechanism of EPO and NF-κB signaling pathway to retard apoptosis of erythroid progenitor cells.

When EPO binds to its receptor, NF-κB is dissociated from IκB and translocates to its binding site on the Bcl-x promoter. When the expression of Bcl-x increases, apoptosis is retarded via mitochondrial function.

CHAPTER 6
DISCUSSION

In the present work, *in vitro* culture demonstrated ineffective erythropoiesis of erythroid progenitor cells derived from β-thalassemia/HbE peripheral blood (Figures 18). This result was consistent with *in vitro* culture of β-thalassemic CD34+ cell derived from bone marrow (6). It has been shown that the fold-increase in cell numbers in β-thalassemic marrow was less than normal marrow at day 7 and β-thalassemia marrow CD34+ progeny was more differentiated on day 4, with predominantly basophilic erythroblasts and polychromatophilic erythroblast at day 7, and prominently polychromatophilic erythroblast at day 11 and 14. In addition, the polychromatophilic erythroblasts had morphologically disrupted cytoplasmic membranes and the few orthochromatic erythroblasts and enucleated RBC produced in β-thalassemia cultures were pale and vacuolated (6). In this study, a few numbers of erythroblasts with abnormal morphology were observed in serum culture of erythroid progenitors isolated from peripheral blood of β-thalassemia/Hb E (Figure 19). However, macrophages, as previously described (6), were not found in this studied culture system. This was probably due to the different source of stem cells, bone marrow vs. peripheral blood, and types and concentrations of the added cytokines. Since *in vitro* culture system showed ineffective erythropoiesis in thalassemia, further study on enhanced apoptosis and its signaling pathway was conducted.

There are many evidences supporting that thalassemic marrow erythroid precursors underwent apoptosis more than that of normal erythroid precursors, and extent of apoptosis was related to ineffective erythropoiesis in thalassemic diseases (4-6). The Bcl-2 and Bcl-x_L expression was shown to suppress apoptosis of cultured erythroid precursor cells. Bcl-x_L expression was highly EPO-dependent in the early stage erythroblasts. *In vivo* evidence indicated that circulating erythroblasts from β-thalassemia intermedia constantly expressed the Bcl-x_L transcript, not Bcl-2 and Bcl-

x_S expression (181). This implies that Bcl-x and EPO maintain the viability of erythroid precursor cells and mature erythroblasts in ineffective erythropoiesis state. In this study, *in vitro* culture of erythroid progenitor cells derived from peripheral blood of β-thlassemia/Hb E demonstrated that EPO retarded apoptosis of thalassemic erythroid progenitor cells via NF-κB involvement (Figures 25, 26 and 29). Bcl-x expression also correlated with NF-κB expression and apoptosis retardation in normal erythroid progenitor cell in the presence of EPO (Figures 21, 26-28 and 30). It has been reported that NF-κB plays role in proliferation and differentiation of normal erythroid progenitor cells (16). It is possible that at early stage of thalassemic cell growth there are accumulated machinery, including Bcl-x, for the maintenance of cell survival via internal or external signalling factors. However, further investigations are still required to define the mechanism by which Bcl-x and EPO maintain the viability of thalassemic erythroid precursor cells and mature erythroblast. It might be envisioned to improve survival of thalassemic erythroid cells.

In general, inactive NF-κB associates with IκB protein and retains in the cytoplasm. Activation of signaling pathway leads to proteolysis of IκB by proteasome, allowing active NF-κB translocates into the nucleus. CsA is a noncompetitive inhibitor of the 20S proteasome and thus prevents IκB degradation so that activation of the NF-κB pathway was inhibited (149). CsA also inhibits proteasome activity *in vitro* and suppress lipopolysaccharide-induced IκB degradation in murine macrophages by stabilizing the ubiquinated forms of IκBα (148). This finding suggests that a target of CsA in inhibiting the NF-κB pathway is the protease activity of the proteasome rather than kinases or ubiquitin ligases that regulate the signal-induced phosphorylation and ubiquitination of IκB, respectively. Similar results are seen in Jurkat cells, as well as human and mouse primary T lymphocytes, where CsA interferes with the degradation of IκBα following phorbol-ester and ionomycin stimulation without altering IκBα phosphorylation (149). The proteolytic processing of the NF-κB precursor p105, which also requires the 26S proteasome complex, is not affected by CsA treatment (150). In addition, CsA also prevents NF-κB via inhibition of calcineurin activity. CsA forms complex with the cellular protein cyclophilin, resulting in inhibition of calcineurin activity, a calcium- and calmodulin-dependent serine/threonine

phosphatase. Calcineurin is required for activation of the transcription factor NF-AT, which binds to the *IL-2* promoter and is critical for regulating IL-2 expression in T lymphocytes. In addition, calcineurin can activate the NF-κB pathway (148). In Jurkat cells, transfection of an expression vector containing a Ca^{2+}-independent calcineurin protein results in increased DNA binding and trans-activation by NF-κB. Hence, CsA inhibition of calcineurin activity can prevent NF-κB activation.

Nuclear factor-κB is expressed in cells derived from normal human BFU-E and the high levels of NF-κB factors, p105/p50, p100/p52, and p65 expressed in early cells derived from BFU-E, decline during maturation (16). We now demonstrated that both normal and thalassemic erythroid progenitor cells expressed NF-κB p65. (Figures 27-28 and 29, respectively). The role of NF-κB on apoptosis of erythroid progenitor cells in the presence of EPO and IFN-γ was explored by using CsA, which inhibit dissociation of IκB protein inhibitor. The result supported that inactivation of NF-κB led to apoptosis of both normal and thalassemic erythroid progenitor cells, as indicated by increased percentage of annexin V-positive cells. This study also demonstrated that the degree of apoptotic cells was depended on the concentration of CsA from 5-100 μM (Figures 21 and 25, Tables 12 and 15). The relatively high activity of NF-κB was shown in early erythroid progenitor cells (25). It was probably that the expression of NF-κB was EPO-dependent and IFN-γ-independent. Thus, confocal microscopy assay showed very high intensity of rhodamine in the normal and thalassemic cells cultured with EPO (Figures 28 and 29, respectively), and 10 μM of CsA showed partially suppression of NF-κB activation, as Western blotting showed small amount of NF-κB in the nuclear fraction of CsA-treated normal cell (Figure 27).

The result showed that CsA induced the different degree of apoptosis in normal and thalassemic ECFC as shown in Figure 21 and 25, respectively. In the presence of EPO colony-forming capacity of normal erythroid progenitor cells was more than that in the presence of IFN-γ (Figure 22). This implies that signaling pathways to retard apoptosis and to induced proliferation of EPO and IFN-γ were not the same.

Nuclear factor-κB also functions in signaling pathways induced by EPO in neuronal cells (182). The EPOR activates Jak2, and then nuclear translocation of NF-

κB is induced. However, the role of NF-κB in the apoptosis of erythroid progenitor cells remains unclear. Choi et al. have demonstrated that short incubations of erythroid progenitor cells with IFN-γ in the absence of erythropoietin delayed the apoptosis of mature erythroids (71). The present study showed that increasing concentrations of CsA, which disturbs the translocation of NF-κB to the nucleus, enhanced the apoptosis of cells cultured with EPO or IFN-γ. Furthermore, NF-κB was prominently expressed only in the presence of EPO, and NF-κB translocated from the cytoplasm to the nucleus when ECFC apoptosis was retarded in the presence of EPO, but not IFN-γ. Thus, EPO might retard the apoptosis of erythroid progenitor cells by NF-κB activation and probably through Jak2, which is distinct from the effects of IFN-γ. Bcl-x protects cells from apoptosis by blocking the release of cytochrome c from mitochondria (45, 183). Depriving ECFCs of EPO reduced Bcl-x expression and induced apoptosis via caspase 3 activation (33). The expression of Bcl-x is EPO-dependent and increased during the terminal differentiation of ECFCs (33). The *Bcl-x* gene possesses an NF-κB binding site (30, 31), and NF-κB plays a regulatory role on *Bcl-x* gene expression (184, 185). This study showed that Bcl-x expression in ECFCs was induced by EPO and reduced by CsA. This indicates that NF-κB translocation is required for EPO to enhance the expression of Bcl-x. However, NF-κB translocated in ECFCs cultured with EPO, but not with IFN-γ. Thus, IFN-γ retarded the apoptosis of ECFCs by enhancing Bcl-x expression without NF-κB translocation. IFN-γ directly inhibits NF-κB-mediated transactivation within the nuclei without affecting the nuclear translocation or DNA binding of NF-κB (186). The IFN-γ receptor acts through the Jak1 and Jak2 kinases (123, 124). Jak2 also associates with the EPO receptor and becomes tyrosine phosphorylated following stimulation of responsive cells by EPO (125, 126). Therefore, by interaction with Jak2 without NF-κB translocation, IFN-γ might mimics the signaling induced by EPO to reduce ECFC apoptosis.

Other studies have shown that shortly after the ΔΨm falls, superoxide anion radicals are generated by an uncoupled respiratory chain within a few hours (48). During the process of apoptosis, ROS produced by mitochondria accelerate cellular shrinkage (187). The reduction of ΔΨm and subsequent ROS generation are key

phenomena in regulating the pathway of apoptosis via Bcl-x (48). Bcl-x also inhibits the mitochondrial permeability transition and retards the apoptosis of many cells types. Mitochondria and ROS production also play an essential role in the regulation of apoptosis of erythroid progenitor cells (59). When CsA was added to cultured ECFCs, the cells with low ΔΨm and high ROS slightly increased in the presence of either EPO or IFN-γ. These results are unusual because cell viability was maintained in the presence of IFN-γ plus CsA. This could be due to the fact that IFN-γ is protecting the cells from apoptosis through the other pathway that is not affected by CsA. This might be another effect of CsA, which is a transient inhibitor of permeability transition that prevents the ΔΨm dissipation accompanied in early apoptosis (44, 188).

Finally, the NF-κB signalling pathway required for EPO to retard ECFC apoptosis was proposed as shown in (Figure 30). When EPO binds to its receptor, NF-κB is dissociated from IκB and translocates to its binding site on the Bcl-x promoter. When the expression of Bcl-x increases, apoptosis is retarded via mitochondrial function. This raises the notion that stimulating NF-κB could improve the survival of erythroid progenitor cells and this strategy would be compatible with using EPO therapy to activate its signal transduction. However, IFN-γ retards apoptosis via a unique pathway, which is independent of both NF-κB signaling and mitochondrial function. The physiological significance of this phenomenon still requires clarification. During an inflammatory response, erythropoiesis might be hampered by various cytokines and microorganisms. At such times, short incubations with IFN-γ might play a crucial role as a “back-up” to maintain the survival of erythroid progenitor cells by using a signaling pathway that is distinct from that of EPO.

CHAPTER 7
CONCLUSION

Normal erythropoiesis is a tightly controlled process requiring EPO to enhance proliferation, differentiation, and to protect against apoptosis of erythroid progenitor cells by increasing the expression of Bcl-x. NF-κB, which protects many cell types from apoptosis, has specific binding site on *Bcl-x* gene of erythroid progenitor cells. NF-κB highly expressed in early normal erythroid progenitor cells and decreased during erythroid maturation. IFN-γ had dual effects in erythroid progenitor cells: induction and protection of apoptosis, depending on stage of the cells. This study showed that EPO and IFN-γ maintain the survival of normal erythroid progenitor cells via apoptotic retardation but with different mechanisms. The role of NF-κB as a second messenger for cytokine responses, as well as in the activation of erythropoietic genes, suggests that it may have an important role in human erythropoiesis. Apoptotic retardation of normal erythroid progenitor cells by NF-κB was studied using CsA, which inactivates NF-κB by inhibit NF-κB dissociation from its inhibitor. This inhibits NF-κB translocation from the cytoplasm to the nucleus, where it binds to specific binding site of target genes. CsA treatment showed that apoptosis of normal erythroid progenitor cells cultured in the present of EPO was retarded less than that of IFN-γ and colony formation capacity was also more reduced. Further study demonstrated that the translocation of NF-κB was observed in the cell cultured with EPO, not with IFN-γ. However, Bcl-x expression was decreased in the CsA-treated cells cultured with both EPO and IFN-γ. In addition, CsA disturbed mitochondrial function, which also involves in apoptosis of the cells. These results support the hypothesis that EPO retards apoptosis of normal erythroid progenitor cells via NF-κB activation and Bcl-x involvement, whereas IFN-γ retarded apoptosis of the cells via other mechanism.

Thalassemic erythroid progenitor cells underwent accelerated apoptosis as detected by an increase in DNA cleavage and an increase in externalized phosphatidylserine on the outer plasma membrane. Apoptosis was suggested to occur at polychromatophilic erythroblast. Apoptotic erythroid progenitor cells were increased approximately fourfold above normal. The tight correlation of ineffective erythropoiesis and marrow erythroid apoptosis was demonstrated. The degree of apoptosis and ineffective erythropoiesis appeared to be controlled by the accumulation of an excess globin chains in early erythroid precursors. These findings led to propose that the mechanism of ineffective erythropoiesis was enhanced apoptosis. However, the molecular mechanism of apoptosis is not known. This study demonstrated *in vitro* ineffective erythropoiesis of thalassemic erythroid progenitor cells collected from peripheral blood correlated with the hemoglobin level of β-thalassemia/Hb E patients. EPO and IFN-γ could maintain the survival of thalassemic erythroid progenitor cells via apoptotic retardation as in normal cells. The involvement of NF-κB expression and apoptosis in thalassemia was demonstrated by using CsA treatment. The expression of NF-κB in erythroid progenitor cells cultured in the presence of EPO was prominent and greater than that in the presence of IFN-γ. CsA could induce apoptosis of thalassemic erythroid progenitor cells as dose-dependent manner. The expression of NF-κB was reduced correlated with the increased apoptosis of erythroid progenitor cells in the presence of EPO, not IFN-γ. This implied that EPO retarded apoptosis of thalassemic erythroid progenitor cells via NF-κB activation as in normal cells.

REFERENCES

1. Wickramasinghe SN, Hughes M, Fucharoen S, Wasi P. The fate of excess β-globin chains within erythropoietic cells in α-thalassaemia 2 trait, α-thalassaemia 1 trait, haemoglobin H disease and haemoglobin Q-H disease: an electron microscope study. Br J Haematol 1984;56(3):473-82.
2. Wickramasinghe SN, Fucharoen S, Wasi P. Studies of erythropoietic cells in heterozygotes and homozygotes for haemoglobin Constant Spring and in heterozygotes for both haemoglobin Constant Spring and α-thalassaemia 1 trait: extent of globin chain precipitation and cell cycle distribution. Clin Lab Haematol 1986;8(3):187-98.
3. Wickramasinghe SN, Bush V. Observations on the ultrastructure of erythropoietic cells and reticulum cells in the bone marrow of patients with homozygous β-thalassaemia. Br J Haematol 1975;30(4):395-9.
4. Centis F, Tabellini L, Lucarelli G, Buffi O, Tonucci P, Persini B, et al. The importance of erythroid expansion in determining the extent of apoptosis in erythroid precursors in patients with β-thalassemia major. Blood 2000;96(10):3624-9.
5. Pootrakul P, Sirankapracha P, Hemsorach S, Moungsub W, Kumbunlue R, Piangitjagum A, et al. A correlation of erythrokinetics, ineffective erythropoiesis, and erythroid precursor apoptosis in thai patients with thalassemia. Blood 2000;96(7):2606-12.
6. Mathias LA, Fisher TC, Zeng L, Meiselman HJ, Weinberg KI, Hiti AL, et al. Ineffective erythropoiesis in β-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol 2000;28(12):1343-53.

7. Sawada K, Krantz SB, Dai CH, Koury ST, Horn ST, Glick AD, et al. Purification of human blood burst-forming units-erythroid and demonstration of the evolution of erythropoietin receptors. J Cell Physiol 1990;142(2):219-30.
8. Muta K, Krantz SB. Apoptosis of human erythroid colony-forming cells is decreased by stem cell factor and insulin-like growth factor I as well as erythropoietin. J Cell Physiol 1993;156(2):264-71.
9. Muta K, Krantz SB, Bondurant MC, Wickrema A. Distinct roles of erythropoietin, insulin-like growth factor I, and stem cell factor in the development of erythroid progenitor cells. J Clin Invest 1994;94(1):34-43.
10. Caux C, Moreau I, Saeland S, Banchereau J. Interferon-γ enhances factor-dependent myeloid proliferation of human CD34+ hematopoietic progenitor cells. Blood 1992;79(10):2628-35.
11. Brugger W, Mocklin W, Heimfeld S, Berenson RJ, Mertelsmann R, Kanz L. Ex vivo expansion of enriched peripheral blood CD34+ progenitor cells by stem cell factor, interleukin-1β (IL-1β), IL-6, IL-3, interferon-γ, and erythropoietin. Blood 1993;81(10):2579-84.
12. Shiohara M, Koike K, Nakahata T. Synergism of interferon-γ and stem cell factor on the development of murine hematopoietic progenitors in serum-free culture. Blood 1993;81(6):1435-41.
13. Choi JW, Kim Y, Fujino M, Ito M. Significance of fetal hemoglobin-containing erythroblasts (F blasts) and the F blast/F cell ratio in myelodysplastic syndromes. Leukemia 2002;16(8):1478-83.
14. Chytil M, Verdine GL. The Rel family of eukaryotic transcription factors. Curr Opin Struct Biol 1996;6(1):91-100.
15. Karin M. How NF-κB is activated: the role of the IκB kinase (IKK) complex. Oncogene 1999;18(49):6867-74.
16. Zhang MY, Sun SC, Bell L, Miller BA. NF-κB transcription factors are involved in normal erythropoiesis. Blood 1998;91(11):4136-44.
17. Valtieri M, Venturelli D, Care A, Fossati C, Pelosi E, Labbaye C, et al. Antisense *myb* inhibition of purified erythroid progenitors in development and differentiation is linked to cycling activity and expression of DNA polymerase alpha. Blood 1991;77(6):1181-90.

18. Bondurant MC, Yamashita T, Muta K, Krantz SB, Koury MJ. c-*myc* expression affects proliferation but not terminal differentiation or survival of explanted erythroid progenitor cells. J Cell Physiol 1996;168(2):255-63.

19. Claudio E, Segade F, Wrobel K, Ramos S, Bravo R, Lazo PS. Molecular mechanisms of TNF-α cytotoxicity: activation of NF-κB and nuclear translocation. Exp Cell Res 1996;224(1):63-71.

20. Lee AH, Hong JH, Seo YS. Tumour necrosis factor-α and interferon-γ synergistically activate the RANTES promoter through nuclear factor κB and interferon regulatory factor 1 (IRF-1) transcription factors. Biochem J 2000;350 Pt 1:131-8.

21. Arsura M, FitzGerald MJ, Fausto N, Sonenshein GE. Nuclear factor-κB/Rel blocks transforming growth factor β1-induced apoptosis of murine hepatocyte cell lines. Cell Growth Differ 1997;8(10):1049-59.

22. Bittorf T, Buchse T, Sasse T, Jaster R, Brock J. Activation of the transcription factor NF-κB by the erythropoietin receptor: structural requirements and biological significance. Cell Signal 2001;13(9):673-81.

23. Emilia G, Donelli A, Ferrari S, Torelli U, Selleri L, Zucchini P, et al. Cellular levels of mRNA from *c-myc*, *c-myb* and *c-fes onc*-genes in normal myeloid and erythroid precursors of human bone marrow: an in situ hybridization study. Br J Haematol 1986;62(2):287-92.

24. Kirsch IR, Bertness V, Silver J, Hollis GF. Regulated expression of the c-myb and c-myc oncogenes during erythroid differentiation. J Cell Biochem 1986;32(1):11-21.

25. Liu JJ, Hou SC, Shen CK. Erythroid gene suppression by NF-κB. J Biol Chem 2003;278(21):19534-40.

26. Wang Z, Liebhaber SA. A 3'-flanking NF-κB site mediates developmental silencing of the human ζ-globin gene. Embo J 1999;18(8):2218-28.

27. Grossmann M, Metcalf D, Merryfull J, Beg A, Baltimore D, Gerondakis S. The combined absence of the transcription factors Rel and RelA leads to multiple hemopoietic cell defects. Proc Natl Acad Sci USA 1999;96(21):11848-53.

28. Makarov SS. NF-κB as a therapeutic target in chronic inflammation: recent advances. Mol Med Today 2000;6(11):441-8.
29. Mattson MP, Camandola S. NF-κB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 2001;107(3):247-54.
30. Chen F, Demers LM, Vallyathan V, Lu Y, Castranova V, Shi X. Involvement of 5'-flanking κB-like sites within Bcl-x gene in silica-induced Bcl-x expression. J Biol Chem 1999;274(50):35591-5.
31. Glasgow JN, Wood T, Perez-Polo JR. Identification and characterization of nuclear factor κB binding sites in the murine bcl-x promoter. J Neurochem 2000;75(4):1377-89.
32. Chen C, Edelstein LC, Gelinas C. The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x_L. Mol Cell Biol 2000;20(8):2687-95.
33. Gregoli PA, Bondurant MC. The roles of Bcl-x_L and apopain in the control of erythropoiesis by erythropoietin. Blood 1997;90(2):630-40.
34. Gregory T, Yu C, Ma A, Orkin SH, Blobel GA, Weiss MJ. GATA-1 and erythropoietin cooperate to promote erythroid cell survival by regulating bcl-x_L expression. Blood 1999;94(1):87-96.
35. Vander Heiden MG, Chandel NS, Li XX, Schumacker PT, Colombini M, Thompson CB. Outer mitochondrial membrane permeability can regulate coupled respiration and cell survival. Proc Natl Acad Sci USA 2000;97(9):4666-71.
36. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972;26(4):239-57.
37. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281(5381):1309-12.
38. Halliwell B, Gutteridge, JMC. Free Radicals in biology an Medicine: Oxford University press; 1999.
39. Buetler TM, Cottet-Maire F, Krauskopf A, Ruegg UT. Does cyclosporin A generate free radicals? Trends Pharmacol Sci 2000;21(8):288-90.

40. Joza N, Kroemer G, Penninger JM. Genetic analysis of the mammalian cell death machinery. Trends Genet 2002;18(3):142-9.
41. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281(5381):1305-8.
42. Susin S, et al. Molecular characterization of mitochondrial apoptosis inducing factor. Nature 1999;397:441-6.
43. Ormerod MG, Paul F, Cheetham M, Sun XM. Discrimination of apoptotic thymocytes by forward light scatter. Cytometry 1995;21(3):300-4.
44. Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. Immunol Today 1997;18(1):44-51.
45. Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, et al. Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 1996;184(3):1155-60.
46. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;275(5303):1129-32.
47. Johnson LV, Walsh ML, Chen LB. Localization of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci USA 1980;77(2):990-4.
48. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, et al. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 1995;182(2):367-77.
49. Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med 1995;181(5):1661-72.
50. Cossarizza A, Kalashnikova G, Grassilli E, Chiappelli F, Salvioli S, Capri M, et al. Mitochondrial modifications during rat thymocyte apoptosis: a study at the single cell level. Exp Cell Res 1994;214(1):323-30.
51. Eastman A, Barry MA. The origins of DNA breaks: a consequence of DNA damage, DNA repair, or apoptosis? Cancer Invest 1992;10(3):229-40.

52. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119(3):493-501.

53. Bedner E, Smolewski P, Amstad P, Darzynkiewicz Z. Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. Exp Cell Res 2000;259(1):308-13.

54. de Murcia G, Menissier de Murcia J. Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem Sci 1994;19(4):172-6.

55. Chiarugi A, Moskowitz MA. PARP-1-a perpetrator of apoptotic cell death? Science 2002;297(5579):200-1.

56. Darzynkiewicz Z, Bender, E., Smolewski P. Flowcytometry in analysis of cell cycle and apoptosis. Seminars in Hematology 2001;38(3):179-193.

57. Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983;130(4):1910-7.

58. Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescin. J Leukoc Biol 1990;47(5):440-8.

59. Matsushima T, Nakashima M, Oshima K, Abe Y, Nishimura J, Nawata H, et al. Receptor binding cancer antigen expressed on SiSo cells, a novel regulator of apoptosis of erythroid progenitor cells. Blood 2001;98(2):313-21.

60. Stamatoyannopoulos G, Grosveld, F. Hb switching. In: Stamatoyannopoulos G, editor. The molecular basis of blood diseases. 3ed. Philadelpdia: W.B. Saunders; 2001. p. 135-82.

61. Steinberg M, Benz EJ. Hemoglobin synthesis, structure and function. In: Hoffman R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, editors. Haematology: principles and practice. 2ed. New York: Churcill Livingstone; 1995. p. 464.

62. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood 1993;81(11):2844-53.

63. Loken M, Shah VO, Dattilio KL, Civin C. Flow cytometry analysis of human bone marrow: I. Normal erythroid development. Blood 1987;69(1):255-263.

64. Robinson J, Sieff C, Delia D, Edwards PA, Greaves M. Expression of cell-surface HLA-DR, HLA-ABC and glycophorin during erythroid differentiation. Nature 1981;289(5793):68-71.

65. Koury MJ, Sawyer ST, Brandt SJ. New insights into erythropoiesis. Curr Opin Hematol 2002;9(2):93-100.

66. Dai M, Mantel CR, Xia ZB, et al. An expansion phase precedes terminal erythroid differentiation of hematopoietic progenitor cells from cord blood in vitro and is associated with up-regulation cyclin E and cyclin-dependent kinase 2. Blood 2000;96:3985-3987.

67. Matushansky I, Radparvar F, Skoultchi AI. Reprogramming leukemic cells to terminal differentiation by inhibiting specific cyclin-dependent kinases in G1. Proc Natl Acad Sci USA 2000;97(26):14317-22.

68. Matushansky I, Radparvar F, Skoultchi AI. Manipulating the onset of cell cycle withdrawal in differentiated erythroid cells with cyclin-dependent kinases and inhibitors. Blood 2000;96(8):2755-64.

69. Hsieh FF, Barnett LA, Green WF, Freedman K, Matushansky I, Skoultchi AI, et al. Cell cycle exit during terminal erythroid differentiation is associated with accumulation of $p27^{Kip1}$ and inactivation of cdk2 kinase. Blood 2000;96(8):2746-54.

70. Papayannopoulou T, Abkowitz, J., D'Andrea, A. Biology of erythropoiesis, erythroid differentiation, and maturation. In: Hoffman R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, editors. Hematology: Basic principle and practice. 3 ed. Philadelphia: Churchill Livingstone; 2000. p. 202-219.

71. Choi I, Muta K, Wickrema A, Krantz SB, Nishimura J, Nawata H. Interferon γ delays apoptosis of mature erythroid progenitor cells in the absence of erythropoietin. Blood 2000;95(12):3742-9.

72. Endo T, Odb A, Satoh I, Haseyama Y, Nishio M, Koizumi K, et al. Stem cell factor protects c-kit+ human primary erythroid cells from apoptosis. Exp Hematol 2001;29(7):833-41.

73. Erslev AJ. Erythropoietin titers in health and disease. Semin Hematol 1991;28(3 Suppl 3):2-7; discussion 7-8.

74. Koury MJ, Bondurant MC. Control of red cell production: the roles of programmed cell death (apoptosis) and erythropoietin. Transfusion 1990;30(8):673-4.

75. Kelley LL, Koury MJ, Bondurant MC, Koury ST, Sawyer ST, Wickrema A. Survival or death of individual proerythroblasts results from differing erythropoietin sensitivities: a mechanism for controlled rates of erythrocyte production. Blood 1993;82(8):2340-52.

76. Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. Fetal anemia and apoptosis of red cell progenitors in Stat5a$^{-/-}$5b$^{-/-}$ mice: a direct role for Stat5 in Bcl-x_L induction. Cell 1999;98(2):181-91.

77. Motoyama N, Kimura T, Takahashi T, Watanabe T, Nakano T. Bcl-x prevents apoptotic cell death of both primitive and definitive erythrocytes at the end of maturation. J Exp Med 1999;189(11):1691-8.

78. Gregoli PA, Bondurant MC. Function of caspases in regulating apoptosis caused by erythropoietin deprivation in erythroid progenitors. J Cell Physiol 1999;178(2):133-43.

79. Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 1991;349(6306):257-60.

80. De Maria R, Testa U, Luchetti L, Zeuner A, Stassi G, Pelosi E, et al. Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood 1999;93(3):796-803.

81. De Maria R, Zeuner A, Eramo A, Domenichelli C, Bonci D, Grignani F, et al. Negative regulation of erythropoiesis by caspase-mediated cleavage of GATA-1. Nature 1999;401(6752):489-93.

82. Zermati Y, Garrido C, Amsellem S, Fishelson S, Bouscary D, Valensi F, Varet B, Solary E, Hermine O. Caspase activation is required for terminal erythroid differentiation. journal experimental Medicine 2001;193(2):247-254.

83. Iscove NN. The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. Cell Tissue Kinet 1977;10(4):323-34.

84. Hara H, Ogawa M. Erythropoietic precursors in mice under erythropoietic stimulation and suppression. Exp Hematol 1977;5(2):141-8.

85. Adamson JW, Fialkow PJ. The pathogenesis of myeloproliferative syndromes. Br J Haematol 1978;38(3):299-303.

86. Dessypris EN, Baer MR, Sergent JS, Krantz SB. Rheumatoid arthritis and pure red cell aplasia. Ann Intern Med 1984;100(2):202-6.

87. Tsuda H, Sawada T, Kawakita M, Takatsuki K. Mode of action of erythropoietin (Epo) in an Epo-dependent murine cell line. II. Cell cycle dependency of Epo action. Exp Hematol 1989;17(3):218-22.

88. Spivak JL, Pham T, Isaacs M, Hankins WD. Erythropoietin is both a mitogen and a survival factor. Blood 1991;77(6):1228-33.

89. Koury MJ, Bondurant MC. Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 1990;248(4953):378-81.

90. Bondurant M, Koury, MJ. Origin and developmnt of blood cells. In: Lee G, Eoserster, J., Lukens, J., Paraskevas, F., Greer, JP., Rodgers, GM., editor. Wintrobe. 10 ed. Baltimore: Williams & Wilkins; 1999. p. 145-168.

91. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. Blood 1991;77(12):2583-90.

92. Uddin S, Kottegoda S, Stigger D, Platanias LC, Wickrema A. Activation of the Akt/FKHRL1 pathway mediates the antiapoptotic effects of erythropoietin in primary human erythroid progenitors. Biochem Biophys Res Commun 2000;275(1):16-9.

93. Myklebust JH, Blomhoff HK, Rusten LS, Stokke T, Smeland EB. Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34(+) hematopoietic progenitor cells. Exp Hematol 2002;30(9):990-1000.

94. Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin AS, Jr., Mayo MW. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. Mol Cell Biol 2000;20(5):1626-38.

95. Burow ME, Weldon CB, Melnik LI, Duong BN, Collins-Burow BM, Beckman BS, et al. PI3-K/AKT regulation of NF-κB signaling events in suppression of TNF-induced apoptosis. Biochem Biophys Res Commun 2000;271(2):342-5.

96. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998;281(5381):1322-6.

97. Silva M, Grillot D, Benito A, Richard C, Nunez G, Fernandez-Luna JL. Erythropoietin can promote erythroid progenitor survival by repressing apoptosis through Bcl-x_L and Bcl-2. Blood 1996;88(5):1576-82.

98. Silva M, Benito A, Sanz C, Prosper F, Ekhterae D, Nunez G, et al. Erythropoietin can induce the expression of Bcl-x_L through Stat5 in erythropoietin-dependent progenitor cell lines. J Biol Chem 1999;274(32):22165-9.

99. Chittenden T, Flemington C, Houghton AB, Ebb RG, Gallo GJ, Elangovan B, et al. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. Embo J 1995;14(22):5589-96.

100. Minn AJ, Kettlun CS, Liang H, Kelekar A, Vander Heiden MG, Chang BS, et al. Bcl-x_L regulates apoptosis by heterodimerization-dependent and -independent mechanisms. EMBO J 1999;18(3):632-43.

101. Anderson DM, Williams DE, Tushinski R, Gimpel S, Eisenman J, Cannizzaro LA, et al. Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. Cell Growth Differ 1991;2(8):373-8.

102. Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, et al. Molecular cloning of mast cell growth factor, a hematopoietin that is

active in both membrane bound and soluble forms. Cell 1990;63(1):235-43.

103. Flanagan JG, Chan DC, Leder P. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the *Sld* mutant. Cell 1991;64(5):1025-35.

104. Heinrich MC, Dooley DC, Freed AC, Band L, Hoatlin ME, Keeble WW, et al. Constitutive expression of steel factor gene by human stromal cells. Blood 1993;82(3):771-83.

105. Wu HK, U., Besmer, P., Lodish, HF. Interaction of the erythropoietin and stem cell factor receptors. Nature 1995;377:242.

106. Jacobs-Helber SM, Penta K, Sun Z, Lawson A, Sawyer ST. Distinct signaling from stem cell factor and erythropoietin in HCD57 cells. J Biol Chem 1997;272(11):6850-3.

107. Muta K, Krantz SB, Bondurant MC, Dai CH. Stem cell factor retards differentiation of normal human erythroid progenitor cells while stimulating proliferation. Blood 1995;86(2):572-80.

108. Nishio M, Oda A, Koizumi K, Satoh I, Sato Y, Endoh T, et al. Stem cell factor prevents Fas-mediated apoptosis of human erythroid precursor cells with Src-family kinase dependency. Exp Hematol 2001;29(1):19-29.

109. Chung IJ, Dai C, Krantz SB. Stem cell factor increases the expression of FLIP that inhibits IFNγ -induced apoptosis in human erythroid progenitor cells. Blood 2003;101(4):1324-8.

110. Wojda U, Noel P, Miller JL. Fetal and adult hemoglobin production during adult erythropoiesis: coordinate expression correlates with cell proliferation. Blood 2002;99(8):3005-13.

111. Miller BA, Perrine SP, Bernstein A, Lyman SD, Williams DE, Bell LL, et al. Influence of steel factor on hemoglobin synthesis in sickle cell disease. Blood 1992;79(7):1861-8.

112. Peschle C, Gabbianelli M, Testa U, Pelosi E, Barberi T, Fossati C, et al. c-kit ligand reactivates fetal hemoglobin synthesis in serum-free culture of stringently purified normal adult burst-forming unit-erythroid. Blood 1993;81(2):328-36.

113. Bhanu NV, Trice TA, Lee YT, Miller JL. A signaling mechanism for growth-related expression of fetal hemoglobin. Blood 2004;103(5):1929-33.

114. Taylor ML, Metcalfe DD. Kit signal transduction. Hematol Oncol Clin North Am 2000;14(3):517-35.

115. Ratajczak J, Majka M, Kijowski J, Baj M, Pan ZK, Marquez LA, et al. Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells. Br J Haematol 2001;115(1):195-204.

116. Sui X, Krantz SB, Zhao ZJ. Stem cell factor and erythropoietin inhibit apoptosis of human erythroid progenitor cells through different signalling pathways. Br J Haematol 2000;110(1):63-70.

117. Lawson AE, Bao H, Wickrema A, Jacobs-Helber SM, Sawyer ST. Phosphatase inhibition promotes antiapoptotic but not proliferative signaling pathways in erythropoietin-dependent HCD57 cells. Blood 2000;96(6):2084-92.

118. Kashii Y, Uchida M, Kirito K, Tanaka M, Nishijima K, Toshima M, et al. A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood 2000;96(3):941-9.

119. Zoumbos NC, Djeu JY, Young NS. Interferon is the suppressor of hematopoiesis generated by stimulated lymphocytes *in vitro*. J Immunol 1984;133(2):769-74.

120. Dai CH, Krantz SB, Kollar K, Price JO. Stem cell factor can overcome inhibition of highly purified human burst-forming units-erythroid by interferon γ. J Cell Physiol 1995;165(2):323-32.

121. Taniguchi S, Dai CH, Krantz SB. Specific binding of interferon-γ to high affinity receptors on human erythroid colony-forming cells. Exp Hematol 1997;25(3):193-8.

122. Means RT, Jr., Krantz SB. Inhibition of human erythroid colony-forming units by gamma interferon can be corrected by recombinant human erythropoietin. Blood 1991;78(10):2564-7.

123. Darnell JE, Jr., Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264(5164):1415-21.

124. Watling D, Guschin D, Muller M, Silvennoinen O, Witthuhn BA, Quelle FW, et al. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-γ signal transduction pathway. Nature 1993;366(6451):166-70.

125. Wojchowski DM, He TC. Signal transduction in the erythropoietin receptor system. Stem Cells 1993;11(5):381-92.

126. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 1993;74(2):227-36.

127. Dai CH, Price JO, Brunner T, Krantz SB. Fas ligand is present in human erythroid colony-forming cells and interacts with Fas induced by interferon γ to produce erythroid cell apoptosis. Blood 1998;91(4):1235-42.

128. Dai C, Krantz SB. Interferon γ induces upregulation and activation of caspases 1, 3, and 8 to produce apoptosis in human erythroid progenitor cells. Blood 1999;93(10):3309-16.

129. Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-κB. J Clin Invest 2001;107(3):241-6.

130. Liu ZG, Hsu H, Goeddel DV, Karin M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell 1996;87(3):565-76.

131. Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol 2001;11(9):372-7.

132. Beauparlant P, Hiscott J. Biological and biochemical inhibitors of the NF-κB/Rel proteins and cytokine synthesis. Cytokine Growth Factor Rev 1996;7(2):175-90.

133. Epinat JC, Gilmore TD. Diverse agents act at multiple levels to inhibit the Rel/NF-κB signal transduction pathway. Oncogene 1999;18(49):6896-909.

134. Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. J Clin Invest 2001;107(2):135-42.

135. Grisham MB, Palombella VJ, Elliott PJ, Conner EM, Brand S, Wong HL, et al. Inhibition of NF-κB activation in vitro and in vivo: role of 26S proteasome. Methods Enzymol 1999;300:345-63.

136. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T. The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell 1994;78(5):773-85.

137. Jobin C, Hellerbrand C, Licato LL, Brenner DA, Sartor RB. Mediation by NF-κB of cytokine induced expression of intercellular adhesion molecule 1 (ICAM-1) in an intestinal epithelial cell line, a process blocked by proteasome inhibitors. Gut 1998;42(6):779-87.

138. Traenckner EB, Wilk S, Baeuerle PA. A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. EMBO J 1994;13(22):5433-41.

139. DiDonato JA, Mercurio F, Karin M. Phosphorylation of IκBα precedes but is not sufficient for its dissociation from NF-κB. Mol Cell Biol 1995;15(3):1302-11.

140. Maggirwar SB, Harhaj E, Sun SC. Activation of NF-κB/Rel by Tax involves degradation of IκBα and is blocked by a proteasome inhibitor. Oncogene 1995;11(5):993-8.

141. Sun SC, Maggirwar SB, Harhaj E. Activation of NF-κB by phosphatase inhibitors involves the phosphorylation of IκBα at phosphatase 2A-sensitive sites. J Biol Chem 1995;270(31):18347-51.

142. Chiao PJ, Miyamoto S, Verma IM. Autoregulation of IκBα activity. Proc Natl Acad Sci USA 1994;91(1):28-32.

143. Finco TS, Beg AA, Baldwin AS, Jr. Inducible phosphorylation of IκBα is not sufficient for its dissociation from NF-κB and is inhibited by protease inhibitors. Proc Natl Acad Sci USA 1994;91(25):11884-8.

144. Muroi M. A clinicopathological study on extensive lymph node dissection for thoracic esophageal cancer. Nippon Kyobu Geka Gakkai Zasshi 1995;43(3):287-99.

145. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science 1995;270(5234):286-90.

146. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS, Jr. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 1995;270(5234):283-6.

147. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. Nature 1998;396(6706):77-80.

148. Frantz B, Nordby EC, Bren G, Steffan N, Paya CV, Kincaid RL, et al. Calcineurin acts in synergy with PMA to inactivate IκB/MAD3, an inhibitor of NF-κB. EMBO J 1994;13(4):861-70.

149. Meyer S, Kohler NG, Joly A. Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-κB activation. FEBS Lett 1997;413(2):354-8.

150. Marienfeld R, Neumann M, Chuvpilo S, Escher C, Kneitz B, Avots A, et al. Cyclosporin A interferes with the inducible degradation of NF-κB inhibitors, but not with the processing of p105/NF-κB1 in T cells. Eur J Immunol 1997;27(7):1601-9.

151. Venkataraman L, Burakoff SJ, Sen R. FK506 inhibits antigen receptor-mediated induction of c-rel in B and T lymphoid cells. J Exp Med 1995;181(3):1091-9.

152. Kahan BD. Immunosuppressive therapy. Curr Opin Immunol 1992;4(5):553-60.

153. Borel JF, Baumann G, Chapman I, Donatsch P, Fahr A, Mueller EA, et al. In vivo pharmacological effects of ciclosporin and some analogues. Adv Pharmacol 1996;35:115-246.

154. Matsuda S, Koyasu S. Mechanisms of action of cyclosporine. Immunopharmacology 2000;47(2-3):119-25.

155. Sawafuji K, Miyakawa Y, Kizaki M, Ikeda Y. Cyclosporin A induces erythroid differentiation of K562 cells through p38 MAPK and ERK pathways. Am J Hematol 2003;72(1):67-9.

156. Sawafuji K, Miyakawa Y, Weisberg E, Griffin JD, Ikeda Y, Kizaki M. Aminopeptidase inhibitors inhibit proliferation and induce apoptosis of K562 and STI571-resistant K562 cell lines through the MAPK and GSK-3β pathways. Leuk Lymphoma 2003;44(11):1987-96.

157. Beveridge T, Gratwohl A, Michot F. Cyclosporin A pharmacokinetics after a single dose in man and serum levels after multiple dosing in recipients of allogeneic bone marrow grafts. Curr Ther Res 1981;30:5-16.

158. Copeland KR, Yatscoff RW, McKenna RM. Immunosuppressive activity of cyclosporine metabolites compared and characterized by spectroscopy and nuclear megnetic resonance. Clin Chem 1990;36:225-229.

159. Borel JF. Pharmacology of cyclosporine (sandimmune). IV. Pharmacological properties in vivo. Pharmacol Rev 1990;41(3)259-371.

160. Weatherall D. The thalassemias. In: Stamatoyannopoulos G MP, Perlmutter RM, Vermus H., editor. The molecular basis of blood diseases. 3 ed. Iowa: Blackwell Science; 2001. p. 183-226.

161. Orkin SH, Kazazian HH, Jr., Antonarakis SE, Ostrer H, Goff SC, Sexton JP. Abnormal RNA processing due to the exon mutation of β^{E}-globin gene. Nature 1982;300(5894):768-9.

162. Finch CA, Deubelbeiss K, Cook JD, Eschbach JW, Harker LA, Funk DD, et al. Ferrokinetics in man. Medicine (Baltimore) 1970;49(1):17-53.

163. Polliack A, Yataganas X, Thorell B, Rachmilewitz EA. An electron microscopic study of the nuclear abnormalities in erythroblasts in β-thalassaemia major. Br J Haematol 1974;26(2):201-4.

164. Wickramasinghe SN, McElwain TJ, Cooper EH, Hardisty RM. Proliferation of erythroblasts in β-thalassaemia. Br J Haematol 1970;19(6):719-27.

165. Wickramasinghe SN, Hughes M, Wasi P, Fucharoen S, Modell B. Ineffective erythropoiesis in haemoglobin E β-thalassaemia: an electron microscope study. Br J Haematol 1981;48(3):451-7.

166. Issaragrisil S, Tang-naitrisorana Y, Piankijagum A, Fucharoen S, Wasi P. Study of hematopoietic progenitors in patients with thalassemia: the effect of splenectomy. Birth Defects Orig Artic Ser 1988;23(5B):323-9.

167. Rigas DA, Koler RD. Decreased erythrocyte survival in hemoglobin H disease as a result of the abnormal properties of hemoglobin H: the benefit of splenectomy. Blood 1961;18:1-17.

168. Nathan DG, Gunn RB. Thalassemia: the consequences of unbalanced hemoglobin synthesis. Am J Med 1966;41(5):815-30.

169. Gabuzda TG, Nathan DG, Gardner FH. The metabolism of the individual C^{14} labeled hemoglobins in patients with H-thalassemia, with observations on radiochromate binding to the hemoglobins during red cell survival. J Clin Invest 1965;44:315-25.

170. Gabuzda TG. Hemoglobin H and the red cell. Blood 1966;27(4):568-79.

171. Pearson HA, Mc FW. Erythrokinetics in thalassemia. II. Studies in Lepore trait and hemoglobin H disease. J Lab Clin Med 1962;59:147-57.

172. Rees DC, Williams TN, Maitland K, Clegg JB, Weatherall DJ. Alpha thalassaemia is associated with increased soluble transferrin receptor levels. Br J Haematol 1998;103(2):365-9.

173. Huebers HA, Beguin Y, Pootrakul P, Einspahr D, Finch CA. Intact transferrin receptors in human plasma and their relation to erythropoiesis. Blood 1990;75(1):102-7.

174. Fessas P, Yatachanas, X. Intraerythroblastic instability of hemoglobin β-4 (Hgb H). Blood 1968;31(3):323-31.

175. Sawada K, Krantz SB, Kans JS, Dessypris EN, Sawyer S, Glick AD, et al. Purification of human erythroid colony-forming units and demonstration of specific binding of erythropoietin. J Clin Invest 1987;80(2):357-66.

176. Sawada K, Krantz SB, Dessypris EN, Koury ST, Sawyer ST. Human colony-forming units-erythroid do not require accessory cells, but do require direct interaction with insulin-like growth factor I and/or insulin for erythroid development. J Clin Invest 1989;83(5):1701-9.

177. Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res 1989;17(15):6419.

178. Higuchi M, Singh S, Chen H, Aggarwal BB. Protease inhibitors differentially regulate tumor necrosis factor-induced apoptosis, nuclear factor-κB activation, cytotoxicity, and differentiation. Blood 1995;86(6):2248-2256.

179. Trubiani O, De Fazio P, Pieri C, Mazzanti L, Di Primio R. Nuclear matrix provides linkage sites for translocated NF-κB: morphological evidence. Histochem Cell Biol 2000;113(5):369-77.

180. Romano MF, Lamberti A, Bisogni R, Garbi C, Pagnano AM, Auletta P, et al. Amifostine inhibits hematopoietic progenitor cell apoptosis by activating NF-κB/Rel transcription factors. Blood 1999;94(12):4060-6.

181. Frogheri L, Doore, F.,Bofigli, S., Pistida, P., Guiso, L., Longinott, M. Bcl-x expression in circulating erythroblasts from patients with ineffective erythropoiesis. Hematologica 1998 (Abstract).

182. Digicaylioglu M, Lipton SA. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-κB signalling cascades. Nature 2001;412(6847):641-7.

183. Franke TF, Cantley LC. Apoptosis. A Bad kinase makes good. Nature 1997;390(6656):116-7.

184. Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, et al. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFκB activation in primary hippocampal neurons. J Biol Chem 1999;274(13):8531-8.

185. Dixon EP, Stephenson DT, Clemens JA, Little SP. Bcl-x short is elevated following severe global ischemia in rat brains. Brain Res 1997;776(1-2):222-9.

186. Suk K, Chang I, Kim YH, Kim S, Kim JY, Kim H, et al. Interferon gamma (IFNγ) and tumor necrosis factor alpha synergism in ME-180 cervical cancer cell apoptosis and necrosis. IFNγ inhibits cytoprotective NF-κB through STAT1/IRF-1 pathways. J Biol Chem 2001;276(16):13153-9.

187. Cohen GM, Sun XM, Snowden RT, Ormerod MG, Dinsdale D. Identification of a transitional preapoptotic population of thymocytes. J Immunol 1993;151(2):566-74.

188. He L, Lemasters JJ. Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function? FEBS Lett 2002;512(1-3):1-7.

APPENDIX

APPENDIX

Appendix 1 Raw hematologic data of Thai normal subjects.

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)
1	BW	M	4.09	13.1	36.9	90.2	31.9	35.4	ND
2	CK	F	4.18	12.8	37.4	89.4	30.7	34.4	1.3
3	KP	M	5.15	14.9	42.2	81.9	29	35.4	1.8
4	NS	F	4.55	13.6	40.6	89.1	29.9	33.6	1.2
	X		4.49	13.6	39.3	87.7	30.4	34.7	1.4
	SD		0.48	0.9	2.5	3.9	1.2	0.9	0.3
	Min		4.09	12.8	36.9	81.9	29	33.6	1.2
	Max		5.15	14.9	42.2	90.2	31.9	35.4	1.8

Appendix 2 Raw hematologic data of β-thalassemia/HbE patients. All patients are splenectomized subjects, except SN.

1. Erythropoiesis assessment

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)
1	SN	F	5.44	8.8	29.4	54.0	16.2	29.9	Not done
2	SaS	F	2.93	6.0	20.2	69.2	20.6	29.7	18.0
3	SP	F	2.91	6.2	21.0	72.3	21.2	29.4	25.1

2. The study of dose effect of EPO and IFN-γ on viability of thalassemic ECFCs

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)
1	WP	M	2.66	5.5	20.6	77.3	20.7	26.7	Not done

3. The study of dose effect of CsA on viability of thalassemic ECFCs

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)
1	AT	M	3.85	7.1	24.3	63.1	18.4	29.1	3.9
2	PT	F	3.77	7.4	25.1	66.4	19.6	29.6	Not done

4. The study of dose effect of CsA on apoptotic induction of thalassemic ECFCs

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)
1	TL	M	2.85	5.5	19.1	67.1	19.3	28.8	33.9
2	AS	M	3.4	6.4	21.2	62.2	18.7	30.1	6.1
3	PK	M	2.97	5.8	19.6	66.1	19.4	29.3	36.1

5. The study of the relative apoptotic induction by CsA in normal and thalassemia

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Retic (%)
1	RH	F	2.59	5.0	17.2	66.3	19.3	29.1	25.1
2	TL	M	2.85	5.5	19.1	67.1	19.3	28.8	33.9
3	PK	M	2.97	5.8	19.6	66.1	19.4	29.3	36.1
4	NpS	M	3.25	6.5	21.3	65.5	20.1	30.7	22.1
5	NthS	F	2.93	6.3	21.5	73.4	21.5	29.3	26.9
6	SsP	F	4.65	7.3	25.6	55.0	15.8	28.7	4.6

6. Investigation of NF-κB expression in thalassemic ECFCs by confocal microscopy

No	ID	Sex	RBC ($x10^6$/mL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC G/dL)	Retic (%)
1	PK	M	2.97	5.8	19.6	66.1	19.4	29.3	36.1
2	SaS	F	2.93	6.0	20.2	69.2	20.6	29.7	18.0
3	KS	F	3.12	6.0	21.6	69.3	19.2	27.7	26.5

Appendix 3 Some CD markers on cell surface of hematopoietic cells.

CD	Positive staining
CD2	- Thymocytes (95%), mature peripheral T cells (almost all), NK cells (80-90%), thymic B cells (50%)
CD3	- Thymocytes, peripheral T cells, NK cells
CD11b	- Follicular dendritic cells, granulocytes, macrophages, myeloid cells beginning with promyelocytes, NK cells, some B/T cells
CD14	- Macrophages/monocytes (90%), granulocytes-weak (30%); dendritic cells, B cell
CD15	- Myeloid cells (90%); activated B and T cells; eosinophils stain intensely
CD16	- NK cells (10-20%), granulocytes, monocytes (rare), alveolar macrophages (common), T cells (some)
CD19	- Pre B, B cells; follicular dendritic cells
CD24	- All B cells, granulocytes, epithelial cells
CD34	- Hematopoietic progenitor cells
CD36	- Platelets, macrophages, endothelial cells, early erythroid cells, megakaryocytes
CD45	- All hematopoietic cells, stronger in lymphocytes (10% of surface area)
CD56	- NK cells (80-90%), activated T cells
CD66b	- Granulocytes
CD71	- All proliferating cells plus iron requiring cells: reticulocytes, erythroid precursors

Appendix 4 Documentary proof of ethical clearance

No. 3/2002

Documentary Proof of Ethical Clearance

The Committee on Human Rights Related to

Human Experimentation

Mahidol University, Bangkok

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Title of Project : **Regulation of Erythropoiesis and Apoptosis in Thalassemia**

Principal Investigator : **Dr. Pranee Fucharoen**

Name of Institution : **Thalassemia Research Center, Institute of Science and Technology for Research and Development**

Approved by the Committee on Human Rights Related to Human Experimentation

Signature of Chairman : S. Danchaivijitr

(Professor Dr. Somwang Danchaivijitr)

Signature of Head of Institute : P.M.

(Professor Dr. Pornchai Matangkasombut)

Date of Approval : 11 FEB 2002

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