CLONING AND EXPRESSION OF BIOLOGICAL ACTIVE HUMAN ERYTHROPOIETIN IN MAMMALIAN CELLS

NIWED KULLAWONG

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Niwed Kullawong

CLONING AND EXPRESSION OF BIOLOGICAL ACTIVE HUMAN ERYTHROPOIETIN IN MAMMALIAN CELLS

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ABSTRACT

Erythropoietin (EPO) is a hematopoietic growth factor which stimulates erythropoiesis (red blood cell production). One widely used therapeutic hormone is a well-known recombinant human erythropoietin (rHuEPO) produced from genetically engineered Chinese Hamster Ovary cells. This 34-kDa glycoprotein is produced primarily by the kidney. During the post-translational modification, glycosylation occurs with the addition of 3 *N*-linked (at Asn-24, -38 and -83) and one *O*-linked (at Ser-126) acidic oligosaccharides. rHuEPO has been used for the treatment of anemia-associated diseases including rheumatoid arthritis, AIDS, and cancer. This recombinant product is prescribed on the assumption that it will increase patients' Hb level, reduce the need for transfusion and tumor progression, and thus improve quality of life and survival.

This work has established a CHO based expression system to produce active rHuEPO applicable for large scale pharmaceutical production. After cloning, selection and 4-rounds of DHFR/MTX-mediated gene amplification, the stable CHO cell lines' efficiency in secreting rHuEPO were established. The expressed rHuEPO migrated in SDS-PAGE as a broad band of 40-45 kDa which was comparable with rHuEPO reference (HEMAX®). This protein was shown to have all three N-linked glycosylation sites and its carbohydrate structure termini were capped with the functional sialic acid residuals. By analysis of *in vitro* interaction to its receptor (EPO-R), rHuEPO was proven to be biologically active by colorimetric MTT assay after stimulation of factor-dependent TF-1 cell proliferation in dose-dependent manner. Moreover, normocytic BALB/c mice injection was employed to confirm its bioactivity *in vivo*. Without any purification, the rHuEPO expressed in this study showed its principal function in physiological conditions by significantly increasing % reticulocyte in testing animal. Thus, these cell lines can be applied for large-scaled production in the biopharmaceutical industry in Thailand in the future.

KEY WORDS: ERYTHROPOIETIN (EPO) / GENE EXPRESSION / GENE AMPLIFICATION / DIHYDROFOLATE REDUCTASE / METHOTREXATE

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การสร้างและคัดเลือกเซลล์ที่แสดงออก ERYTHROPOIETIN ของมนุษย์ในเซลล์สัตว์เลี้ยงลูกด้วยนม (CLONING AND EXPRESSION OF BIOLOGICAL ACTIVE HUMAN ERYTHROPOIETIN IN MAMMALIAN CELLS)

นิเวศน์ กุลวงค์ 4636871 MBMG/M

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บทคัดย่อ

Erythropoietin (EPO) เป็นฮอร์โมนที่ผลิตมาจากไต ทำงานเกี่ยวข้องกับการเร่งการสร้างเม็ดเลือดแดงในร่างกาย recombinant human erythropoietin (rHuEPO) ที่ใช้กันทั่วไปซึ่งสร้างมาจาก Chinese hamster ovary (CHO) cells ด้วยเทคนิคการตัดต่อทางพันธุกรรม เป็นฮอร์โมนบำบัดโรค EPO เป็นโปรตีนที่มีขนาด 34 kDa ซึ่งมีคาร์โบไฮเดรตเป็นส่วนประกอบสำคัญโดยในโมเลกุลประกอบด้วยโครงสร้างน้ำตาลชนิด N-linked อยู่สามตำแหน่งที่กรดอะมิโน Asn 24 38 และ 83 และชนิด O-linked อยู่หนึ่งตำแหน่งที่กรดอะมิโน Ser 126 rHuEPO ถูกใช้ในผู้ป่วยที่มีภาวะโลหิตจางจากโรคต่างๆ รวมทั้ง โรคข้ออักแสบรูมาตอยด์ เอดส์และมะเร็ง เนื่องจากสามารถเพิ่มระดับฮีโมโกลบิน ลดการเปลี่ยนถ่ายเลือด เพิ่มคุณภาพชีวิตและเพิ่มอัตราการรอดชีวิตของผู้ป่วยได้ งานวิจัยนี้ได้พัฒนาระบบการผลิต rHuEPO จาก CHO cells ซึ่งเหมาะสำหรับการผลิตในเชิงอุตสาหกรรมในอนาคต หลังจากผ่านกระบวนการสร้างและคัดเลือก และขบวนการเพิ่มการแสดงออกของยีนแล้วสามารถสร้างกลุ่มของเซลล์ยั่งยืนที่ผลิต rHuEPO ได้สำเร็จ โปรตีนที่สร้างได้เคลื่อนที่ใน SDS-PAGE ในลักษณะแบนกว้างซึ่งเทียบเคียงกับ rHuEPO อ้างอิง (HEMAX®) โดยพบโครงสร้างน้ำตาลชนิด N-linked ได้ทั้งสามตำแหน่ง และพบว่ามี sialic acid เป็นส่วนประกอบสำคัญของโครงสร้างน้ำตาลเหล่านี้ด้วย ส่วนการศึกษาการทำงานของโปรตีน พบว่าสามารถกระตุ้นการเพิ่มจำนวนของเซลล์ต้นกำเนิดเม็ดเลือดแดง (TF-1 cells) ผ่านทางตัวรับ (EPO receptor) ได้ในหลอดทดลอง ส่วนการกระตุ้นการสร้างเซลล์เม็ดเลือดแดงในหนูทดลอง (BALB/c) นั้นพบว่าสามารถเพิ่มจำนวนเม็ดเลือดแดงตัวอ่อน (reticulocyte) ได้และเทียบเคียงได้กับโปรตีนอ้างอิง จึงกล่าวได้ว่าการศึกษานี้ได้ผลิตเซลล์ยั่งยืนที่สามารถนำไปใช้ในการบวนการชีวอุตสาหกรรมต่อไปได้

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LIST OF ABBREVIATIONS

A_{260}	=	absorbance at 260 nonometers
A_{280}	=	absorbance at 280 nonometers
A	=	adenine
aa	=	amino acid(s)
Arg	=	arginine
Asn	=	asparagine
BFU-E	=	burst-forming unit-erythroid
BHK	=	baby hamster kidney
bp	=	base pair(s)
^{o}C	=	degree Celcius
C	=	cytosine
cDNA	=	complementary DNA
CHO	=	Chinese Hamster Ovary murine cell line
CMV	=	cytomegalovirus
Cys	=	cysteine
dATP	=	deoxyadenosine-5'-triphosphate
dCTP	=	deoxycytosine-5'-triphosphate
dGTP	=	deoxyguanosine-5'-triphosphate
DHFR	=	dihydrofolate reductase
dTTP	=	deoxythymidine-5'-triphosphate
DEAE	=	diethylaminoethyl
DIG	=	digoxigenin
DMEM	=	Dulbecco's Modified Egle Medium
DMSO	=	dimethyl sulfoxide
DTT	=	dithiothreitol

LIST OF ABBREVIATIONS (Continued)

E. coli	=	*Eschericia coli*
EDTA	=	ethylene diamine tetraacetic acid
EPO	=	erythropoietin
EPO-R	=	erythropoietin receptor
FBS	=	fetal bovine serum
FCS	=	fetal calf serum
G	=	guanine
g	=	gram(s)
xg	=	x gravity or centrifugal force
GM-CSF	=	granulocyte/macrophage colony-stimulating factor
GST	=	glutathione S transferase
Hb	=	hemoglobin
Hct	=	hematocrit
HIF-1	=	hypoxia inducible factor-1
hr	=	hour(s)
IgG	=	immunoglobulin G
IL	=	interleukin
kb	=	kilobase(s)
kDa	=	kilodalton(s)
MAA	=	*Maackia amurensis* agglutinin
mcl-1	=	Induced myeloid leukemia cell differentiation protein
MEM	=	Minimal essential medium
mg	=	milligram(s)
mg/ml	=	milligrams/milliliter
min	=	minute(s)
μg	=	microgram(s)
μl	=	microliter(s)

LIST OF ABBREVIATIONS (Continued)

ml	=	milliliter(s)
μM	=	micromolar
mM	=	millimolar
Mr	=	relative molecular mass
MTT	=	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl-tetrazolium bromide
MTX	=	methotrexate
MW	=	molecular weight
nM	=	nanomolar
nt	=	nucleotide(s)
OD	=	optical density
P/S	=	penicillin/streptomycin
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
pg	=	picogram(s)
pmol	=	picomole
PMSF	=	phenylmethylsulfonyl fluoride
pO_2	=	oxygen pressure
PVDF	=	polyvinylidene difluoride
RBCs	=	red blood cells
rHuEPO	=	recombinant human erythropoietin
rpm	=	revolution per minutes
RT-PCR	=	reverse transcription-polymerase chain reaction
RTase	=	reverse transcriptase
sc	=	subcutaneous
SDS	=	sodium dodacylsulfate
sec	=	second(s)
Ser	=	serine
SH2	=	Src homology-2

LIST OF ABBREVIATIONS (Continued)

SH-PTP	=	SH2-containing protein-tyrosine phosphatase
STAT	=	signal transducer and activator of transcription
STD	=	standard
T	=	thymine
v/v	=	volume / volume
w/v	=	weight / volume
NFκB	=	nuclear factor kappa B

CHAPTER I
INTRODUCTION

1.1. Hunan Erythropoietin (EPO)

1.1.1. Overview

The production of red blood cells (RBCs) is controlled by an intricate interaction between various factors and cytokines. A positive correlation of hypoxia and anaemia with erythropoiesis has been documented through clinical observation, direct relationship of hypoxia to RBC count. EPO is the principal growth factor responsible for the regulation of RBC production during steady-state conditions and for accelerating the recovery of the circulating RBCs after acute blood loss. In early age, therapeutic EPO was isolated and purified from urine of patients with aplastic anemia (Miyake et al., 1977). Until the blooming of recombinant technology, EPO DNA probes were constructed and used to isolate and clone its corresponding gene in 1985 (Jacobs et al., 1985). The US Food and Drug Administration approved EPO use in 1989, and it is now widely used for the treatment of anemia associated with renal failure, cancer, prematurity, chronic inflammatory disease and human immunodeficiency virus (HIV) infection (Jelkmann, 1992).

1.1.2. Biochemistry and molecular biology

1.1.2.1. Gene mapping

Human EPO gene was mapped to chromosome 7pter-q22 (Watkins et al., 1986). The human EPO gene spans over a 3.6 kb in the human genome and comprises five exons and four introns (Lin et al., 1985). The 3.9 kb of the 5' flanking sequence contains a classical canonical CAAT box, TATA boxes and other transcriptional regulatory elements and the 1.8-kb extended 3' flanking region contains the following regulatory elements: nitrogen-regulatory/oxygen-sensing consensus sequences, tissue-specific regulatory elements, a lymphokine-responsive element, as well as binding

sites for AP and Sp1 (Lee-Huang et al., 1993). To date, EPO genes have been isolated from many species as shown in multiple amino acid sequence alignment (Figure 1). Analysis of these EPO homologues revealed that high degree of homology is not restricted to the amino acid coding regions but also in the 3' uncoding regions (Lee-Huang et al., 1993).

1.1.2.2. Molecular structure

The human EPO gene encodes a 193-amino acid residue propolypeptide divided into a 27-amino acid leader sequence and a 166-amino acid mature protein with a calculated molecular mass of 18,399 Da (Lin et al., 1985) (Figure 2). However, EPO in blood circulation consists of 165 amino acids resulted from post-translational cleavage of the last residue on its carboxyl terminus (Arg-166). Human EPO contains two intra-chain disulfide bonds (Cys7-161 and Cys29-33), one O-linked (Ser-126) and three N-linked (Asn-24, Asn-38 and Asn-83) glycosylation sites. Circular dichroism spectral analysis has proposed that its secondary structure contains 50% of α-helix moiety. The remaining structure is mainly random and no obvious β–sheet structure could be observed (Lai et al., 1986) (Figure 3, A). The tertiary EPO structure is defined by four antiparallel α-helices similar that of growth hormone (Wen et al., 1994).

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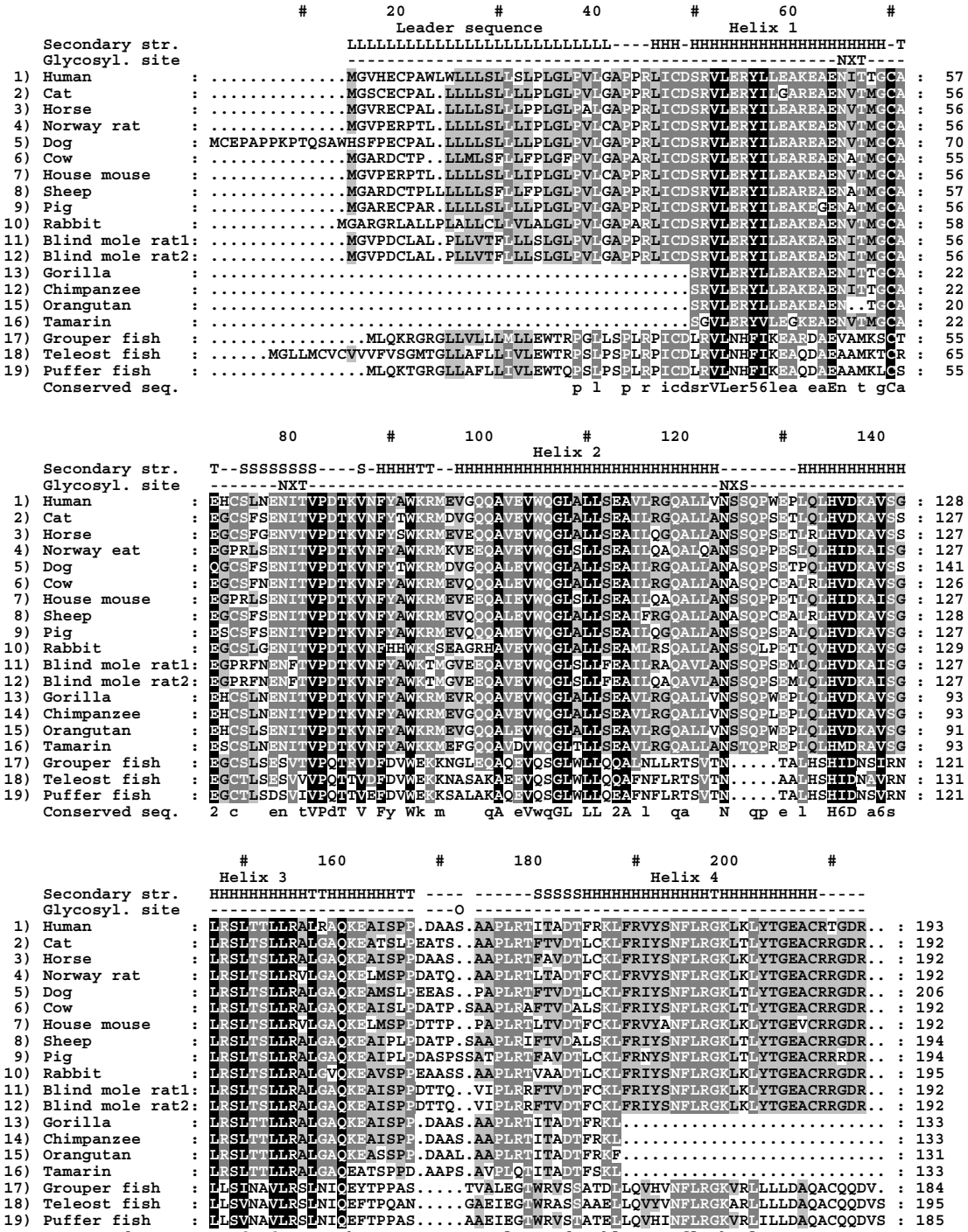
Leader sequence
Helix 1
Helix 2
Helix 3
Helix 4
Secondary str.
Glycosyl. site
1) Human
2) Cat
3) Horse
4) Norway rat
5) Dog
6) Cow
7) House mouse
8) Sheep
9) Pig
10) Rabbit
11) Blind mole rat1
12) Blind mole rat2
13) Gorilla
14) Chimpanzee
15) Orangutan
16) Tamarin
17) Grouper fish
18) Teleost fish
19) Puffer fish
Conserved seq.

Figure 1 Multiple amino acid sequence alignment (MSA) of human EPO and other species showing sequence similarity, conserved positions, glycosylation sites

The sequences alignment was performed by Window-based AlignX program (with blosum62) (a component of Vector NTI Suite 6.0). The secondary structure (L; leader sequence, H; helix, S; strand and T; turn), N-linked- (N-X-T/S), and O-linked (O) glycosylation sites are based on the human EPO structure. All sequences were retrieved from Genbank on NCBI server:

1) NP_000790.1 *Homo sapiens* (Human)
2) NP_001009269.1 *Felis catus* (Cat)
3) BAC55239.1 *Equus caballus* (Horse)
4) NP_058697.1 *Rattus norvegicus* (Norway rat)
5) NP_001006647.1 *Canis familiaris* (Dog)
6) NP_776334.1 *Bos taurus* (Cow)
7) NP_031968.1 *Mus musculus* (House mouse)
8) CAA80848.1 *Ovis aries* (Sheep)
9) NP_999299.1 *Sus scrofa* (Pig)
10) AAG36962.1 *Oryctolagus cuniculus* (Rabbit)
11) CAG29400.1 *Spalax galili* (Blind mole rat1)
12) CAG29397.1 *Spalax golani* (Blind mole rat2)
13) AAM76633.1 *Gorilla gorilla* (Gorilla)
14) AAM76632.1 *Pan troglodytes* (Chimpanzee)
15) AAM76634.1 *Pongo pygmaeus* (Orangutan)
16) AAM76636.1 *Saguinus oedipus* (Cotton-top tamarin)
17) AAW29029.1 *Epinephelus coioides* (Orange-spotted grouper fish)
18) AAR25698.1 *Tetraodon nigroviridis* (Teleost fish)
19) AAQ72467.1 *Takifugu rubripes* (Puffer fish or Fugu rubripes)

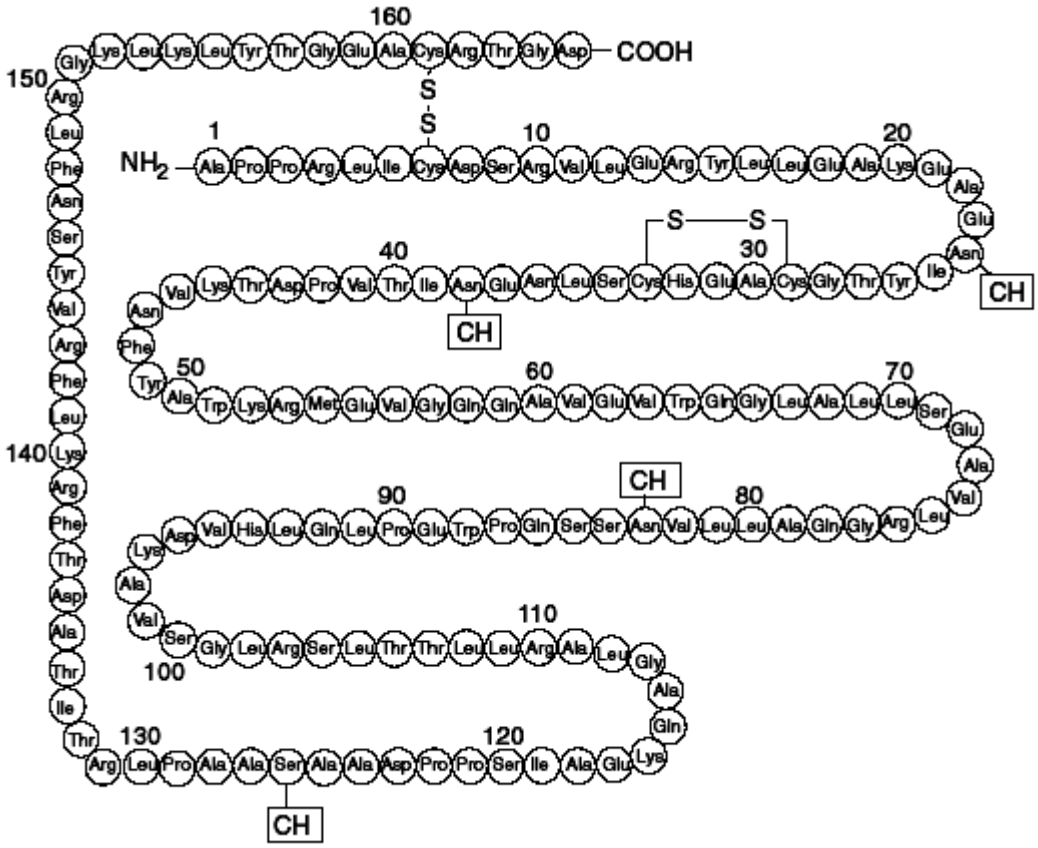

Figure 2 The primary structure of mature EPO (Lappin, 2003)

The 27-amino acid leader sequence and carboxyl Arg-166 are cleaved off. Two disulfide bonds tether the molecule together between Cys-29 and 33 and Cys-6 and 161. Three N-linked sugars are present at Asn-24, 38, and 83, and one O-linked sugar is present at Ser-126.

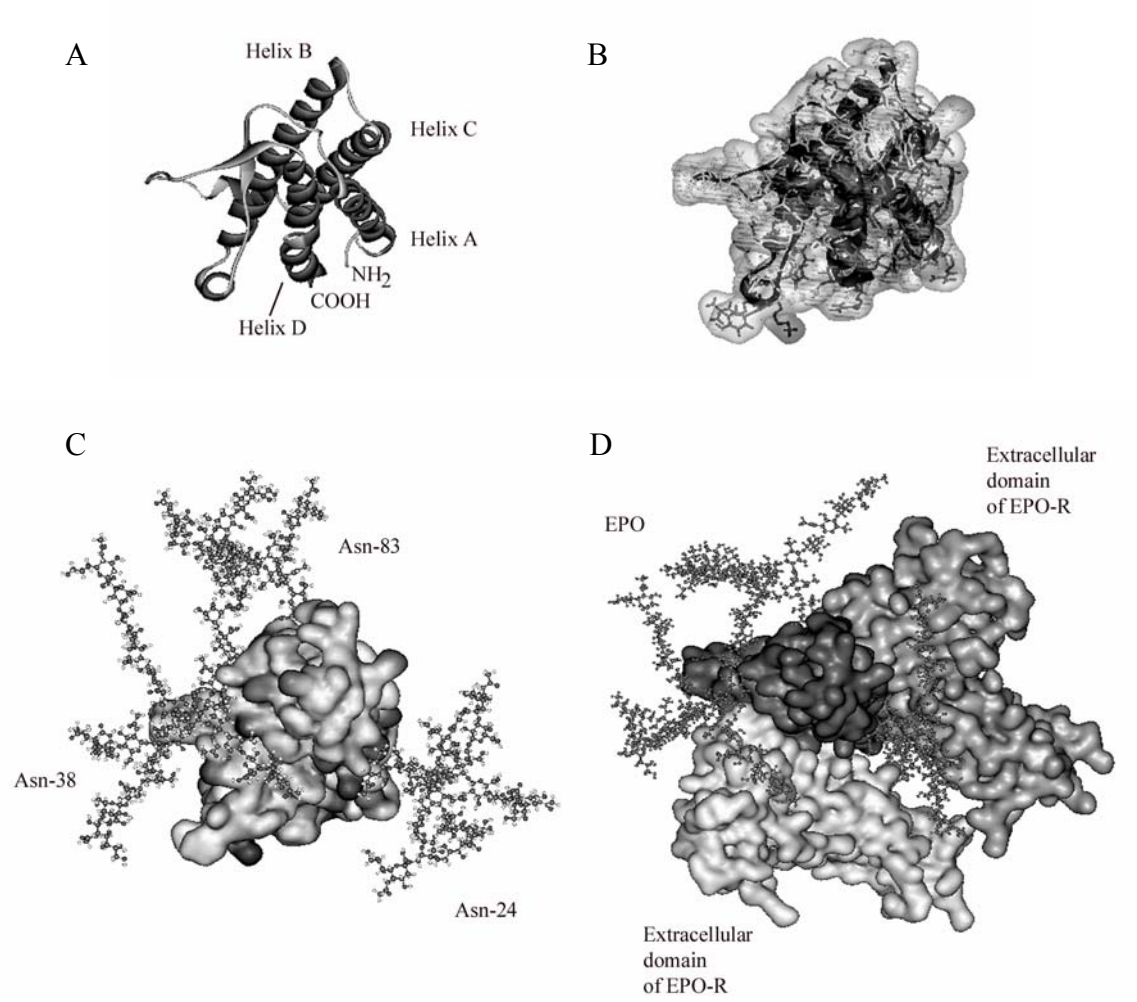

Figure 3 *In silico* simulation of tertiary structure of human EPO

This simulation was generated by using WebLab ViewerPro 3.7 tools, (A) ribbon structure without N-glycans, (B) space filled structure by VDW force, (C) space filled structure with three N-glycans (Tetra-antenary, complex type), (D) EPO binding to extracellular domain of EPO-Rs (modified form X-ray diffraction structure, Syed at al., 1998). *In silico* glycosylation was performed by using web-based tool GlyProt on http://www.dkfz-heidelberg.de/spec/glyprot/php/main.php. This simulation connects only one N-glycan conformation out of a manifold to the protein scaffold. N-glycans are highly flexible molecules that normally exist in a variety of rapidly converting conformations. Also the influence of the attached N-glycan on conformational changes of the protein is not evaluated.

1.1.2.3. Glycosylation and carbohydrate structure in EPO polypeptide

Studies of glycoprotein hormones have demonstrated wide variety of functions for the carbohydrate moieties on the molecule. The predominant sugars found on human glycoproteins include galactose, mannose, fucose, N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and N-acetylneuraminic acid (Neu5Ac); the human form of sialic acid. The carbohydrate chains of EPO may affect its conformation leading to changes in biosynthesis, secretion, solubility, susceptibility to protease and other denaturing conditions. For the first aspect, it has been evidenced that glycans of Asn-38 and Asn-83 play an important role since the prevention of one of these two glycosylation sites by site directed mutagenesis abolishes EPO secretion (Dube et al., 1988; Delorme et al., 1992). For the second point, several studies have shown that the capping of external galactose residuals by sialic acid prevented its binding to hepatic asialoglyprotein binding lectin, a process for EPO destruction (Fukuda et al., 1989; Spivak & Hogans, 1989; Imai et al., 1990; Higuchi et al., 1992). The proper glycosylation of EPO is essential for its function *in vivo*. Removal or modification of the glycan chain results in altered *in vivo* and *in vitro* activity. The N-linked carbohydrates of human EPO have three main functional units: the main core, the branched portion and the terminal component (Figure 4) (Ng et al., 2003). The sugar chains comprise 40% of the molecular weight of recombinant human EPO (Sasaki et al., 1987; Takeuchi et al., 1988). At present, there are three forms of rHuEPO available for clinical use. They are classified as epoetin alfa, epoetin beta, and epoetin omega according to the manufacturing method. Epoetin alfa and beta are both produced in CHO cells, whereas epoetin omega is produced in baby hamster kidney (BHK) cells. Oligosaccharide structures of recombinant proteins appear to be dependent on expression methods and culture conditions (Storring et al., 1998; Kanazawa et al., 1999). Determination of charge state for the oligosaccharide structures from rHuEPO was demonstrated by weak anion exchange chromatography based on the method of Guile et al. (1994) as shown in Figure 5. From the carbohydrate composition studies, the major carbohydrate units of EPO are tetra-antennary saccharides with or without N-acetyl-lactosamine repeats.

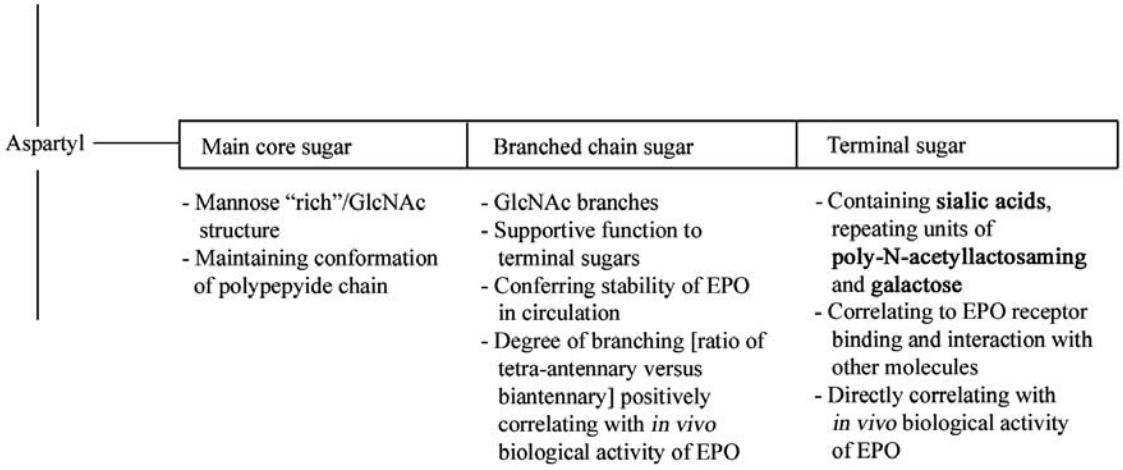

Figure 4 Outline of the functional units from N-glycosylation moiety of EPO.

Carbohydrate branch attached to Asn residues on EPO molecule is divided into main core-, branch chain- and terminal sugar, respectively (Ng et al., 2003).

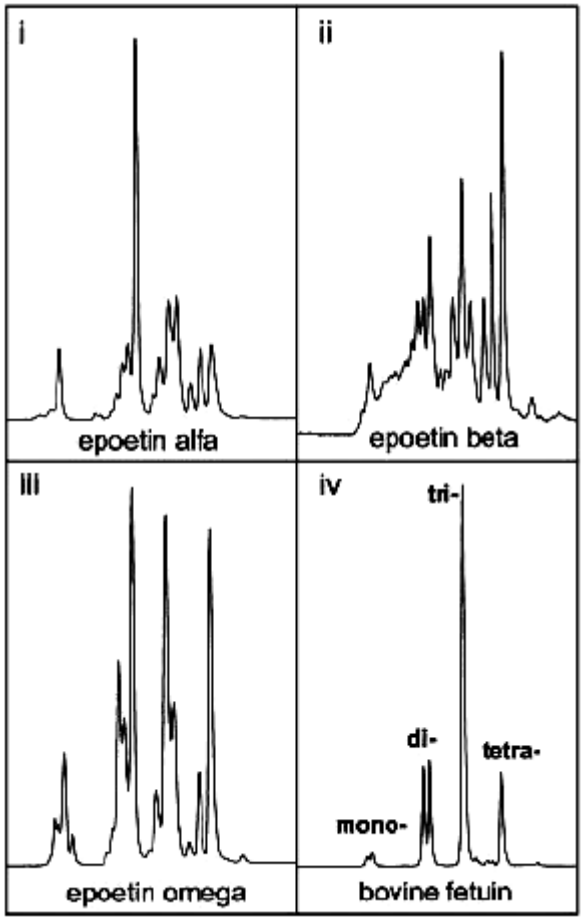

Figure 5 Determination of oligosaccharide complexity of rHuEPOs

Determination of charge state for the oligosaccharide structures from rHuEPO was demonstrated by weak anion exchange chromatography (Skibeli et al., 2001).

Panel i: glycans from epoetin alfa (20 pmol).

Panel ii: glycans from epoetin beta (20 pmol).

Panel iii: glycans from epoetin omega (20 pmol).

Panel iv: glycans from bovine fetuin (20 pmol).

1.1.2.4. Sialiation

Sialic acids are typically found at the outermost ends of N-glycans, O-glycans, glycosphingolipids of mammalian glycoprotein and occasionally capping the side chains of GPI anchors. Furthermore, the number of sialic acid residuals and branching pattern of the N-linked oligosaccharides modify the pharmacodynamics and biological activity of EPO *in vivo*. The degree of sialiation of the oligosaccharides from 4 batches of rHuEPO from transfected CHO cells was determined by Rice et al. (1992) to be 80–88%. However, they did not report on the characteristics of the cell culture from which the EPO was purified nor did they explore the culture parameters that could give rise to variability in the degree of sialiation. In addition, CHO cells do not express sialyl-α2→6 transferase, all of these saccharides expressed from these cells is sialyated by α2→3 sialyl linkages (Takeuchi et al., 1988).

1.1.3. EPO production

1.1.3.1. Sites of production

The red cell mass is continuously adjusted to optimal size as an oxygen carrier by a feedback mechanism and EPO is the hormone mediator of this feedback (Jacobs et al., 1985). Kidney has been proven to be the primary site of EPO production in adult whereas the liver is the primary production site in the fetus before birth (Wintour et al, 1996). By using *in situ* hybridization (Koury et al., 1988) or RT-PCR (Mujais et al., 1999), peritubular (interstitial or endothelial) and tubular location are the cellular site of production in mice.

1.1.3.2. Control of production

Regulation of EPO gene expression occurs mainly at the transcriptional level, although it is tissue specific, developmentally controlled, and inducible (Lee-Huang et al., 1993). A 256-bp of 3' flanking sequence was shown to bind four or more different nuclear factors and this region functioned as a hypoxia-inducible enhancer (Semenza et al., 1991). In addition, Wang & Semenza (1993) have identified a 120-kD DNA-binding protein, hypoxia-inducible factor 1 (HIF-1) that bound to part of this region.

1.1.4. Mechanism of action

1.1.4.1. EPO receptor (EPO-R)

A gene for a human EPO receptor has been isolated and mapped to the p region of chromosome 19 (Budarf et al., 1990). The cDNA of this receptor predicts to encode a 55 kDa, 508 amino acid residual transmembrane protein comprised of a 24 amino acid signal peptide, a 226 amino acid external segment, a 22 amino acid transmembrane segment, and a 236 amino acid cytoplasmic domain (Youssoufian et al., 1993). Similarities between the primary and tertiary structure of the receptor of EPO, IL-2, IL-3, IL-4, IL-6, GM-CSF, growth hormone and prolactin have suggested the occurrence of a cytokine receptor "superfamily" (Bazan, 1989). The gene encoding EPO receptor comprises of eight exons and seven introns, encoding a 507 amino acid peptide (66 kDa), for human EPO-R. In the human gene, exons 1–5 encode the 251-amino acid extracellular domain, exon 6 encodes a 20-amino acid membrane-spanning region, and exons 7 and 8 encode the 236-amino acid intracellular or cytoplasmic domain (Sawyer et al. 1989). Functional receptors for EPO, in fact, have been found in both erythroid blood cell line and non-erythroid blood cell lines, such as myeloid cells, lymphocytes and megakariocytes, and also in a wide variety of non-haematopoietic cells, such as endothelial cells, mesangial, myocardial and smooth muscle fibre cells. In the central nervous system (CNS), neurons express EPO-R and astrocytes produce EPO (Sakanaka et al., 1998) which support anti-apoptotic activity in these neuronal cells.

1.1.4.2. EPO responsive cells

Erythropoiesis is the process whereby a fraction of primitive multipotent hemopoietic stem cells becomes committed to the red cell lineage. The EPO receptor is apparently expressed primarily on the CFU-E and the pronormoblast stage of erythroid cell development (Sawada et al., 1990). CFU-E expresses EPO-R with the highest level among the erythroid cell lineage and the number of EPO receptors per cell gradually decreases during erythroid cell differentiation. Furthermore, it has been shown that reticulocyte and mature erythrocyte do not contain EPO receptors (Sawada et al., 1990; Wickrema et al., 1992).

1.1.4.3. Signal transduction mechanism

EPO-induced intracellular signaling occurs through a rapid tyrosine phosphorylation of several proteins even though the EPO receptor does not possess endogenous tyrosine kinase activity. First, intracellular signaling occurs by activating the JAK2 tyrosine kinase, which is pre-associated with the EPO receptor constitutively in the transmembrane region (see Figure 6). Upon EPO bind to receptor, eight tyrosine residues in the cytoplasmic domain of the EPO receptor are phosphorylated. Tyrosine phosphorylation of the EPO-R creates "docking sites" for SH2 domain(s) in signaling molecules such as the protein tyrosine phosphatases (SH-PTP), phosphoinositide 3-kinase (PI3 kinase), and STAT5 (Klingmuller et al., 1997). Acting via its receptor (EPO-R), EPO up-regulates bcl-2 and inhibits apoptosis of erythroid cells and rescues neurons from hypoxic damage (Acs et al., 2003). Moreover, addition of exogenous EPO to tumor cell lines expressing EPO-R increased nuclear DNA binding activity of NFκB and increased the expression of the antiapoptotic genes bcl-1, bcl-xL, and mcl-1 (Batra et al., 2003).

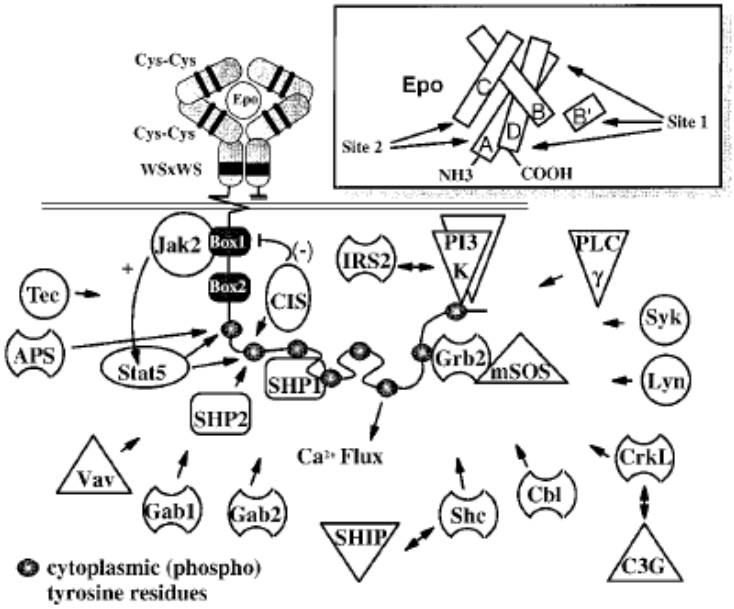

Figure 6 Signal transduction pathway of EPO-R

Upon binding sites 1 and 2 of a single EPO molecule, Jak2 is activated by autophosphorylation and phosphorylates eight EPO receptor cytoplasmic (P)Y sites. Twenty-one (or more) signal transduction factors then are recruited and activated. Factors known to associate with activated Epo receptor complexes include STAT5, Cis1, three PTKs (Lyn, Syk, and Tec), two PTPs (SHP1 and 2), three phospholipid-modifying enzymes (PI3-K, PLC-g, and SHIP), eight molecular adaptors (Grb2, Shc, Cbl, CrkL, APS, IRS-2, and Gab1 and 2), and three nucleotide exchange factors (Vav, C3G, and mSOS) (Wojchowski et al., 1999).

1.1.5. Heterologous EPO expression

In order to obtain recombinant proteins, many kinds of heterologous gene expression systems have been used. The most useful hosts are bacteria, such as *Escherichia coli* (Shibui & Nagahari, 1992; Kujau et al., 1998), yeasts such as *Schizosaccharomyces pombe* (Smerdon et al., 1998) and *Saccharomyces cerevisiae* (Wood et al., 1985), and mammalian cells, such as Chinese hamster ovary cells (Ma et al., 1988) and mouse myeloma cells NS0 (Bebbington et al., 1992). The production of recombinant proteins in mammalian cells offers many advantages over that in prokaryotic cells. In most cases, mammalian cell systems do not need refolding of the product and are able to introduce posttranslational modifications which are often essential for their full biological activity (Jenkins & Curling, 1994).

Many recombinant proteins used in medicine are relatively small and simple in their structure, thus biologically functional proteins can be produced in prokaryotes such as *E. coli*. However, some human proteins of medical interest are more complicated in that biological function requires posttranslational modification. For example, EPO is extensively glycosylated with the carbohydrate portion accounting for 40% of its molecular mass. It has been shown that the carbohydrate portion of EPO is important for biological function. EPO produced in *E. coli* or yeast is inactive or very weakly active *in vivo*, while that produced in COS or CHO are fully active. The synthesis of recombinant human EPO (rHuEPO) from CHO cells has been well characterized (Sasaki et al., 1987; Takeuchi et al., 1988).

1.1.6. Clinical interest of EPO

Upregulation expression of EPO is occured in a number of adaptive and pathological conditions. For example, various types of secondary polycythemias are associated with the production of higher EPO than normal levels. The increasing of EPO level may be an adaptive response associated with conditions that produce tissue hypoxia, such as living at high altitude, chronic obstructive pulmonary disease, cyanotic heart disease, sleep apnea, high-affinity hemoglobinopathy, smoking or localized renal hypoxia (Jelkmann, 1992; Krantz, 1991; Porter & Goldberg, 1993). In

addition, certain renal tumor and hepatomas are also associated with high EPO levels and erythrocytosis (Kew & Fisher, 1986; Montagna et al., 1994). All of these conditions were associated with erythrocytosis.

Deficient (or inefficient) EPO production relative to hemoglobin level is also associated with certain forms of anemia. These include anemia of renal failure and end-stage renal disease, anemia of chronic disorders (chronic infection and rheumatoid arthritis), autoimmune disease, AIDS, and malignancy (Kendall, 2001). Many of these conditions are associated with the generation of IL-1 and a factor that has been shown to be an inhibitor of EPO activity (Jelkmann et al., 1992; Jelkmann, 1994). However in certain anemias are found EPO-independent. These include aplastic anemia, iron deficiency anemia, the thalassemia, megaloblastic anemia, pure red cell aplasia, and myelodysplastic syndromes.

1.1.7. Assays for EPO

For all of assay methods, the amount of EPO in a sample is determined by comparison to reference standard. EPO activities are usually expressed in international units (IU). One unit of EPO was originally defined as the activity that produced the same effect as 5 micromoles of cobalt. Significant differences in EPO activity were seen when assayed by different methods such as *in vivo, in vitro* and various immunological assays.

1.1.7.1. Immunological assay

Many different kinds of immunological assays for EPO have been used for investigations reported in the scientific literature. These include agar double diffusion, hemagglutination-inhibition (HI), radioimmunoassay (RIA), enzyme immunoassays, immunoradiometric, enzyme-linked immunoabsorbent assays (ELISA), and chemiluminescence immunoassays. Commercial EPO assays have employed the principles of hemagglutination-inhibition, radioimmunoassay, enzyme immunoassay and chemiluminescence immunoassay.

1.1.7.2. In vitro bioassay

In *in vitro* bioassay, EPO activity is assessed in short-term cultures of erythroid tissue taken from rats or mice, either from bone marrow, spleen or 12-15 day fetal mouse liver. One of the following three end points have been used for evaluation: (a) ^{59}Fe-incorporation into heam (Fried et al., 1957), (b) incorporation of precursor into RNA, DNA or glycosylated side chains, e.g., ^{125}I-deoxyuridine, ^{3}H-thymidine of ^{14}C-glucosamine uptake, or (c) growth of erythroid colonies from marrow erythroblasts (CFU-Es). In addition, recent investigations of rHuEPO activity estimations have been carried out using cell culture assay based on different cell lines, including AS-E2 (Miyazaki et al., 1997), TF-1 (Kitamura et al., 1989), HCD57, UT-7 and UT-7/EPO (Wen et al., 1994). The *in vitro* bioassays are less laborious and more sensitive than the *in vivo* bioassay. Their use has been gradually displaced by immunoassays that are both more sensitive and more specific. Nevertheless, the activity reported by *in vitro* assay may not accurate particularly for the unglycosylated EPO which is totally inactive *in vivo*.

1.1.7.3. In vivo bioassay

EPO *in vivo* bioassays measure the biological activity of EPO in an intact animal. The effect of the test material on the rate of red cell production as estimated by the reticulocyte count or by percentage of radioactive iron (^{59}Fe) incorporated into peripheral red blood cells is directly related to the amount of EPO injected. However, this assay will not detect normal or subnormal amount of EPO in human serum. Despite the primitive nature of the test, its expense, and laboriousness, this is the standard reference assay for others methods because it is the only test that measures the true *in vivo* biological activity of EPO.

1.2. Mammalian Expression Systems

1.2.1. COS-1 cells

COS cells are African green monkey kidney cells (CV-1) transformed with an origin-defective SV40 virus which has integrated into COS cell chromosomal DNA. Therefore, COS cells produce wild-type SV40 large T antigen but not viral particle. Since SV40 large T antigen is the only viral protein required in *trans* for viral replication, SV40 origin-containing plasmids replicate in these cells to a high copy number (10,000 to 100,000 copies/cells) with in 48 hr post-transfection. Under the control of appropriate promoter, COS cells will produce the protein at relatively high levels over a short period of time. COS-1 cell line is one of original three cell lines isolated (COS-1, COS-3 and COS-7). It contains only two integrated copies of the viral DNA (Gluzman, 1981).

1.2.2. CHO cells

Many amplifiable selection markers are now available for use in mammalian cells. Although various cell lines can be used for gene amplification, many protocols rely upon the use of CHO cells. The advantage of CHO cells for heterologous gene expression are (1) amplified genes that are intregrated into host chromosome may be stably maintained, even in the absence of continued drug selection; (2) a variety of proteins have been shown to express at high level; and (3) CHO cells can be adapted for suspension culturing and could be scaled up to >5,000 liters.

1.2.3. DHFR/MTX gene amplification system

Cell line development may include both sub-cloning the cell line to select higher producing clones and use of gene amplification. Drug resistance has been a tool of choice in the biopharmaceutical industry to induce gene amplification. For high-level expression of recombinant proteins, one of the most widely used mammalian expression systems is the gene amplification procedure offered by the use of dihydrofolate reductase-deficient (*dhfr*−) CHO cells (Reff, 1993; Trill et al., 1995). This system is based on *dhfr* gene coding for the DHFR enzyme that catalyzes the

conversion of folate to tetrahydrofolate (FH_4). This precursor is necessary for the *de novo* synthesis of purines, pyrimidines, and glycine (Goeddel, 1990) (Figure 7). Methotrexate (MTX) is a drug which is similar (i.e., an analog) to folate and binds to DHFR enzyme thereby inhibiting the production of dihydrofolate (FH_2) and tetrahydrofolate (FH_4). *dhfr*− CHO cells which up take an expression vector containing wild type *dhfr* gene can develop resistance to MTX by amplifying the *dhfr* gene. On the other hand, with insufficient levels of DHFR, cells are deprived of nucleoside precursors (hypoxanthine and thymidine) and die. Because the amplification unit is much larger than the size of the *dhfr* gene, the specific gene of interest colinked to the *dhfr* gene in the same expression vector or adjacently resides in the host chromosome is normally coamplified (Kaufman et al., 1983).

The emergence of a wide variety of biological expression systems for the large-scale production of therapeutic proteins has shifted the focus from vectors to host organisms. Although expression systems now span bacteria, fungi, plants, insects, and mammalian cells, the vast majority of recombinant-derived biopharmaceuticals at the present time have been produced in *E. coli* and in mammalian cells. This promises to change as the economic benefits of the newer systems permit the development of a new generation of proteins heretofore considered unfeasible for commercial development. Despite the impressive results which have been observed for many newer systems, there are many commercial considerations which suggest that CHO cell expression systems may continue to dominate in the manufacture of biopharmaceuticals for a long time to come.

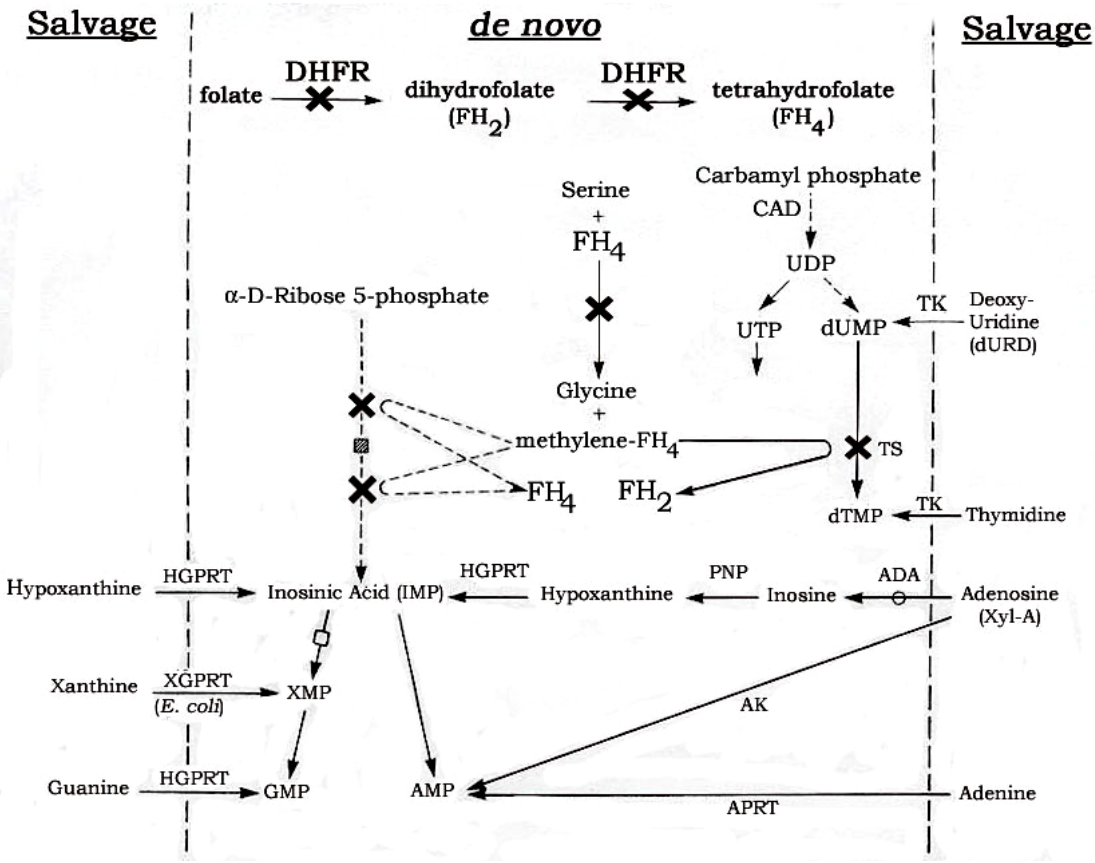

Figure 7 Inhibition by MTX in purine and pyrimidine biosynthesis pathways.

Dihydrofolate reductase (DHFR), enzyme in *de novo* biosynthesis pathway, is inhibited by MTX resulting in inhibition of dihydrofolate (FH_2) and tetrahydrofolate (FH_4) biosynthesis. The cross symbols (×) indicate reaction inhibited by MTX supplementation. This figure was modified from Kaufman (1990).

CHAPTER II
OBJECTIVES

In spite of the high demand of rHuEPO for clinical application in Thailand, the use of this hormone is limited. This is mainly due to the very expensive price of the commercial rHuEPO that must be imported. Although the human EPO cDNA has been cloned for many years leading to the production of rHuEPO, no attempt in establishing system to produce rHuEPO for domestic use has been reported despite of available of the good expression system and technology providing large-scale isolation of rHuEPO for wide-range therapeutic application. Consequently, this study aims to develop and establish mammalian cell expression system for:

1. expression and characterization of rHuEPO overproduced in COS-1 cells,
2. establishment of stable CHO cells lines over-secreting biological active rHuEPO, and
3. biochemical and biological characterization of rHuEPO expressed in stably transfected CHO cells.

CHAPTER III
MATERIALS

3.1. Chemicals

1) (±) Amethopterin (Methotrexate; MTX) (Sigma)
2) Chloroquine diphosphate (Sigma)
3) DMSO (Sigma)
4) HEPES (Research Oracnis)
5) Adenosine (Sigma)
6) Thymidine (Sigma)
7) 2'-deoxyadenosine (Sigma)
8) MTT (Sigma-Aldrich Life Science)

3.2. Enzymes

1) Improm-II™ reverse transcriptase (Promega, USA)
2) *Taq* DNA polymerase (Promega, USA)
3) *Cla* I Restriction endoribonuclease (Promega, USA)
4) Neuraminidase (EC 3.2.1.18) (Sigma)

3.3. Mammalian Cell Lines

1) *dhfr*$^{-}$/Chinese Hamster Ovary (CHO) cells (Kaufman et al., 1991)
2) African Green Monkey kidney (COS-1) cells
3) Erythroleukemic TF-1 cell (ATCC® No. CRL-2003)

3.4. Culture Media

1) Dulbeco's Modified Egle Medium (DMEM) (Gibco BRL, USA)
2) Minimum Essential Medium Alpha Medium (MEMα^{-}) (Gibco BRL, USA)

3) OptiMEM I Reduced-Serum Medium (Gibco BRL, USA)
4) CD CHO Medium (Gibco BRL, USA)
5) SFC-30 Medium CHO Express (PromoCell GmbH, Germany)
6) RPMI 1640 medium (Gibco BRL, USA)
7) Fetal bovine serum (FBS) (Gibco BRL, USA)
8) Fetal craft serum (FCS) (PAA Laboratories GmbH, Austria)
9) Penicillin/Streptomycin (P/S) (100X) (Gibco BRL, USA)
10) 200 mM L-glutamine (100X) (Gibco BRL, USA,)
11) 100 mM Sodium pyruvate, (Gibco BRL, USA)
12) Trypsin/EDTA (Gibco BRL, USA)

3.5. Mammalian Expression Plasmid

1) pED-EPO (Figure 8)

3.6. Antibodies

1) Mouse monoclonal IgG anti-human Epo antibody (Santa Cruz Biotechnology)
2) Horse radish peroxidase (HRP)-conjugated monoclonal anti-mouse IgG antibody (Sigma, USA)

3.7. Kits

1) Lipofectamin™ 2000 Kit (Invitrogen)
2) TRI REAGENT® (Molecular Reseaech Center)
3) Centriplus YM-10 (Amicon® Bioseparations)
4) PNGase F (New England BioLabs, USA)
5) DIG Glycan Detection Kit (Roche Applied Science)
6) ECL plus (Amersham Pharmacia Biotech)

3.8. Animal

1) Male BALB/c mice (National Laboratory Animal Center, Mahidol University (NLAC-MU), Thailand

3.9. Computer software

1) Microcal Origin 5.0 Professional

3.10. Miscellaneous

1) Prestained SDS-PAGE Standard, Board Range (BIO-RAD)
2) Immun-Blot™ PVDF membrane (BIO-RAD)
3) Epoetin alfa (HEMEX®, BIO SIDUS S.A., Argentina)
4) Recombinant human GM-CSF (Sigma)
5) Microhaematocrit tubes (Na-hep.) (BAND GMBH)

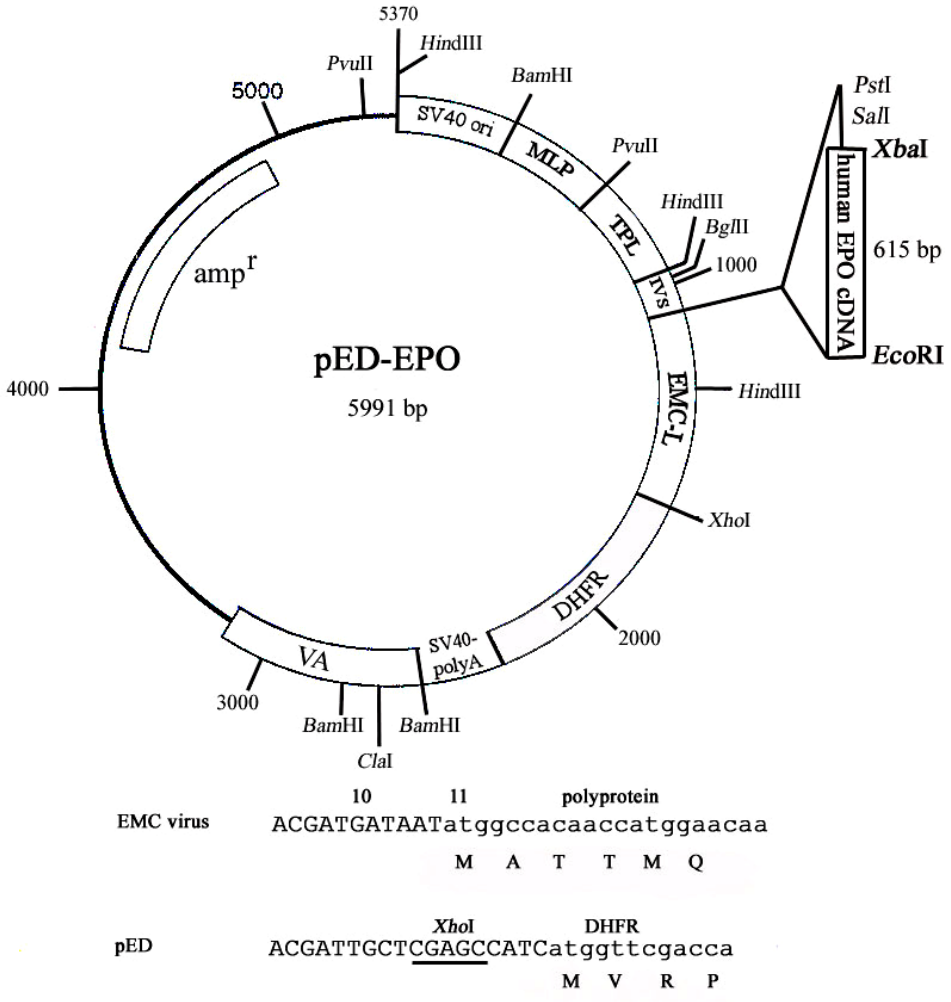

Figure 8 Physical map of pED-EPO mammalian expression vector (Modified from Kaufman et al., 1991)

The expression vector contains the simian virus 40 (SV40) origin of replication and enhancer element, the adenovirus major late promoter (MLP), the tripartite leader from adenovirus late mRNA (TPL) and a hybrid intron composed of the 5'splice site from the first leader of adenovirus major late mRNAs and a 3' splice site from an immunoglobulin gene (IVS). The HuEPO cDNA is followed by the 5' untranslated leader from encephalomyocarditis (EMC) virus, a murine DHFR-coding region, the SV40 late polyadenylation signal (SV 40-pA) and the adenovirus *VAI* RNA gene (VA).

CHAPTER IV
METHODS

4.1. Transient Expression in COS-1 Cells

COS-1 cells were widely used for rapid characterization of the mammalian expression plasmid vector and usually used for transient production of protein of interest at the high level. In this study, COS-1 expression system was used to characterize the expression plasmid whether it is functional prior to establishing stable cell lines.

4.1.1. COS-1 cell culture and culture maintenance

As a convenient procedure when working with mammalian cell cultures, all equipments and solutions were sterile. Culture media and working solution were pre-wormed at 37^{o}C in waterbath for 15-30 min before use. All COS-1 cell incubations were performed at 37^{o}C, 10% CO_2 incubator and in a humidified condition. And the following procedures for cell culture were performed with sterile technique. In order to culture COS-1 cells, a vial of frozen cells stored in liquid N_2 was rapidly recovered by gentle shacking in a 37^{o}C waterbath just until the medium was thawed. Cell suspension was transferred to a 100-mm culture dish and then resuspended thoroughly with 10 ml complete DMEM medium {DMEM (Gibco) supplemented with 10% FBS (Gibco), 1% P/S solution (Gibco) [50 units/ml penicillin, 50 mg/ml streptomycin] and 2 mM L-glutamine (Gibco)}. The COS-1 cells were normally grown approximately 2-3 days until cell density reached the desired confluence. For trypsinization, after cells grown in 100-mm culture dish have reached 100% confluence, the cells were washed once with 7 ml PBS, pH 7.4, then overlaid with 2.5 ml 0.25% (w/v) trypsin/0.2% EDTA solution for 3-5 min or until cells detached from plate. The cells were then re-suspended thoroughly with 8 ml complete DMEM medium by pipetting. The trypsinized cells were seeded into a new plate with fresh complete medium. Seeding

concentration or split ratio used depended on purpose of experiment, 1:5 split ratio for transfection or 1:10 split ratio for cell maintenance.

4.1.2. DEAE-dextran-mediated transfection

In our laboratory, this is the most general and reproducible method for plasmid DNA transferring into COS-1 cells. A day before transfection, COS-1 cells were split from a confluent plate to 1:5 split ratio into new plates to generate 60-70% confluent plates at the time of transfection. At the transfection date, transfection solution [400 µl 1 M Tris, pH 7.3, 8 µg purified circular plasmid DNA (pED–EPO#2) and 2.8 ml serum free-DMEM] was prepared. Prior to transfection, cells were washed twice with 7 ml PBS, pH 7.4 and transfection solution was mixed thoroughly with 0.8 ml DEAE-dextran solution {2.5 mg/ml DEAE-dextran in serum free-DMEM [DMEM (Gibco) supplemented with 1% P/S solution (Gibco) and 2 mM L-glutamine (Gibco)]}. Cells then were overlaid with 4 ml transfection solution and incubated at 37^{o}C for 6 hr followed by washing twice with 7 ml PBS, pH 7.4. The washed cells were treated with 2 ml 10% DMSO solution (137 mM NaCl, 5 mM KCl, 0.7 mM NH_2HPO_4, 6 mM D-glucose, 21 mM HEPES, 10% DMSO; pH 7.1) for 2 min. After solution was aspirated, cells were treated with 5 ml complete DMEM supplemented with 0.1 mM chloroquin and incubated at 37^{o}C for 2.5 hr and then washed once with 7.0 ml PBS, pH 7.4. Finally, cells were fed with complete DMEM medium. At this point the incubation timing was started (h_0) and continued to harvest time.

4.1.3. Total RNA isolation by TRI reagent®

As RNA sample for RT-PCR, total RNA was isolated from experimental plate. Cells were trypsinized, transferred to a 1.5-ml microcentrifuge tube, washed once with 1 ml PBS, and pelleted by centrifugation at 2,000 rpm for 5 min at 4^{o}C. Cell pellet was homogenized with 1.0 ml of TRI reagent® by pipetting. The homogenate was incubated at room temperature for 5 min, mixed with 0.2 ml chloroform, vigorously mixed by vortex for 15 sec and then stored at room temperature for 15 min. The mixture was fractionated by centrifugation at 12,000 xg for 15 min at 4^{o}C. After centrifugation, the solution was separated into three phases, upper clear aqueous phase, middle phase and lower red phenol-chloroform phase containing RNAs, DNAs

and proteins, respectively. An upper aqueous phase was transferred into a fresh 1.5-ml microcentrifuge tube. Total RNA was precipitated down by adding 0.5 ml isopropanol, standing at room temperature for 8 min and centrifugation at 12,000 rpm for 8 min at room temperature, after that supernatant was discarded. The obtained RNA pellet was washed once with 1.0 ml 75% ethanol solution, centrifuged at 7,500 rpm for 5 min at room temperature. After removing ethanol out, the RNA pellet was air dried and dissolved in DEPC-treated water for 15 min. The RNA solution was stored at –80^{o}C after use.

4.1.4. Determination of RNA concentration and purity

The quantity and quality of isolated RNAs were determined by double-beam spectrophotometer (U-2000, Hitachi, Ltd., Japan) at the absorbance of 260 and 280 nm. The RNA concentration was calculated from absorbance of 260 nm (A_{260}) by the following formula:

$$\text{Single-stranded RNA: concentration } (\mu g/\mu l) = \frac{A_{260} \text{ x dilution factor x } 40}{1000}$$

The RNA purity was determined by the absorbance ratio of A_{260}/A_{280}. A ratio should be 1.8-2.0 which represents high purity of RNAs.

4.1.5. Reverse transcription-polymerase chain reaction (RT-PCR)

4.1.5.1. First strand cDNA synthesis by reverse transcription

To synthesize the first strand cDNAs of mRNAs isolated from transfected COS-1 cells, approximately 2.7 μg of isolated total RNA was subjected to reverse transcription using ImProm-II™ Reverse Transcription System (Promega) according to the supplied procedure. In brief, the reaction containing RNA was heat-denatured briefly at 70^{o}C for 5 min in the presence of 3.6 nM Oligo (dT) primer, 3.6 units/μl Recombinant RNasin® Ribomuclease Inhibitor (Promega) in reaction volume of 5.5 μl and the reaction was then chilled on ice basket for 5 min. All solution was then added into reverse transcription reaction in the presence of 1X ImProm-II™ Reaction

Buffer, 2 mM $MgCl_2$, 2 mM each of dATP, dTTP, dCTP and dGTP and 1 μl of ImProm-II™ reverse transcriptase.

4.1.5.2. Polymerase chain reaction

To PCR amplified EPO cDNA, a set of specific primers for human EPO [GST-EPO-Sense, 5' - CTCTGGGGATCCCAGTCCTGGGCGCCCCAC - 3' (*Bam*H I site was underlined) and hEPO anti/*Eco*R I, 5' - TATGAATTCGTGGACACACCTGGTGGTCA-
TCTG - 3'(*Eco*R I site was underlined)], 10 μl of the first-strand cDNA template were added into the reaction containing 1X *Taq* DNA polymerase PCR buffer (Promega), 2 mM $MgCl_2$, 2 mM each of dATP, dTTP, dCTP and dGTP and 2.5 units *Taq* DNA polymerase in reaction volume of 50 μl. To reduce non-specific amplification, the hot-start method was applied to the reaction. Amplification was carried out as followed: 30 sec at 95°C, 30 cycles of 30 sec at 94°C, 45 sec at 55°C and 30 sec at 72°C, 7 min at 72°C. The reaction was analyzed by DNA electrophoresis.

4.1.6. Sodium dodacylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

4.1.6.1. Preparation of cell lysate and cultured medium samples

All cultured medium samples were centrifuged at 5000 rpm for 10 min at 4°C to collect its supernatant before SDS-PAGE sample preparation. The testing samples were mixed with 4X SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 40% (v/v) glycerol, and 400 mM DTT) in 3:1 ratio. The samples were heated to 100°C, mixed by vortex, placed on ice box for 2 min, and centrifuged at 12,000 rpm for 10 min at room temperature. The samples waiting for loading were placed on room temperature until it was ready to load into the gel.

4.1.6.2. SDS-PAGE

Protein samples were resolved in 15% SDS-PAGE after treatment in 1X SDS-PAGE sample buffer. SDS-PAGE gels were prepared as described by Laemmli (1970) and the gels were prepared in PROTEIN II (BIORAD). Proteins in the gels were separated in 1X Tris-glycine running buffer [25 mM Tris-HCL, 193 mM glycine and 0.1% (w/v) SDS] at a constant voltage of 80 volts until the running dye migrated out off the gels. In some experiments, a duplicate gel was performed, stained with Coomassie blue staining solution [0.25% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) methanol and 10% (v/v) acetic acid] for either 2 hr or overnight. The excess dye was destained with solution I [50% (v/v) methanol and 10% (v/v) acetic acid] until the desired intensity and low background were obtained. Then gel was then fixed with solution II [10% (v/v) methanol and 10% (v/v) acetic acid] for 1 hr or overnight.

4.1.6.3. Western blotting and detection

Proteins on the gel were electro-transferred to a PVDF membrane (BIO-RAD) with a constant voltage of 40 volts in low temperature environment ($4^{o}C$) for overnight (approximately 12 to16 hr). The blotted membrane was blocked in blocking solution {5% non-fat dried milk in Phosphate-buffered saline-Tween (PBS-T) [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and 0.2% Tween 20]} for 2 hr at room temperature, then incubated with blocking solution containing the 1:2000 dilution of primary antibody (mouse monoclonal anti-human EPO antibody, Santa Cruz Biotechnology) for 2 hr at room temperature. The membrane was then washed three times in PBS-T for 10 min each and incubated with blocking solution containing the 1:4000 dilution of secondary antibody (anti-mouse IgG conjugated with horseradish peroxidase) for 1.5 hr. After washing with PBS-T, three times for 10 min each, the blot was visualized under chemiluminescence (ECL plus Western Blotting Detection System, Amersham) according to the manufacturer's instructions.

4.2. Stable Expression in CHO Cells

4.2.1. CHO cell culture and culture maintenance

Before transfection, parental CHO/*dhfr*$^-$ cells were grown in complete MEMα$^+$ {[MEMα$^-$ (Gibco) with 10% FBS (Gibco), 1X P/S solution (Gibco) and 2 mM L-glutamine (Gibco)] supplemented with 10 μg/ml adenosine (Sigma), 10 μg/ml 2'-deoxyadenosine (Sigma) and 10 μg/ml thymidine (Sigma)}. After transfection, the transfected (*dhfr*$^+$) cells and all gene-amplified clones were maintained in complete MEMα$^-$ medium [MEMα$^-$ with 10% FBS (Gibco), 1% P/S solution (Gibco) (50 units/ml) and 2 mM L-glutamine (Gibco) (without four nucleosides)] supplemented with 10 nM of dihydrofolate reductase (DHFR) inhibitor methotrexate (MTX) (Sigma) as selective medium. MTX supplemented in the culture medium was used to maintain the expression level of DHFR in the cell and was variable and depended on the round of amplification as in Table 1.

4.2.2. Lipofectamine™ 2000 reagent-mediated transfection

A day before transfection, a 100% confluent plate of CHO/*dhfr*$^-$ cells was split into a new 100-mm culture dish with a cell concentration of $4x10^5$ cells/ml and then grown at 37°C in 5% CO_2 incubator with humidified atmospheres for approximately 22 to 24 hr to obtain 90-95% confluent plate. At the transfection date, 14 μg of *Cla* I digested-plasmid DNA and 10 μg of carrier circular DNA were mixed together resulted in 24 μg sample DNA. For one transfection, DNA sample was mixed with 1.5 ml of serum-free MEMα$^-$ medium [MEMα$^-$ (Gibco) supplemented with 2 mM L-glutamine and 1X P/S solution] in a sterile 15-ml centrifuge tube and then incubated at room temperature for 5 min. At the same time, the transfection solution was prepared by mixing 60 μl of Lipofectamine™ 2000 reagent together with 1.5 ml of serum-free MEMα$^-$ medium in a sterile 15-ml centrifuge tube and then incubated at room temperature for 5 min. For one plate, transfection solution was mixed into DNA solution by trapping, dropped thoroughly onto a 90-95% confluent plate (prewashed twice with 7.0 ml of 37°C pre-warmed PBS, pH 7.4). After the plate was incubated for 6 hr, the transfection solution was replaced with fresh complete MEMα$^+$ medium. At

24 hr post-transfection time, the cells were trypsinized and resuspended with 10 ml of medium, and then split into a new 100-mm culture dish with 1:50 and 1:100 dilutions in complete MEMα^- medium supplemented with 10 nM of MTX. Addition of MTX was aimed to reduce the background from survival of un-transfected CHO/*dhfr*$^-$ cells. The cells were continuously grown on 37^{o}C CO_2 incubator for 1.5 to 2 weeks. During the incubation time, the cell growth and cell density were observed daily and cultured medium was replaced with fresh medium weekly.

4.2.3. Clonal selection and propagation

At approximately 1.5 to 2 weeks after incubation with selective drug MTX, an individual colony (originated from a single cell) of interest (with about 500 cells or more) was marked under inverted microscope. Before collecting, the plate was washed once with 7 ml of 37^{o}C pre-warmed PBS, pH 7.5 followed by removing of the remaining PBS out by aspiration. A sterile cloning cylinder was placed to cover the colony of interest, and pressed down to ensure a good seal was made between the bottom of cloning ring and culture dish. Then, a single colony of marked clone was treated separately with 50 µl of 37^{o}C pre-warmed 0.25% (w/v) trypsin/0.2% EDTA solution followed by incubation at 37^{o}C for 3 to 5 min or until the cell began to detach. After incubation, protease activity of trypsin was inactivated by adding 50 µl of the serum-containing complete medium. Then, cells were resuspended by pipetting up-down and further sub-cultured to a well of 96-well plate containing 150 ml of complete MEMα^- medium supplemented with a specified concentration of MTX with regular monitoring. Cultured medium was fed every day until confluence. The cells were sequentially split and allowed to grow in the higher surface area of culture plate, in 24-well, 6-well plate and 100-mm culture dish, respectively. Master stocks of individual cell lines were perpared in freezing medium (complete MEMα^- medium) and stored under liquid N_2.

4.3. EPO Over-expression-Mediated by DHFR/MTX Gene Amplification System

As MTX is a specific inhibitor of DHFR, the cells are cultured in increasingly higher levels of MTX during gene amplification. Those CHO cells that copies of the DHFR gene have increased, and therefore higher levels of the enzyme, are selected. Only one clone that efficiently secreted rHuEPO into the culture medium in each round of amplification was selected, and further cultured in the complete MEMα^- supplemented with higher MTX concentration accordingly to the Table 1 (in chapter "RESULTS"). Many MTX-resistant clones, showing active cell division, were selected and subjected to propagation procedure as previously described. After propagation step, each clone was analyzed to determine rHuEPO secretion level by using criteria according to the Table 2. In brief, cells were seed into the 6-well culture plate with the same cell concentration. After the cell growing time, cultured medium was replaced with fresh medium. The culture medium was collected after an incubation time was reached. Cell counting was used to determine growth ratio of each clone after trypsinization. The growth ratio was used to minimize the variation between cell growth and protein secretion level of each clone.

4.4. Characterization of Protein Expressed From CHO Cells

4.4.1. Protein sample preparation

Unpurified protein sample obtained from the culture medium in the presence of 10% serum wase not suitable for characterization. In order to obtain the expressed EPO in serum-free medium for biochemical characterizations (PNGase F digestion and sialic acid detection), Opti-MEM (serum-free) and CD CHO medium (protein-free) (Gibco) were applied to culture the transfected CHO cells (#2.5) and culture medium was collected after 24-hr incubation. To obtain rHuEPO test samples for biological characterization (*in vitro* and *in vivo* biological activities), the 3rd round amplified-clone (1.2#2.5.4.6.4) was fed with Opti-MEM (serum-free) medium (Gibco), SFC-30 (serum-free) medium (PromoCell GmbH) medium or CD CHO (protein-free) medium (Gibco), and grown for 24 hr. Conditioned culture medium was collected and analyzed by SDS-PAGE and western blot analysis. The cultured medium

was then concentrated by using Centriplus® YM10 column (Millipore). The concentrated samples were compared to rHuEPO reference (HEMAX®) and roughly estimated its amount by dot blot or western analysis (data not shown).

4.4.2. Deglycosylation by PNGase F Digestion Kit

Briefly, the protein sample was boiled for 10 min in 1X Glycoprotein denaturing buffer [0.5% (w/v) SDS and 1% β-mercaptoethanol] to denature proteins. The denatured samples were then incubated in 1X G7 buffer (50 mM sodium phosphate, pH 7.5), 1% Nonidet P-40 and PNGase F (New England BioLabs) at 37°C for optimized incubation time. After digestion, samples were subjected to 15% SDS-PAGE and western blot analysis as previously described.

4.4.3. Neuraminidase (sialidase) digestion

Protein samples were digested with *Salmonella typhimurium* neuraminidase in 1X Digestion buffer (40 mM $C_2H_3NaO_2$, 0.8 mM $CaCl_2$, pH 5.0) at 37°C for 24 hr. The enzyme concentration used in the reaction was firstly optimized by digesting a constant amount of protein at 37°C for 24 hr. The digested samples were further analyzed the presence of un-digested $\alpha(2\rightarrow3)$-linked neuraminic acids (sialic acids) in the molecule by DIG Glycan Differentiation Kit.

4.4.4. Neuraminic acid (sialic acid) detection by DIG Glycan Differentiation Kit

4.4.4.1. SDS-PAGE, immuno-blotting and detection

The test and control samples were subjected to 15% SDS-PAGE and electro-blotted onto PVDF membrane at 4°C for overnight. This membrane was blocked in blocking solution [1 ml of 10X Blocking reagent in 9 ml of Tris buffered saline (TBS) (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) for 2 hr at room temperature. The membrane was then washed twice with 50 ml of TBS and once with 50 ml of Buffer 1 (0.05 M Tris-HCl, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.5) for 10 min each. The membrane was incubated with MAA (*Maackia amurensis* agglutinin) solution [1:200, 30 μl of digoxigenin-labeled MAA solution in 6 ml Buffer 1] for 60

min at room temperature. Excess MAA was removed by washing twice with 50 ml of TBS and once with 50 ml of TBS-T (0.2% Tween 20 in TBS). The blot was then incubated with secondary antibody solution [1:1,000, 6 μl of anti-digoxigenin-alkaline phosphatase (AP) solution in 6 ml of TBS-T] at room temperature for 60 min and washed three times with 50 ml of TBS-T. The brown-color signal was developed by incubating for 5 min with developing solution (200 μl of NBT/X-phosphate reagent in 10 ml Buffer 2), and terminated by washing the membrane with deionized water.

4.4.5. *In vitro* biological activity assay in TF-1 cells

4.4.5.1. TF-1 cell culture and culture maintenance

TF-1 is a growth factor-dependent human cell line that was originally isolated from the bone marrow cells of a 35-year-old Japanese man with erythroleukemia (AML M6) in 1987 (Kitamura et al., 1989). This cell line is proliferatively responsive to several hematopoietic growth factors, including EPO and GM-CSF. TF-1 cells were grown as cell suspension in either 25-ml or 75-ml cell culture flask with complete culture medium [RPMI 1640 medium (Gibco) supplemented 10 mM HEPES (Research Oracnis), 10% FCS, 2% P/S solution (100 mg/ml penicillin, 100 units/ml streptomycin), 2.5 g/L of D-glucose, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate (Gibco)] with optimal density ~ 0.5-1.0 x 10^6 cells/ml at 37^{o}C, 5% CO_2 incubator with humidified condition (according to Product Information Sheet for CRL-2003). In order to maintain this cell line as undifferentiated cell, 2 ng/ml of recombinant human GM-CSF (rHuGM-CSF) (Sigma) were supplemented into the culture medium.

4.4.5.2. Colorimetric MTT assay for cell proliferation

Measurement of cell viability and proliferation after stimulation of rHuGM-CSF, EPO reference and EPO expressed was based on the reduction of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (Sigma-Aldrich) reduced by metabolically active TF-1 cells, in part by the action of mitochondrial succinate dehydrogenase enzyme. The resulting intracellular purple formazan crystal could be solubilized by the addition of a detergent (SDS) and quantified by spectrophotometry.

A linear relationship between cell number and absorbance was established, enabling accurate quantification of changes in cell proliferation. In the experiments, after incubation TF-1 cells in the presence or absence of growth factors (rHuGM-CSF, rHuEPO) in 6-well culture plate with the total volume of 1.0 ml, 200 μl MTT reagent (5 mg/ml) was added into each well followed by gentle rocking. The plate was incubated at 37^{o}C for approximately 4 hr. After incubation, 800 μl of detergent solution (10% SDS) were added to lyses the cells and solubilize the colored crystals followed by 12-hr incubation at 37^{o}C. The colored samples were read using a spectrophotometer (Novaspec® II, Pharmacia) at a wavelength of 570 nm. The absorbance produced was directly proportional to the number of viable cells. This method was modified from Page et al. (1988).

4.4.6. *In vivo* biological activity assay in BALB/c mice

4.4.6.1. Laboratory animal and injection

All mature male BALB/c mice were housed under controlled condition. Thirty mice were divided into three groups of ten (N=10) for negative control (NC), positive control (PC) and test (T) groups, respectively, by minimizing the differences of body weight and all pre-injection parameters. Each mouse was marked individually with the different color tag. Two days before injection date (day 0), blood sample was collected individually into heparinized microheaematocrit tube by tail-bleeding. Multiple doses of 30 IU/0.2 ml/mouse/day was injected subcutaneously (*sc*) on days 3, 4, 5 and 6 into the respective animal and blood collection were carried out on day 7. In test (T) and positive control (PC) groups, all mice were injected subcutaneously (*sc*) by rHuEPO expressed in SFC-30 medium and commercial rHuEPO reference (HEMAX®), respectively. In negative control (NC) group, the mice were injected with PBS, pH 7.5, containing 75 μg/ml of albumin (BSA). Complete blood cell count (CBC) and reticulocyte count were performed at the day of blood collection by automated blood count machine (ADVIA120, Bayer). The injection procedure was modified from Ramos et al. (2003).

CHAPTER V
RESULTS

EPO is an example of peptide hormone by which post translation modification by addition of carbohydrate moiety to specific asparagine residues plays an essential role to its physiological function. Thus selecting appropriate host cell to producing recombinant EPO is among the most critical step that greatly influent the success in producing functionally active EPO applicable for clinical use. CHO cells are among the most widely use heterologous gene expression system for producing therapeutic proteins particularly those require correct glycosylation modification including EPO. This thesis work aims to develop CHO based expression system to produce biologically active recombinant human EPO applicable for large scale pharmaceutical production. To achieve this goal, an appropriate recombinant expressing EPO must be constructed. The plasmid will be first characterized by transient expression in the COS-1 cells prior to use to generate constitutively expressing and secreting rHuEPO in CHO stable transfected cell line. The expressed proteins were then tested for its biochemical properties by western blot analysis, PNGase F digestion, sialic acid detection and *in vitro* and *in vivo* biological activities. Actually, the medium used to culture the EPO-expressing cells in this study were serum-containing, serum-free and protein-free medium. Each medium was used to culture the cells in different situation, such as serum containing medium was used as general medium when growing or maintaining the cells and during gene amplification while serum- or proteins-free medium was used to grow cell when the proteins would be the samples for characterization or for *in vitro* and *in vivo* biological activity assay. For several sub-clones after each round of gene amplification, they were named according to the original clone (#2.5) with supplement indication with n, when n means the sub-clone No. n in that round of amplification, for example, 2.5.4 means the sub-clone No. 4 in the first round of amplification and 2.5.4.6 means the sub-clone No. 6 in the second

round of gene amplification. In addition, the original clone for each round of amplification was separately amplified with two concentration of MTX. In order to indicate the MTX concentration used, the number of concentration in the term of μM was indicate prior to the clone No., for example, 0.2#2.5.4 and 1.2#2.5.4.6.4 means the sub-clone No.4 of the original clone (No. 2.5) amplified in 0.2 μM MTX and the sub-clone No.4 of the 2-round-amplified sub-clone (No.2.5.4.6) amplified in 1.2 μM MTX, respectively.

5.1. Transient Expression and Analysis of Recombinant Human Erythropoietin (rHuEPO) in COS-1 Cells

To verify whether the recombinant plasmid pED-EPO is capable of directing synthesis of erythropoietin, transient DNA transfection into COS-1 cells was used as a method to monitor the synthesis of the expect protein. COS-1 cell is a green African monkey kidney cell line engineered to constitutively express SV-40 large T antigen. This factor is required for extra-chromosomal replication of plasmid DNA containing *SV-40 ori*gin of replication e.g. pED vector (Kaufman et al., 1991). In this study, COS-1 cells were transfected with a recombinant plasmid containing EPO cDNA from human hepatocyte (pED-EPO#2) (Tirasophon, unpublished data) by DEAE-dextran mediated transfection method for 6 hours. After transfection the cells were fed with fresh complete DMEM medium and allowed to recover. First the expression of EPO was determined by RT-PCR to ensure that the recombinant plasmid can drive transcription of the target gene. Total RNA were isolated at 72-hr post-transfection using TRI-REAGENT and the RNAs were reverse transcribed into cDNA then PCR amplified with EPO specific primers. From the RT-PCR result shown in Figure 9, a band of PCR product (~550 bp) was detectable (lane 3) in contrast, no amplified band was detected in the absence of reverse transcriptase (lane 2), suggesting that the amplified product in lane 3 was generated from the synthesized cDNA, not genomic DNA. This result confirmed that the recombinant plasmid can direct transcription of EPO mRNA in COS-1 cells. Next, the expression of rHuEPO would be monitored at the protein level.

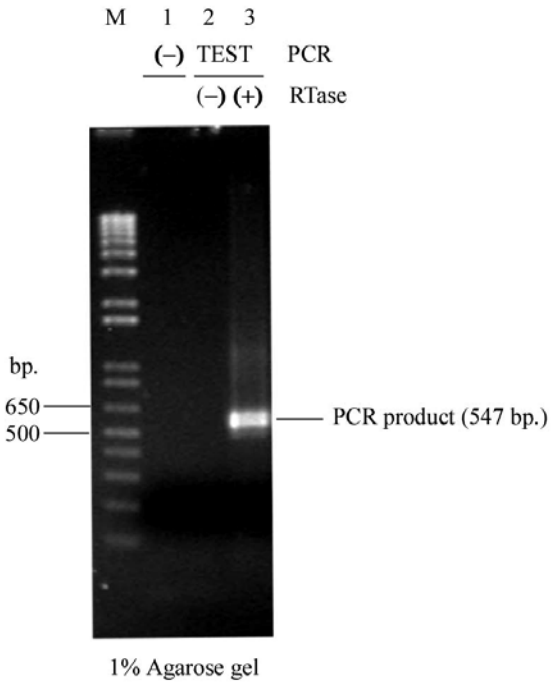

Figure 9 RT-PCR analysis of human EPO mRNA expressed in COS-1 cells

COS-1 cells were transfected with pED-EPO recombinant plasmid. Total RNA were isolated from the transfected cells at 72 hr post transfection and subjected to analyze by RT-PCR using EPO specific primers. PCR product was analyzed in 1% agarose gel electrophoresis.

Lane M = 1 kb plus ladder,
1 = PCR negative control,
2 = RT-PCR control in the absence of reverse transcriptase (RTase)
3 = test sample with an expected band of 547 bp PCR product.

The volume of condition medium or equal amount of protein in cell lysate (prepared in NP-40 lysis buffer) colleted at 24, 48, and 72 hr after transfection were analyzed by 15% SDS-PAGE and immunoblotted using specific mouse monoclonal anti-human EPO antibody. The specific signal of rHuEPO was visualized by chemiluminescence. As shown in Figure 10, multiple bands with sizes varied from 23 to 45 kDa were specifically observed in total cell lysate of the transfected cells but not in mock transfected cell indicating that the recombinant plasmid could direct synthesis of rHuEPO. The signal was detected as early as 24 hr and at maximum level at 48 post transfection however they were hardly detected at 72 hr post transfection. All the recombinant proteins are larger than the predicted size of unglycosylated (~ 23 kDa) but smaller than the fully glycosylated rHuEPO used as positive control suggesting that rHuEPO expressed from COS-1 cells was a glycosylated protein and their heterogeneity could be due to the different degree of glycosylation and/or complexity of carbohydrate moieties added to the polypeptide chain. In contrast, HuEPO secreted into the condition medium appeared as a more compact band (~35 kDa to 45 kDa) of protein compared to proteins in the cell lysate and the protein mobility was comparable to commercial rHuEPO reference (HEMAX®). The protein could be detected at low level at 24 hr post infection and the level was dramatically increased when time was prolonged to 48 and 72 hr. Taken together This result was a good indicators that pED-EPO recombinant plasmid can effectively direct expression and secretion of rHuEPO into culture medium.

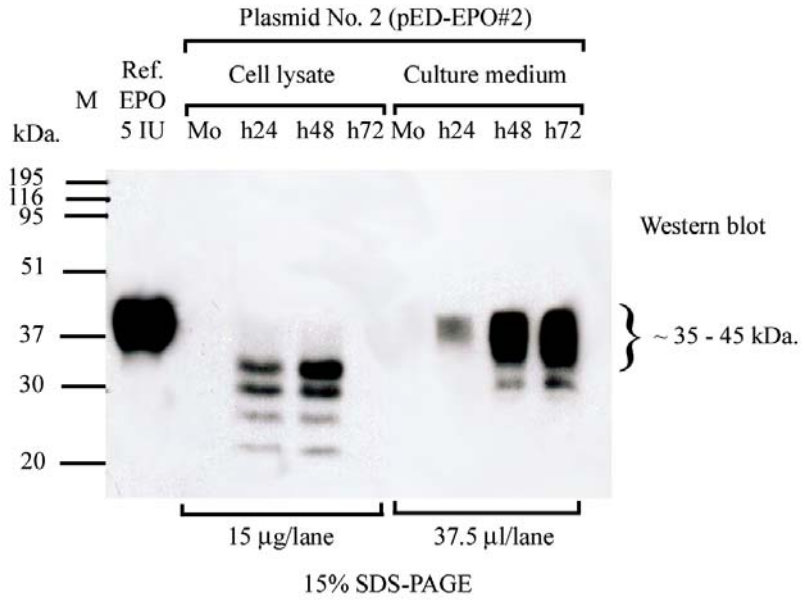

Figure 10 Western blot analysis of protein expressed from COS-1 cells

Total cell lysates and cultured medium were collected 24, 48 and 72-hr post-transfection (indicates as h24, h48 and h72, respectively) were analyzed by western blotting using mouse monoclonal anti-human EPO antibody. The patterns of overexpressed protein were compared to un-transfected cells (Mo) or 5 IU of commercial rHuEPO reference (HEMAX®). Fifteen micrograms of total proteins and 37.5 µl of cultured medium were loaded in each lane. M indicates the migration size of prestained SDS-PAGE molecular weight marker (BIO-RAD).

5.2. Stable Expression of rHuEPO in CHO Cells

To establish stable cell line capable of constitutively express rHuEPO, CHO/*dhfr*$^-$ cells were transfected with linearized pED-EPO#2 expression plasmid using Lipofectamine^{TM} 2000 (Invitrogen^{TM}) as described in the methods. After 24-hr post-transfection, the transfected cells were trypsinized and seeded into 100-mm culture dishes at 1:50 - 1:100 dilutions, in complete MEMα$^-$ supplemented with 10 nM MTX. This low concentration of MTX helps to reduce background from outgrowth of untransfected cells. After 1.5 to 2 weeks, the transfected cell formed colonies in selective medium and were randomly isolated for expanding. These selected clones were further analyzed for erythropoietin synthesis and secretion. The cells were grown in 100-mm culture dishes in complete MEMα containing 10 nM MTX for 48 hr incubation. Steady stage of rHuEPO synthesis in these clones was monitored from total cell extract (as approximate kDa) whereas secreted rHuEPO (as approximate kDa) level was analyzed from the cultured medium (Figure 11). Of 6 individual clones analyzed, all of them express rHuEPO protein but at different level. Significant higher level of rHuEPO was observed in the condition medium particularly the clone #2.5 that appeared to express and secrete the highest level rHuEPO (~ 133 mU/μl). This clone then was used in the first round of gene amplification in the later step.

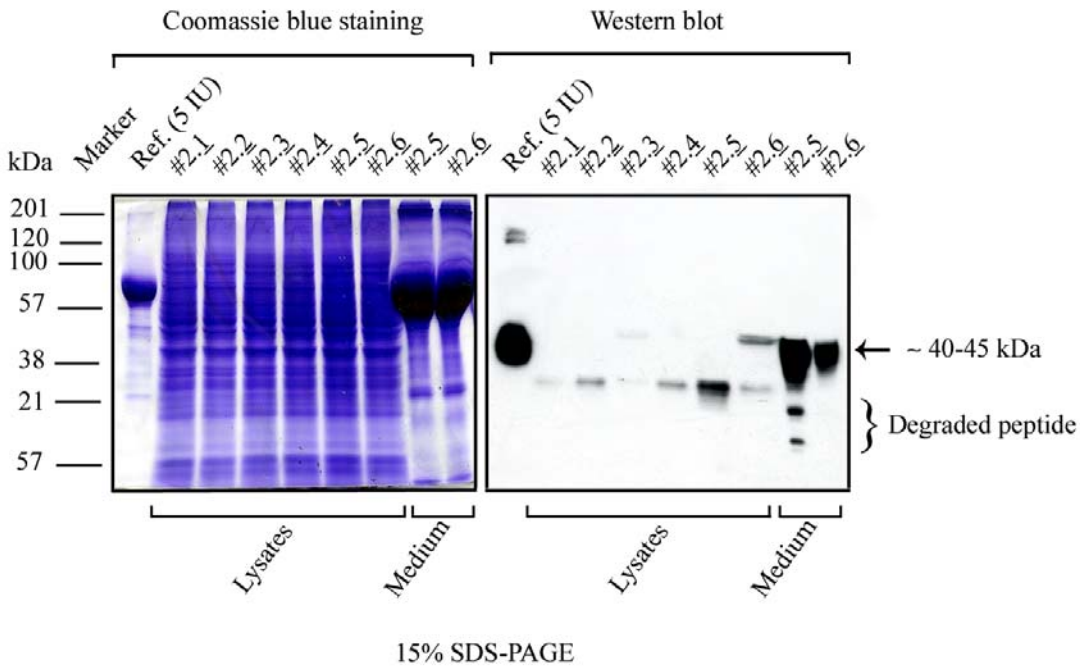

Figure 11 Analysis of Stable expressing clones

CHO/*dhfr*$^{-}$ cells were transfected with linearlized pED-EPO#2 generating 6 of transfected clones (#2.1 to #2.6). Total cell lysate of each clone and condition medium from some selected clones (as indicated) were analyzed in 15% SDS-PAGE. The gel was stained by Coomassie blue staining or probed with mouse anti-human EPO monoclonal antibody. The relative molecular mass (M_r) were indicated by pre-stained MW marker (BIO-RAD) as indicated in kDa. Five IU of rHuEPO reference (HEMAX®) was used as positive control. The broad band of expected EPO positive signal was detectable and indicated with arrow.

5.3. Generation of High Level rHuEPO-Expressing Stable Cell Lines

The recombinant CHO cell line expressing rHuEPO was subjected to the next step to generate the sub-clones that constitutively express higher level of rHuEPO. The DHFR/MTX gene amplification system was used to amplify copy number of EPO expression cassette integrated in chromosome transfected CHO cell. The amplification was performed by increasing the concentration of a DHFR inhibitor, MTX, in the medium at each rounds of amplification. Each round was conducted for approximately one to two months. The highest rHuEPO producing clone was selected for further round of amplification.

In the first round of amplification, the parental clone #2.5 was seeded in complete MEMα^- medium with higher MTX concentration (0.1 and 0.2 μM). After two weeks, subpopulation of the clone that survived in higher concentration of MTX formed colonies of ~500 cells/colony. These survived clones were expected to have co-amplification of copy number of both EPO expression cassette and DHFR more than the un-amplified clone. Clone expansion was done as described in the methods. To determine the level of rHuEPO in each clone, they were seeded in to 6-well plate at $5x10^5$ cells/well and grown for 24 hr. After cells were fed with new medium, the cultured medium was collected at 48 hours later. The expression levels were screened by dot blot analysis (data not shown). Two amplified clones (0.1#2.5.4 and 0.1#2.5.5) were further confirmed for their expression level compared with the un-amplified clone (#2.5) by western blot analysis (Figure 12). Recombinant HuEPO secretion level in these two clones was approximately 5 times higher than the level from parental clone (#2.5). Between these two clones, rHuEPO was secreted from clone 0.1#2.5.4 better than from clone 0.1#2.5.5 particularly when comparing the steady state of rHuEPO synthesis detected from its level retained within the cells. For longer incubation time (72 hr), the protein secretion level was higher, whereas, it seems to be less stable than the shorter incubation time (48 hr). However, the expression level was too low to be detected by Coomassie blue staining.

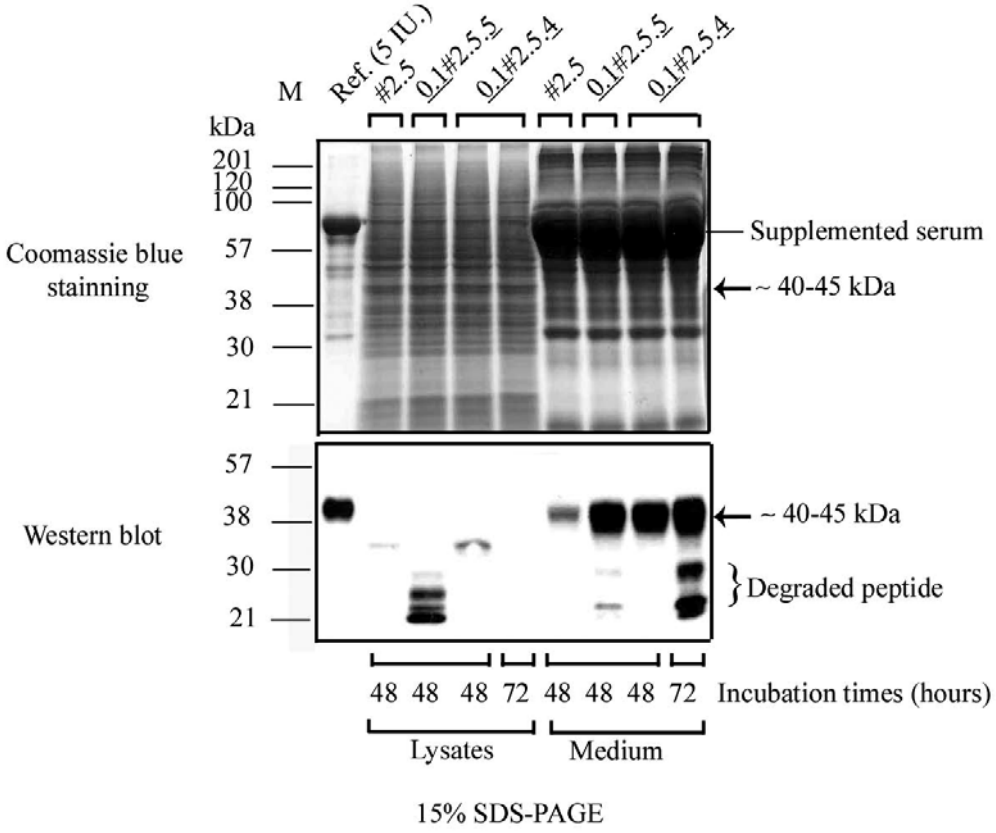

Figure 12 Analysis of EPO secretion level after the first round of amplification

Un-amplified clone (#2.5), 100 nM MTX-amplified clones (0.1#2.5.5 and 0.1#2.5.4) were seeded with approximately $2x10^6$ cells/plate and grown for 48 hr in 37^oC, 5% CO_2 incubator with humidified condition. Fresh medium was replaced and then collected after 48-hr [and 72 hr for clone 0.1#2.5.4] incubation. Forty μg of total proteins from each cell lysate and 30 μl of each condition culture mediums were analyzed and compared by Coomassie blue staining and western blot analysis, using mouse anti-human EPO monoclonal antibody. The M_r was indicated by pre-stained MW marker (BIO-RAD) migration in kDa.

Clone 0.1#2.5.4 was subjected to second round of amplification using higher MTX concentration (0.5 and 0.8 μM). After clone expansion, the candidate clones were screened as summarized in Table 1. From 16 clones screened by western blot analysis, three candidate clones producing highest signal (0.5#2.5.4.4, 0.5#2.5.4.6 and 0.8#2.5.4.5) were further confirmed for its expression level (according to Table 2) by comparing with the previous round-amplified clone by western blot analysis (see Figure 13). The expression level in these three clones was approximately 5 times higher than its parental clone (0.1#2.5.4). Similar gene amplification was performed for the third and fourth rounds using 1.2 and 1.5 μM MTX, and 2.0 and 5.0 μM MTX, respectively. In both round each of which enhance the level of amplification by two fold. As a result, comparisons between the un-amplified clone #2.5 and the fourth-round amplified clone 2.0#2.5.4.6.4.5, the secretion level was increased approximately 100 times (5 x 5 x 2 x 2) within 4 rounds of gene amplification better than the original clone.

Table 1 Summary of screening criteria to determine the expression of rHuEPO in 6-well plate

Round[1]	[MTX][2] (μM)	Seeding conc[n][3] (cells/well)	Growing time[4] (hr)	Incubation time[5] (hr)	No. of tested clones	Method
1	0.1 & 0.2	$5x10^5$	48	24	16	Dot blot
2	0.5 & 0.8	$5x10^5$	24	24	16	WB[6]
3	1.2 & 1.5	$2x10^5$	24	24	28	WB
4	2.0 & 5.0	$2x10^5$	24	20	18	WB

1 Round of gene amplification

2 Methotrexate concentration

3 Amount of cells seeded into each well

4 Times used for growing cells before new medium replacement

5 Times used after new medium replacement

6 Western blot analysis

Table 2 Summary of condition used to confirm the expression of rHuEPO in 100-mm culture dish

Round[1]	[MTX][2] (μM)	Seeding conc[n][3] (cells/plate)	Growing time[4] (hr)	Incubation time[5] (hr)	No. of tested clone	Method
1	0.1 & 0.2	$2x10^6$	48	48	2	WB[6]
2	0.5 & 0.8	$1x10^6$	24	24	3	WB
3	1.2 & 1.5	$0.5x10^6$	24	24	6	WB
4	2.0 & 5.0	$2.5x10^5$	24	24	3	WB

1 Round of gene amplification

2 Methotrexate concentration

3 Amount of cells seeded into 100-mm culture dish

4 Times used for growing cells before new medium replacement

5 Times used after new medium replacement

6 Western blot analysis

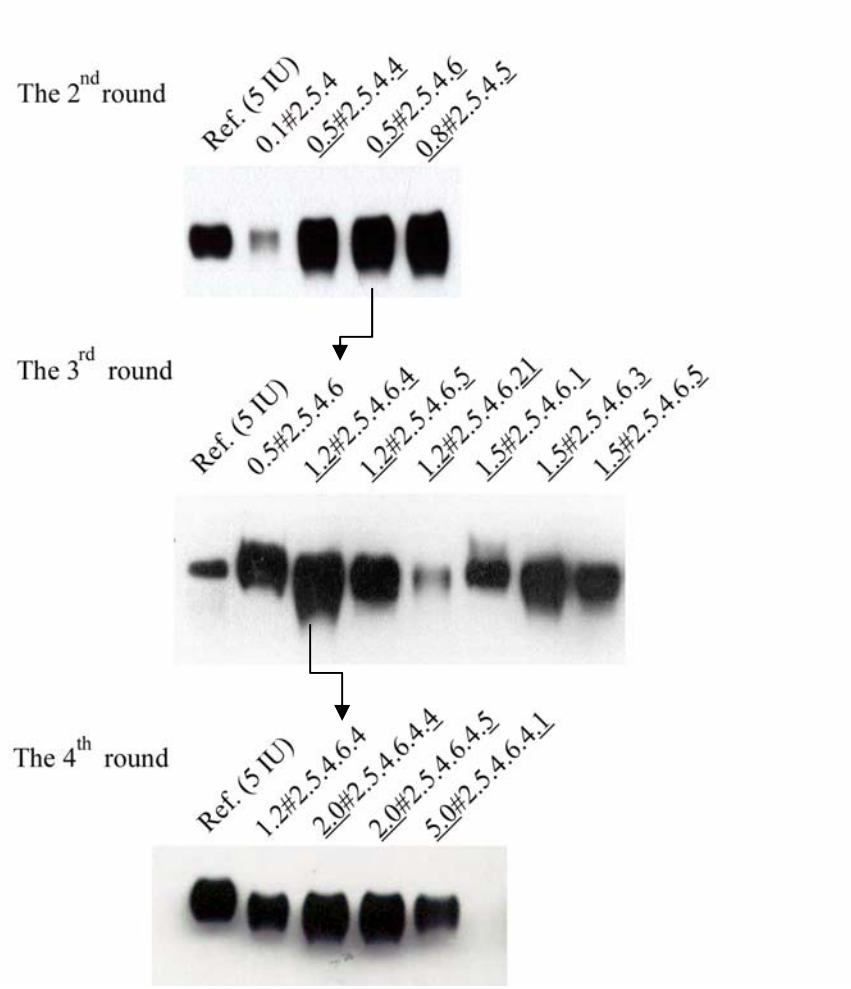

Figure 13 Analysis of EPO secretion level in the other rounds of amplification

EPO secretion level in culture medium from each round of amplification was compared together with the same round and to the previous round. Five IU of rHuEPO reference (HEMAX®) were used as positive control. The arrows indicate the subclone No. in the previous round used for comparison in the next round. The underlines indicate the higher concentration of MTX and the number of subclone in that round. Each blot was obtained form confirmation analysis and sample was prepared as criteria in Table 2.

5.4. N-Linked Glycosylation Characterization by PNGase F Digestion

We next characterized the state of carbohydrate modification of the rHuEPO produced the cell line generated in this study using enzymatic activity of PNGase F. This enzyme catalyzes specific cleavage of the complex carbohydrate from the glycoprotein molecule. Therefore it was used to determine the number of N-glycosylation existing on rHuEPO molecule produced from our stable cell lines. In order to determine the optimal concentration of PNGase F suitable for partial cleavage of the carbohydrate moiety from rHuEPO molecule, concentrated rHuEPO (30 U, determined by western blot) were digested with serial 10-fold dilution of *Flavobacterium meningosepticum* PNGase F ($9.1 – 9.1x10^{-6}$ U/μl) for 5 min (Figure 14, A). PNGase F at $9.1x10^{-2}$ U/μl was the minimal amount of PNGase F that caused partial digestion of rHuEPO in the indicate condition. Hence, this enzyme concentration was then used to digest the rHuEPO at 37°C for various times point (3, 5 10, 15, 30 45 and 60 min) (Figure 14, B). Partial digestion pattern of rHuEPO could be clearly observed within 3-30 min. These digesting conditions were used to digest our rHuEPO samples to compare with the reference rHuEPO, with $9.1x10^{-2}$ U/μl of enzyme for 5, 10 and 30 min. Treatment of the reference rHuEPO with this N-glycanase F resulted in the reduction of apparent migration size down from ~45 kDa to 38, 31, 24 and 23 kDa, respectively (Figure 15). Each intermediate band represents different degree of glycosylations which remains attached to the rHuEPO molecule. The major pattern of rHuEPO produced from our cell line upon partial digestion is resembled to those obtained from the reference sample implying that our rHuEPO contains three N-linked glycosylation sites (and probably one O-linked glycosylation).

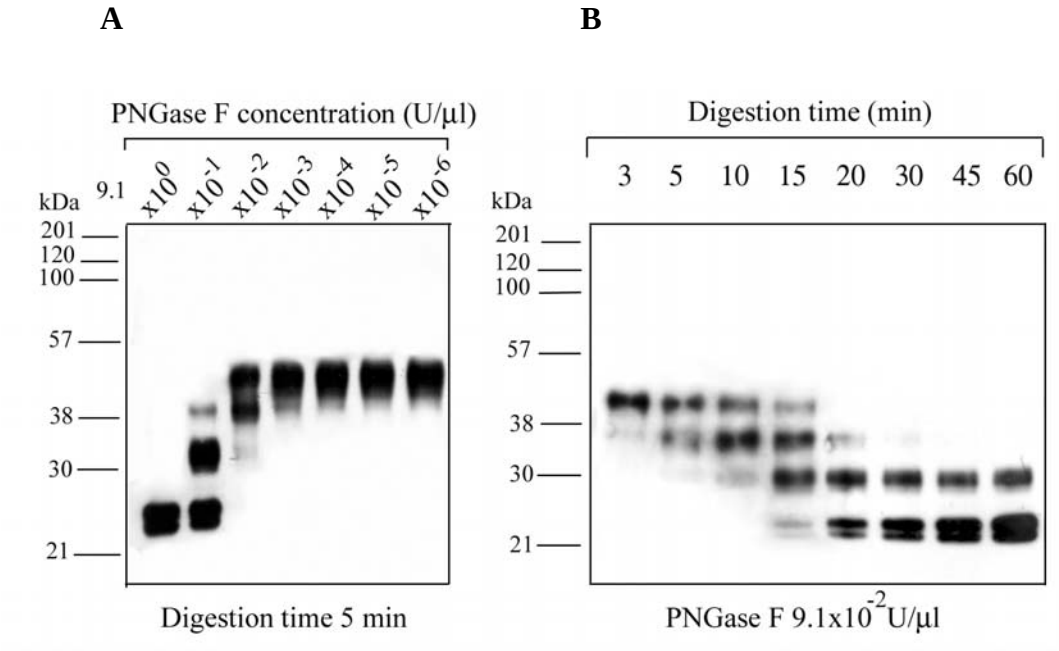

Figure 14 PNGase F digestion optimization

Thirty units of rHuEPO in concentrated condition medium of stable cell line expressing rHuEPO were partially digested with a 10-fold dilution of PNGase F from *F. meningosepticum* (from 9.1 – $9.1x10^{-6}$ U/µl) for 5 min (Panel A). The estimated concentration of $9.1x10^{-2}$ U/µl of PNGase F was employed to digest the recombinant protein for a indicate times (3, 5 10, 15, 30 45 and 60 min) at 37°C (Panel B). Approximately 4.5 units of rHuEPO per lane was analyzed by SDS-PAGE and probed with mouse monoclonal anti-human EPO antibody. The M_r was indicated by pre-stained MW marker (kDa).

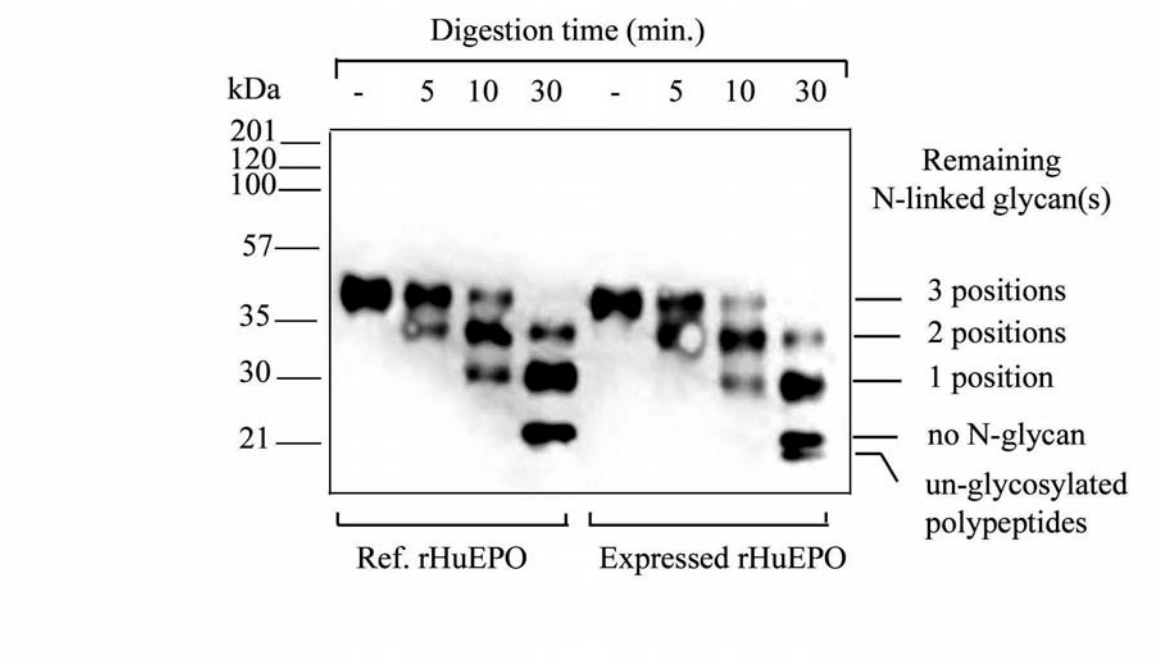

Figure 15 PNGase F digestion analysis for N-linked glycosylation of rHuEPO expressed from CHO cells

Recombinant HuEPO in concentrated condition medium of stable cell line expressing rHuEPO was treated with PNGase F from *F. meningosepticum* (0.09 units/μl) for 5, 10 and 30 minutes at 37°C. The digested pattern of rHuEPO was analyzed in 15% SDS-PAGE by digestion and probed with mouse anti-human EPO monoclonal antibody. Commercial rHuEPO reference (HEMEX®) treated with the same condition was used as positive control. Approximately 4.5 unit of tested or reference rHuEPO was loaded in each lane. The M_r was indicated according to migration of pre-stained MW marker (kDa).

5.5. Sialic Acid Detection by Lectin-Binding Assay

As the *in vivo* biological activity of EPO appears to be dependent on the presence of sialic acid residuals on the termini of sugar moieties attached to the rHuEPO molecule. To ensure that rHuEPO produced from our cell line will be active *in vivo*, it is necessary to investigate whether sialic acid presence on the molecule or does not.

By using MAA (*Maackia amurensis* agglutinin), which is a lectin that specifically recognizes sialic acid linked α(2-3) to galactose in N- and O-linked glycans, sialic acids were identified as the part of carbohydrate component in the study. Sialic acid residual can be eliminated by the activity of sialidase or neuraminidase from *Salmonella typhimurium* (Sigma). Firstly, the optimal neuraminidase concentration was determined by digesting the concentrated samples with various enzyme concentrations decreasing two-fold from 1.07 to 16.67 x 10^{-3} units/ml in total volume of 75 μl as described in the methods (data not shown). The approximately 4,800 units (estimated by Western blot analysis) of concentrated protein samples were digested again with 1.07 units/ml neuraminidase as a negative sample (T-) of sialiation detection. Two different forms of known protein (10 μg each), asialofetuin and fetuin (a 68-kDa glycoprotein which contains three N-glycosidically and three O-glycosidically gebundene linked chains) were treated in the same condition as negative control (NC) and positive control (PC), respectively. All samples (2,880 units each of test samples and 2 μg each of control samples) were separated in 15% SDS-PAGE.

A duplicate SDS-PAGE (9.6 units each of test samples and 2 μg each of control samples) was performed and subjected into another immunoblot. In the first blot, the presence of sialic acid-containing EPO was detected by using specific MAA agglutinin binding to α(2 →3) sialyated galactose moieties (see figure 16, A). In another blot, mouse anti-human EPO monoclonal antibody was used to determine the position of both neuraminidase-digested and un-digested EPO (see figure 16, B).

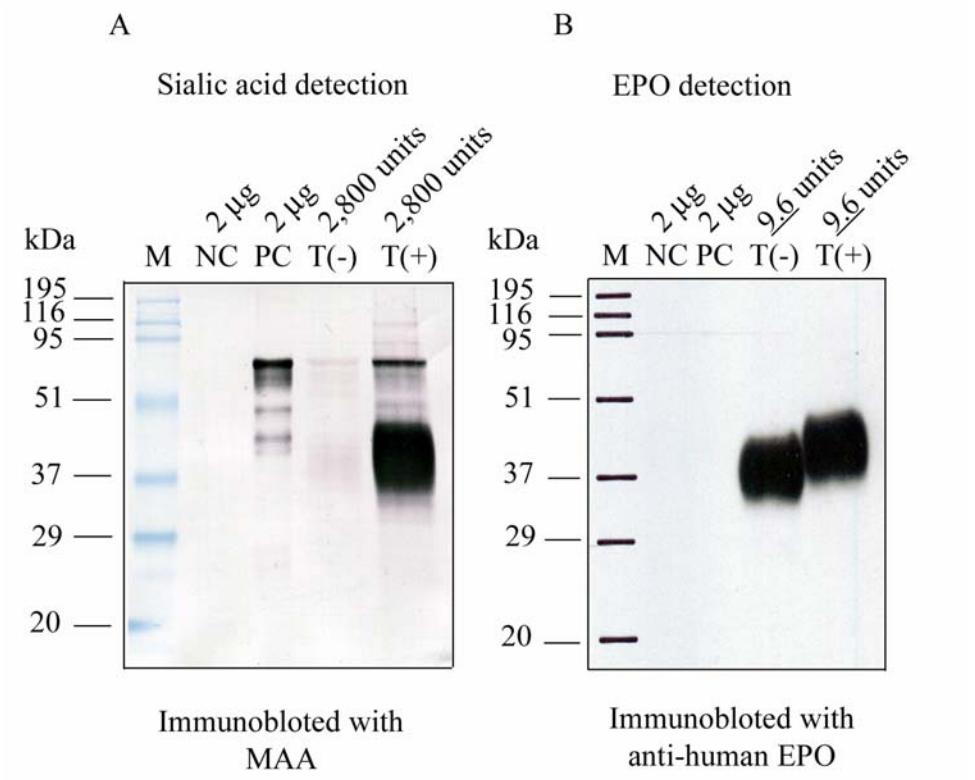

Figure 16 Lectin-binding assay and western blot analysis for sialic acid detection

Approximate 4,800 units of rHuEPO from concentrated condition medium of were digested with or without 1.07 units/ml of *S. typhimurium* neuraminidase. All samples were resolved in 15% SDS-PAGE. Two µg of asialofetuin or fetuin (a 68-kDa glycoprotein) were included in the gel as negative control (NC) and positive control (PC), respectively. Proteins were transferred onto the membrane and probed with MAA (Panel A). A duplicate blot of the same samples was analyzed and probed with mouse monoclonal anti-human EPO antibody (Panel B). T(-) and T(+) mean de-sialiated and sialiated test sample, respectively. The relative molecular mass (M_r) were indicated by pre-stained MW marker (BIO-RAD) migration sizes in the term of kDa.

As a result of sialic acid detection, positive control bands of sialiated fetuin (PC) were detected, whereas the negative control band of asialofetuin (NC) was not observed, proving that the specific signals were generated only in the presence of sialiated glycan targets. In the test samples, lane (T-) and (T+), most of the signal were generated in the undigested sample (T+) but was barely detected in the sialidase-digested sample (T-) suggesting that the expressed EPO contains the sialic acid residuals in the molecule. As in the western blot (using mouse monoclonal anti-HuEPO antibody) result, the EPO specific bands were confirmed and the result showed that digested-EPO (T-) migrates in the SDS-PAGE faster than the un-digested sample (T+). From the EPO detection result in Figure 16 (Panel B), the non-specific sialic acid-positive band migrating higher than EPO was confirmed.

5.6. *In Vitro* Biological Activity in Factor-dependent Human Erythroleukemic Cell Line TF-1

TF-1 is a growth factor-dependent human cell line that was originally isolated from the bone marrow cells of a 35-year-old Japanese man with erythroleukemia (AML M6) in 1987 (Kitamura et al., 1989). This cell line is proliferatively responsive to several hematopoietic growth factors, including EPO and GM-CSF. These cells grow as cell suspension in RPMI-1640 medium supplemented with 10 mM HEPES, 10% FCS, 2% P/S solution (100 mg/ml penicillin, 100 units/ml streptomycin), 2.5 g/L of D-glucose, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate (Gibco BRL)] with optimal density at ca. 0.5-1.0 x 10^6 cells/ml at 37^oC, 5% CO_2 incubator with humidified condition. In order to maintain this cell line with the undifferentiated morphology, 2 ng/ml of recombinant human GM-CSF (Sigma) were supplemented into the culture medium. Without supplementation of GM-CSF, it could not survive and died within one week with the presence of some intracellular granule in culture medium. The survival of these cells was promoted by supplemented growth factors, GM-CSF and EPO, in this study.

5.6.1. Activation of TF-1 cell proliferation by rHuGM-CSF

In order to determine the minimum concentration of GM-CSF to be supplemented into the culture medium of TF-1 cells that required for maintaining viability of this cell, dose response curve of TF-1 proliferation was prepared by stimulating TF-1 cells in the presence of various concentrations of rHuGM-CSF.

In the experiment, the 24-hr growth factor-depleted TF-1 cells were exposed to various dilution (2-fold serial dilution ranging from 25 ng/ml to 25 pg/ml) of rHuGM-CSF with the cell density of $1x10^5$ cells/ml/well in 6-well plate. After 72-hr (days 3) stimulation, cell morphology were examined by inverted microscopy. In all conditions the cell morphology remained intact and viable however their proliferation rates were varied depend on the concentration of rHuGM-CSF present in the medium. Cell proliferation was determined by MTT assay and analyzed by statistical tool (Origin 5.0) as shown in Figure 17. Medium with higher concentrations of rHuGM-CSF stimulated TF-1 proliferation better than medium with the lower concentrations (between 0.025-0.800 ng/ml). However the effect was saturated when the concentration is higher than 0.800 ng/ml corresponding to the reported ED_{50} of 0.123 - 0.220 ng/ml of rHuGM-CSF. From this result, rHuGM-CSF at 25 pg/ml (basal requirement) was selected to supplement into the medium for determining the effect of EPO on TF-1 growth.

5.6.2. Activation of TF-1 cell proliferation by rHuEPO

To assay the activity of rHuEPO produced from our cell lines, a rHuEPO-expressing clone (1.2#2.5.4.6.4) was grown in 3 different serum-free mediums (Opti-MEM, CD CHO and SCF-30 medium) for 24 hr and all condition medium were subjected to tested for TF-1 stimulation by comparing to the rHuEPO referent standard (HEMAX®). A 2-fold serial dilution of each condition medium (from 100 µl to 0.049 µl) was prepared and supplemented into fresh medium in the presence of 25 pg/ml rHuGM-CSF prior to TF-1 cell feeding for analysis of proliferation activation. The viable cell numbers were determined by MTT assay (as describe in "METHODS"). Result in Figure 17 indicates the rHuEPO in each condition medium (OptiMEM, CD CHO and SFC-30) that is capable of activating TF-1 growth in does-dependent manner similar to that of the reference, indicating that rHuEPO produced in these condition medium are biologically active *in vitro*. The maximal (saturated) activity of rHuEPO-SFC (Abs = 0.580) was higher than of rHuEPO-OptiMEM and rHuEPO reference STD (Abs = 0.540) suggesting that at the saturated concentration of testing rHuEPO, EPO expressed in SFC-30 culture medium could stimulate the proliferation of TF-1 cell better than the others. In contrast, EPO expressed in CD CHO culture medium did not show the saturated concentration for maximal activity. Based on this result, the estimated rHuEPO activity in SFC-30 and OptiMEM (rHuEPO-SFC-30 and rHuEPO-OptiMEM) were 6.488±1.264 and 3.595±0.692 units/µl, respectively, calculated by correlating the volume (µl) of medium used (which gave the activity correspond to the reference) with the amount (units) of rHuEPO referent standard.

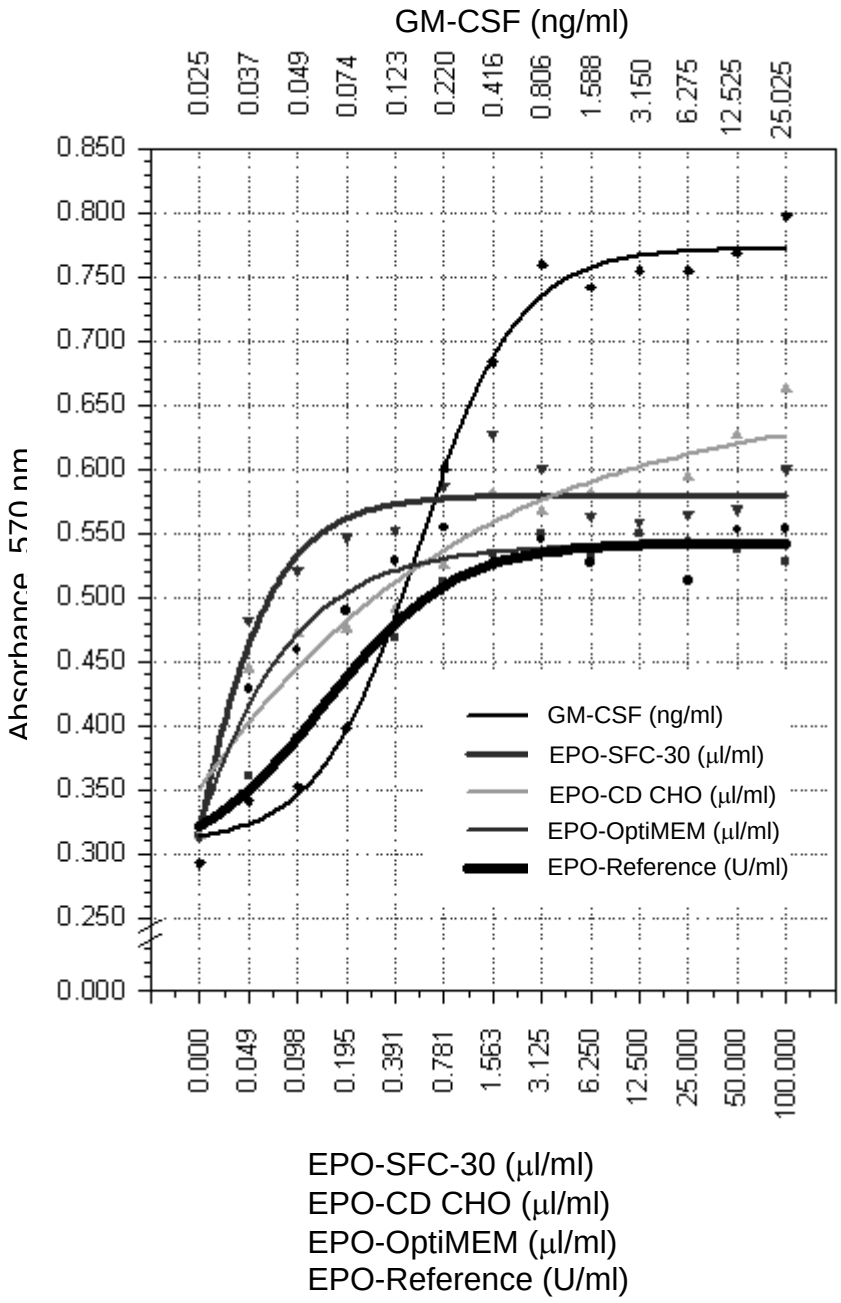

Figure 17 Activation of TF-1 cell proliferation by rHuGM-CSF and rHuEPO

Figure 17 Activation of TF-1 cell proliferation by rHuGM-CSF and rHuEPO (Continued)

A constant amount of $1x10^5$ cells of TF-1 cells was seeded into 6-well plate and cultured in rHuGM-CSF lacking medium for 24 hr prior to switching to the same medium containing various concentration of rHuGM-CSF (ranging from 25 ng/ml to 25 pg/ml) prepared by 2-fold serial dilution, and containing 100 µl of a 2-fold serial dilution of 24-hr culture medium collected from culturing clone 1.2#2.5.4.6.4 in OptiMEM, CD-CHO or SCF-30 medium diluted in corresponding medium containing 25 pg/ml rHuGM-CSF. Whereas 100 units of rHuEPO reference STD (HEMAX®) was also prepared as 2-fold serial dilution in the same medium as indicated above. The cells were cultured for 72 hr then their proliferation was determined by MTT assay showing in the term of absorbance value and the dose-response curve was analyzed by statistical tool (Origin 5.0).

5.7. *In Vivo* Biological Activity in BALB/c Mice

In vivo activity of rHuEPO produced from our stable cell line was elucidated based on ability to stimulate new red blood cell production in animal model. Equivalent activity of rHuEPO from unpurified SFC-30 medium (determined by *in vitro* bioassay) was administrated in BALB/c mice by subcutaneous (*sc*) injection into BALB/c mice (N = 10) for 4 times (at day 3, 4, 5 and 6) after pre-injected blood collection (at day 0). Blood samples were collected at day 7 and subjected to analysis for complete blood count (CBC) and reticulocyte count using automated blood counter (ADVIA120, Bayer). Each parameter compared between pre- and post-administration by rHuEPO was summarized in Table 3. Injection of both commercial rHuEPO and unpurified rHuEPO did not change the red blood cell count, hemoglobin content or hematocrit of the tested animals among each group. The values of these hematological parameters are comparable between pre and post administration in all conditions. However, treatment with rHuEPO from our cell line had dramatic effect on reticulocytes count, the fast-responsive parameter, comparable to that observed in the group treated with reference standard as shown in Figure 18. In negative control (CONTROL) group, reticulocyte count was not altered (3.8±1.2 and 3.8±0.6) after injection of serum albumin confirming that this carrier protein did not affect reticulocyte synthesis. In contrast, erythropoiesis was stimulated upon injecting with control rHuEPO reference. The level of young RBC (reticulocyte) in the blood circulation was increased from 3.8±1.3 % in pre-injection to 17.0±1.6 % in post-injection. This result confirmed that erythrocyte progenitors in these mice responded well to the EPO administration. Similar result was observed in mice injected with unpurified condition medium containing rHuEPO (rHuEPO-SFC-30). The level of reticulocytes in this group was increased from 4.0±1.4% in pre-injection to 14.3±2.2 % in post injection. Which were approximately 3.6 fold increasing compared to 4.5 in positive control (STD). Thus this evidence clearly indicates that rHuEPO produced in our stable cell line are biologically active and comparable with reference.

Table 3 Comparison of the hematological parameters upon rHuEPO administration

Parameters	CONTROL[1]		STD[2]		TEST[3]	
	Pre[4]	Post[5]	Pre	Post	Pre	Post
BW[6] (g)	29.2±2.1	29.7±2.1	28.4±2.1	29.6±2.0	29.3±1.4	30.3±1.7
RBC[7] ($x10^6$ cells/μl)	10.4±0.5	10.9±1.2	10.0±0.9	10.7±2.3	9.8±1.7	10.6±1.2
Hb[8] (g/dl)	14.8±1.2	15.7±0.9	14.7±1.2	14.8±3.3	14.2±2.3	14.7±2.0
Hct[9] (%)	43.9±3.6	47.3±3.0	43.8±3.2	48.4±9.9	42.5±5.2	47.6±5.0
Retic[10] (%)	3.8±1.2	3.8±0.6	3.8±1.3	17.0±1.6	4.0±1.4	14.3±2.2

1 CONTROL, negative control group injected with multiple doses of PBS, pH 7.4, containing 375 μg/ml of bovine serum albumin, fraction V (PIERCE) for 4 days

2 STD, positive control group injected with multiple doses of rHuEPO reference STD in PBS, pH 7.4, containing 375 μg/ml of human albumin for 4 days

3 TEST, testing group injected with multiple doses of rHuEPO expressed in SFC-30 medium (rHuEPO-SFC-30) in PBS, pH 7.4, containing 375 μg/ml of bovine serum albumin, fraction V (PIERCE) for 4 days

4 Pre, pre-injection sample collected at day 0

5 Post, post-injection sample collected at day 7

6 BW, body weight measured by analytical balance METTLER TOLEDO model PB3002 (Switzerland)

7 RBC, red blood cells

8 Hb, hemoglobin

9 Hct, hematocrit

10 Retic, reticulocyte per 100 cells of erythrocyte.

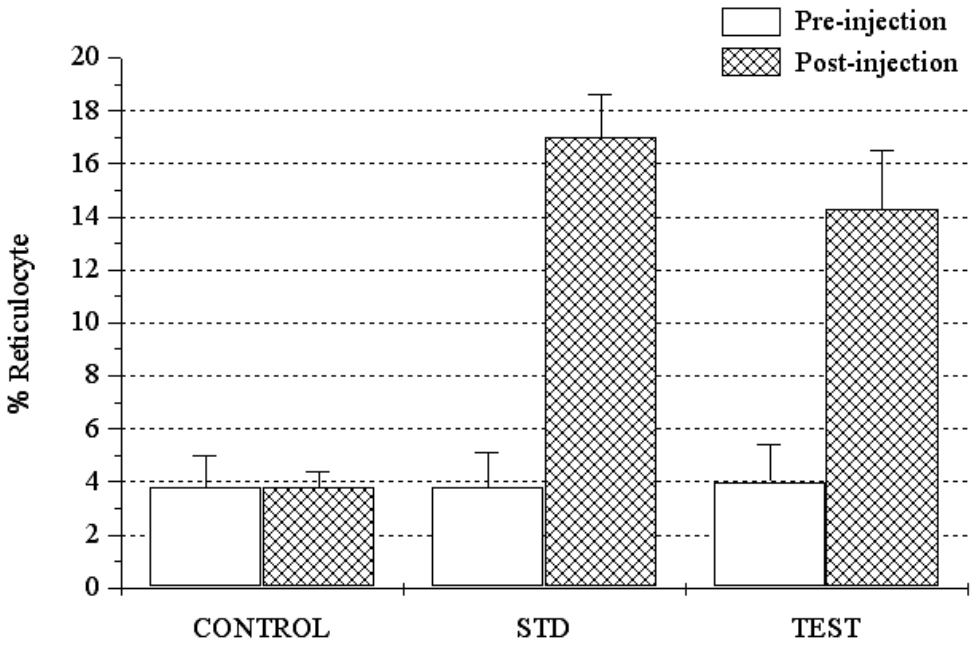

Figure 18 *In vivo* biological activity of rHuEPO in BALB/c mice.

Thirty IU of rHuEPO reference (HEMAX®) (STD), an equivalent units (30 units) of unpurified condition medium (rHuEPO-SFC-30) (TEST), and bovine serum albumin (CONTROL) were subcutaneously (*sc*) injected into BALB/c mice (N=10) at day 3, 4, 5 and 6 after pre-injected blood collection (at day 0). Post-injected blood sample was collected at day 7. Reticulocyte count was performed by automated blood counter (ADVIA120, Bayer). Values indicated in the figure represent mean with error bar determined by (Origin 5.0).

CHAPTER VI
DISCUSSION

Transient expression by means of extrachromosomal replication in COS-1 cell is frequently used to determine the function and integrity of the cloned gene introduced into these cells in the form of recombinant plasmid. This is usually for producing small amount of the recombinant protein sufficient for initial characterization. In this study, the transient expression system was used for characterizing the expression cloned human EPO engineered as bicistronic *epo-dhfr* expression cassette. The expression of this cloned gene was confirmed at the level of gene transcription and protein synthesis as well as secretion by RT-PCR and western blot analysis, respectively. Although time course monitoring of the mRNAs was not performed in this study, detection of plasmid derived EPO mRNA at 72-hr post-transfection implicates that EPO expression could sustain in the cells for relatively long (as in Figure 9). While monitoring EPO expression at the protein level by western blot analysis reflecting the steady stage level of rHuEPO in these cells provided several interesting features. First, high level of protein reacted by anti-HuEPO monoclonal antibody indicated that the recombinant protein was efficiently synthesized. The multiple bands of rHuEPO in the cell lysate (in Figure 10) representing un-glycosylated-, 1-glycosylated-, 2-glycosylated- and 3-glycosylated polypeptide implicated that transferring the carbohydrate moiety during its post-translation modification in ER is rate limiting step for rHuEPO production in COS-1 cells. In addition, the secreted protein in the medium exhibits a larger apparent molecular weight indicative of a further modification including of N-linked glycan processing and O-linked glycosylation (in the Golgi complex).

Second, these heterologous proteins were observed as early 24 hr post-transfection and found at maximum level 48 hr post-transfection. The protein level was rapidly declined and disappeared at 72 hr suggesting that the plasmid encoding rHuEPO may be lost from the cell population thus no more rHuEPO synthesis

occurred in these cells. In consistent with this observation, rHuEPO appeared in the cultured medium was observed at the highest level after 48 hr post- transfection and remained stable (at 72 hr). Third, the recombinant protein migrated with comparable size to that of reference control indicating that the glycosylation modification status of the secreted rHuEPO was complete. However, their broad pattern implies heterogeneity due to slight variation of carbohydrate complexity may exist in the pooled of secreted rHuEPO. This evidence has also been shown in COS-1 expression of other glycoproteins, such as human antithrombin III (ATTIII) (Wasley et al., 1987).

DHFR was frequently and successfully used as genetic marker for clonal selection in many cell types. DHFR catalyzes the conversion of folate to tetrahydrofolate (FH_4), a biocatalyst for the synthesis of glycine, thymidine monophosphate, and for purine biosynthesis (Figure 7). CHO cells deficient in DHFR-expression due to chemical mutagenesis (Urlaub & Chasin, 1980) are therefore unable to grow in medium depleted of nucleosides unless they acquire a functional DHFR-gene through transfection. Growing transfectants can be further selected for gene amplification by step-wise increasing of MTX concentration in the medium. This folic acid analogue (antifolate) binds and inhibits DHFR steriochiometrically, forcing the cells to undergo gene rearrangement and amplification for their survival. By multiple round of selection in increasing MTX concentrations, cell populations in which its genome contain several hundred copies of DHFR-gene can be obtained (Kaufman, 1990).

After transfection of CHO cells with the expression pED-EPO plasmid, many transfected cells can survive in the medium supplemented with 10 nM MTX. All the selected clones found to express rHuEPO in their cell extract, although with varying level, as well as in their culture medium. While recombinant protein in the culture medium looked similar to those observed in COS-1 cell, only single form of rHuEPO (~30 kDa) was found in cell extract of transfected CHO cell indicating that glycosylation modification is not rate limiting step. It is possible that the level of rHuEPO in these transfected CHO cells was relative low compared to those found in COS-1 cells thus requirement of glycosylation for this recombinant protein is not yet exceed the ability of CHO cells. Since most mammalian cells including CHO cells do not support extrachromosomal replication of pED plasmid, thus all these MTX

resistant CHO cells must contain at least one copy of the desired expression cassette(s) was (were) intregrated into their chromosome(s) to produce sufficient level of DHFR. With the advantage of DHFR/MTX gene amplification system, co-intregrated EPO-gene can be co-amplified once the DHFR-gene was amplified upon increasing the concentration of MTX in the medium. One reasons that why EPO-gene was co-amplified with DHFR-gene is that DHFR-gene was located downstream from the EPO gene in the expression cassette (Figure 8). Both genes in this cassette were expressed in the same mRNA transcript as bicistronic mRNA control by the activity of Adenovirus promoter. However they used the different translation initiation complex to drive protein synthesis. The translation of EPO open reading frame (ORF) required the general initiation site whereas the translational initiation of the downstream ORF of DHFR was independent from the upstream ORF. The translation of gene encoding DHFR was promoted by the internal ribosome binding site of EMC virus (EMC-L) within the mRNA (Kaufman et al., 1991). Whenever the functional DHFR level in certain population of the cells was enough to overcome the increased concentration of MTX, it implicated the transcription and translation of DHFR gene that were up-regulated due to the copy number of expression cassette which had been amplified.

The DHFR amplification of one of our initial clone increased their production by approximately 5-fold per round, whereas the induction was gradually decreased in later round of amplifications. During that gene amplification by increasing of MTX concentration, many evidences showed that cellular homeostasis was disturbed from increasing of MTX. These included 1) some clones could not survive in the later step of clonal expansion, 2) many survived clones showed the differences in morphological features during the times of amplification and propagation. Even a single clone was isolated but during expansion, the cloned cells showed some differences, in size and growth rate, among them. Normally, the *dhfr*-mediated gene amplification procedure took approximately 1 to 2 months for each round. During this long period, the cells might go through the diverse molecular mechanisms responsible for gene amplification, and thereby become heterogeneity. The fact that only the DHFR amplification is required for cells to survive upon increasing MTX concentration, it is possible at least in some cases that during gene amplification process cells might have mechanism to delete portion of EPO sequence which is not necessary for MTX

resistant trait. Hence this could result in a poor correlation between EPO production and DHFR level.

For the application of a gene amplification system to industrial processes, one of the most important factors is the selection method employed for obtaining highly productive recombinant CHO cell lines that can stably produce desired recombinant proteins. As a reason, the more rounds and gradually increased MTX concentration were needed to screen the highly different subclone out of the more heterogeneous clone. Throughout this thesis, the terms ‘‘clone’’ and ‘‘subclone’’ were used for consistency. However, as demonstrated here, it should be noted that immortalized mammalian cells have to be considered as heterogeneous, even after very short periods of cultivation. During screening times, the cell numbers before and after cell cultivation were counted to determine growth ratio of these subclones because these subclones contained many heterogeneous cells, which become candidate subclones for the next round. After each subsequent MTX concentration increase, the clonal specific growth rates declined as the population adapted to the new level of selection. Different results in growth ratio on this matter might occur because the recombinant CHO cells resulting from transfection have different sites of integration and their subsequent consequences on host cell function may differ significantly as has shown in the stability analysis of the amplified CHO subclones, which were randomly selected from cell population producing a chimeric antibody (Kim et al., 1998). However, the selection methods have so far been carried out only empirically and have been performed by trial and error.

The function of the oligosaccharides on the EPO molecule is complex. EPO glycosylation has been implicated in the secretion, stabilization, biological activity, antigenicity and circulatory half-life of the protein (Dube et al., 1988; Delorme et al., 1992). The predominant sugars found on human glycoproteins include galactose, mannose, fucose, N-acetylgalactosamine, N-acetylglucosamine, and N-acetylneuraminic acid (Neu5Ac, the human form of sialic acid). Several approaches have been used to determine the carbohydrate moieties on EPO molecule to ensure that rHuEPO from our cell lines are properly produced. One of these methods is the partial and full deglycosylation by glycanase enzyme. In this study, N-glycanase F (PNGase F) was used in partial digestion of the expressed protein. PNGase F

specifically cleaves nearly all types of the Asn-linked carbohydrate chain from glycopeptides or glycoproteins leaving the aspartic acid residual in the polypeptides (Maley et al., 1989). The number of N-linked glycosylation of the expressed proteins have examined to be three N-linked glycosylation sites as the rHuEPO reference (HEMAX®) (Figure 15). The apparent mobility patterns on SDS-PAGE of both proteins were almost identical except that there was an extra band appeared in our expressed rHuEPO as slightly smaller than those observed in the reference sample. Unlike the reference rHuEPO that has been purified and selected for complete modification, our rHuEPO used in this characterization was the crude sample thus it might be composed of mixed population between fully and partial modified rHuEPO. These bands might represent the population of protein that has incomplete O-linked glycosylation. Although we have evidence to support this speculation that this non-O-glycosylated form have previously been reported for rHuEPO expressed in BHK and COS-1 cells (Delorme et al., 1992; Elliott et al., 1994; Tsuda et al., 1990; Mueller et al., 1999). It is possible that the O-glycan might not be added in to O-linked glycosylation site in the expressed recombinant proteins or the O-glycan glycosylation occurred but unstable during long time storage.

The presence of terminal sialic acid on the carbohydrate chains is the most essential part for the full *in vivo*, but not *in vitro*, biological activity of EPO (Fukuda et al., 1989b; Lowy et al., 1960; Erbayraktar et al., 2003). As the important portion for its biological activity *in vivo*, we have monitored the presence of sialic acid by using specific lectin MAA that bound tightly to the sialic acid portion on the carbohydrate termini. This MAA binds specifically to sialic acid $\alpha(2\rightarrow3)$-linked to galactose termini in all carbohydrates. In EPO, there are three N-linked and one O-linked glycans which MAA can bind to all carbohydrate types. Although the sialic acid type of N-glycan produced from CHO cells (N-glycolylneuraminic acid, Neu5Gc) is different from human origin (N-acetylneuraminic acid, Neu5Ac), MAA still binds to all sialic acid types in the same conformation. In the experiment, the result showed the presence of the sialic acid specifically attached to the glycans (Figure 16). Sialidase digestion helped to confirm the specific binding of MAA lectin to the glycans by removing the sialic acid out. Recombinant HuEPO appeared on SDS-PAGE with a small size reduction after de-sialyation, which is in agreement with other studies (Takeuchi et al.,

1990; Tsuda et al., 1990; Kung & Goldwasser, 1997; Hammerling et al., 1996). The signal generated by color reaction was a broad band as the chemiluminascent signal from western blot due to the sialic acid was the part of all carbohydrate termini. From this evidence, sialic acid was characterized in that they were incorporated into probably all glycan positions but were really not all branch termini.

EPO *in vitro* assay gives a measure of how well EPO interacts with its receptor on erythroid precursor cells. Some changes in its native conformation or in the amount of carbohydrate branching can effect their interaction. To proof this hypothesis, an erythroleukemic cell line, TF-1 cells, was employed as a cellular indicator system to determine the biological activity of EPO *in vitro*. TF-1 is a growth factor dependent human cell line that was originally isolated from the bone marrow cells of a patient with erythroleukemia (Kitamura et al., 1989). TF-1 is a useful tool for analyzing the human receptors for IL-3, GM-CSF, and EPO or the signal transduction of these hematopoietic growth factors. TF-1 cells response to many growth factor including GM-CSF and EPO. Among these factors, GM-CSF was found as the most critical one that required for TF-1 growth. Our preliminary data indicated that TF-1 growth can be stimulated efficiently by GM-CSF alone in does dependent manner. As shown in Figure 17, these cells responded to GM-CSF after culture for 72 hr. The cell proliferation was determined by colorimetric MTT assay which the increasing in cell number was proportional to the measured absorbance (Abs). In contrast, supplement of EPO alone to the medium failed to stimulate TF-1 proliferation. Thus to establish system that allow us to monitor the rHuEPO activity in this cell line, the minimal concentration of GM-CSF was determined to be (0.025 ng/ml) and used for cell maintaining during rHuEPO activity test. This concentration was sufficient to maintain the survival of TF-1 cells but insufficient to stimulate proliferation of the cells unless EPO was also presented. After culturing TF-1 cells with various dilutions of EPO, but with the constant amount of 0.025 ng/ml of GM-CSF, these cells showed the proliferating effect, indicating that the EPOs bound to its receptors and stimulated the cell proliferation. The data obtained were clear that TF-1 stimulation was increased gradually when the higher concentration of EPO was supplemented together with a constant amount of 0.025 ng/ml of GM-CSF. However, when the concentration of rHuEPO exceed approximately 3 unit/ml, stimulatory effect on TF-1 proliferation was

found at saturating point (Abs = 0.540 for EPO-Reference and EPO-OptiMEM, and 0.580 for EPO-SFC-30) which was relative lower than those observed in GM-CSF (0.770). This result could be explained by limiting of EPO receptors expressed on the TF-1 cell surface which may be much lesser than that of GM-CSF receptors. This evidence was supported by the previous study of Hermine et al. 1996 that GM-CSF induces a rapid down-modulation of EPO-R at the mRNA and binding site levels with in 4 hr whereas EPO has no effect on GM-CSF receptor (GM-CSF-R) expression. Similar trend in dose response were observed upon replacing the reference rHuEPO with the culture media of the established rHuEPO-expressing CH) cell line. Slight variation on TF-1 stimulation was observed when compared between different types of media used. This may result from the intensity variation of EPO-EPO receptor interaction among the EPO samples cultured from the different culture mediums. Nevertheless this result provides evidence proven that rHuEPO produced by our cell line is biological active *in vitro*.

Once the expressed proteins were proven active *in vitro*, next these proteins were needed to be tested for biological activity *in vivo*. These *in vitro* assay has a serious disadvantage since they are unable to discriminate between intact EPO (sialiated form) and its asialo- or aglycosylated variants (desialiated or asialo forms), which the asialo forms have a much shorter plasma half-life and, therefore, a greatly reduced bioactivity when administered *in vivo*. Compared to activity test *in vitro*, many more physiological factors are known to affect rHuEPO function *in vivo*. These include its circulatory half-life, physiological barriers, and its carbohydrate components (Fukuda et al., 1989a; Spivak & Hogans, 1989; Imai et al., 1990; Higuchi et al., 1992). The presence of sialic acids in the molecule is important for its activity *in vivo* (Dube et al., 1988; Higuchi et al., 1992; Imai et al., 1990). Because the un-sialiated glycoproteins were rapidly removed from blood circulation by galactose-binding proteins expressed on the hepatocyte cell surface in the liver (Fukuda et al., 1989a; Ohara & Yamagata, 1986) resulting in depletion of EPO in the circulation (Fukuda et al., 1989a). In this study, the EPO expressed from these recombinant CHO cells was proven to be sialiated form. With this strong indication, the rHuEPO expressed in SFC-30 medium was believed to be active and could stimulate mouse erythroid progenitor cells resulting in increasing of young RBCs (reticulocytes) in

their blood circulation. The reticulocyte newly released from the bone barrow could be distinguished from normal erythrocyte easily. This cell type can be counted automatically as the percentage of mature erythrocyte. The fact that reticulocyte number is rapidly increased within one week upon EPO administration, thus it is usually used as an indicator to demonstrate the *in vivo* activity of rHuEPO (Ramos et al., 2003). As the obtained result, the % reticulocyte was markedly increased (from 4.0±1.4 % to 14.3±2.2 %) within 5 days after injection with testing rHuEPO. The increment is approximately 3.6 fold which is slightly lower than the result after administrated with the reference standard (4.5 folds), where other parameters of complete blood count appear to be unchanged. While the accuracy of other hematological parameters such RBC count, Hb concentration and Hct, whose accuracy entirely depend on the quality of blood sample being collected for analysis, % reticulocyte count does not depends on the sample quality because it was calculated directly relative to RBC number. The nature of blood from mouse is somewhat different from human blood particularly the rapid clotting nature which made it difficult to obtain good quality of sample.

The emergence of a wide variety of biological expression systems for the large-scale production of therapeutic proteins has shifted the focus from vectors to host organisms. Although expression systems now span bacteria, fungi, plants, insects, and mammalian cells, the vast majority of recombinant-derived biopharmaceuticals at the present time have been produced in *E. coli* and in mammalian cells. This promises to change as the economic benefits of the newer systems permit the development of a new generation of proteins heretofore considered unfeasible for commercial development. In spite of the high demand of rHuEPO for clinical application in Thailand, the use of this hormone is limited. This is mainly due to the very expensive price of the commercial rHuEPO that must be imported. Although the human EPO cDNA has been cloned for many years leading to the mass production of commercial rHuEPO used for the treatment of anemia. These commercial product are available under the names Epogen (Amgen), epogin (Chugai), Epomax (Elanex), Eprex (Janssen-cilag), Neorecormon or Recormon (Roche), and Procrit (Ortho biotech) with variations in the glycosylation pattern of EPO distinguishes these products. Epogen, Epogin, Eprex and Procrit are generically known as epoetin alfa, Neorecormon and

Recormon as epoetin beta and Epomax as epoetin omega. All of them are manufactured from other countries with high cost. In Thailand, no attempt in establishing system to produce rHuEPO for domestic use has been reported despite of available of the good expression system and technology providing large-scale isolation of rHuEPO for wide-range therapeutic application. Despite the impressive results which have been observed for many of the newer systems, there are many commercial considerations which suggest that CHO cell expression systems may continue to dominate the manufacture of biopharmaceuticals for a long time to come.

CHAPTER VII
CONCLUSION

1. The recombinant plasmid engineered to direct synthesis of rHuEPO in mammalian host cells is functional as demonstrated by transient expression in COS-1 cells.
2. Recombinant HuEPO-expressing CHO stable cell lines were established by stable transfection. Dicistronic *dhfr-epo* expression cassette in CHO cells was amplified by DHFR/MTX-mediated gene amplification. After 4 rounds of amplification, EPO secretion level of the best-producing subclone was approximately 100 times higher than the original clone (#2.5).
3. N-linked glycosylation pattern analyzed by PNGase F digestion of the expressed rHuEPO occurred as similar as referent EPO (HEMAX®) and its carbohydrate structure was sialiated with sialic acid residuals as proven by specific lectin MAA binding assay.
4. *In vitro* biological activity of the expressed rHuEPO was determined to be biologically active by stimulating the proliferation of human erythroleukemic TF-1 cell lines.
5. *In vivo* biological activity assay in BALB/c mice was evaluated by increasing reticulocytes in the blood circulation significantly and its activity was comparable with the EPO reference (HEMAX®).

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