Development and Characterization of Hyperglycosylated Recombinant Human Erythropoietin (HGEPO)

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ABSTRACT

Erythropoietin (EPO), a glycoprotein hormone produced from primarily cells of the peritubular capillary endothelium of the kidney, is responsible for the regulation of red blood cell production. We have been investigating the roles of glycosylation site added in the biosynthesis and function of recombinant protein. We constructed three EPO mutants (Δ69, Δ105 and Δ69,105), containing an additional oligosaccharide chains. EPOWT and EPOΔ69 were effectively expressed in transient and stably transfected CHO-K1 cell lines. But, it wasn't detected any protein in the culture medium of EPOΔ105 and EPOΔ69,105 mutants.

The growth and differentiation of EPO-dependent human leukemic cell line (F36E) were used to measure the cytokine dependency and *in vitro* bioactivity of rec-hEPO. MTT assay values were increased by survival of F36E cells at 24h. To analysis biological activity *in vivo*, two groups of ICR-mice (7 weeks old) were injected subcutaneously with 10 IU per mice of rec-hEPO molecules on days 0 and 2. Red blood cell and hematocrit values were measured on 6 days after the first injection. The hematocrit values were remarkably increased in all treatment groups. The pharmacokinetic analysis was also affected in the mice injected with rec-hEPO molecules 2.5 IU by tail intravenous.

Protein samples were detected by Western blotting. An EPOΔ69 protein migrated as a broad band with an average apparent molecular and detected slightly high band. Enzymatic N-deglycosylation resulted in narrow band and was the same molecular size. The biological activity of EPOΔ69 was enhanced to compare with wt-hEPO. The half-life was longer than wt-hEPO. The results suggest that hyperglycosyalted recombinant human erythropoietin (EPOΔ69) may have important biological and therapeutic good points.

(Key words : Erythropoietin, Recombinant, Hyperglycosylation, Pharmacokinetic)

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein produced from a 193-amino acid gene product after an N-terminal leader sequence containing 27 amino acids is cleaved. A carboxy-terminal arginine is lost from this 166-amino acid residue during passage into the circulation leaving a circulating hormone with 165 amino acids. The molecular mass of the EPO peptide is about 18 kDa (Lai *et al.*, 1986). EPO is mainly synthesized in the adult kidney and circulates in blood plasma, and a small portion of it is synthesized by the liver, and possibly by macrophages in the bone marrow (Benjamin and Franklin, 1999). An active hEPO consists of a single 166-amino acid polypeptide chain with three N-glycosylation sites an Asn^{24}, Asn^{38} and Asn^{83}, respectively, and one O-glycosylation site at Ser^{126} (Broudy *et al.*, 1987; Egrie *et al.*, 1986; Sasaki *et al.*, 1987; Takeuchi *et al.*, 1989). The EPO gene has been cloned and sequenced in a number of species including mouse, monkey, rat, human, sheep, pig and cat (McDonald *et al.*, 1986; Magao *et al.*, 1992; Wen *et al.*, 1993; Shoemaker *et al.*, 1986). And it has been made in understanding the functional characterization and molecular analysis of the recombinant EPO.

In earlier studies, we have been studying the recombinant glycoproteins (eCG, hFSH, TPO and EPO). It was found that deglycosylated sites were affected the expression and biological activity of these recombinant derivatives. Thus glycosylation sites play a pivotal role of the function and roles (Lee *et al.*, 2003; Min, 2000; 2001; Min *et al.*, 1996, 1997, 2004; Park *et al.*, 2005; Kim *et al.*, 2007).

In the presented study, we constructed hyperglycosylated EPO mutants and compared the expression quantity. The biological activity of recombinant hEPO was determined by the measuring MTT assay *in vitro*, hematocrit value and pharmacokinetics *in vivo*.

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MATERIALS AND METHODS

Materials

The expression vector pcDNA3 was purchased from Invitrogen Life Technologies (California, USA). CHO-K1 cells and Human leukemic cell line (F-36E) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). General PCR reaction cocktail (Top-Taq™ Polymerase, 10xbuffer, dNTP mixture) purchased from Core Bio System (Seoul, Korea). Restriction enzymes used were bought from Takara and Toyobo (Osaka and Tokyo, Japan). Ham's F-12, Opti-MEM I, serum free CHO-S-SFM II, neomycin analogue G418 and lipofectamine 2000 reagents were bought from Gibco BRL (MD, USA). Fetal bovine serum was from Hyclone laboratories (Utah, USA). Centriplus® Centrifugal Filter Devices purchased Amicon Bio separations (MA USA). The EPO ELISA assay kit purchased from R&D systems Inc (MN, USA). MTT cell growth kit purchased from Chemicon international (USA). ICR (Institute of Cancer Research) mice purchased from Korea Animal Technology (Koatech) (Seoul, Korea). EPO Elisa kit was from R & D system Inc. (MM, USA). All the other reagents used were from Wako Pure Chemicals (Osaka, Japan).

Cloning of EPO Gene and Site-directed Mutagenesis

The hEPO cDNA was amplified by polymerase chain reaction (PCR) using oligonucleotide primers descriced [sense: 5'-ggT ACCATgggggTgCACggTgAgTACTCg; antisense: 5'-TggAATTCACCATgggggTgcACgAATgTCCT]. Primers were added the EcoRI and Kozak sites to the 5'-end XhoI to the 3'-end of EPO cDNA. The cDNA fragment with 582bp predicted for hEPO was amplified from human liver cDNA library. The PCR fragments were ligated into PCR2.1 vector (invitrogen) and digested with *EcoR* I and *Xho* I. The fragments were ligated into *EcoR* I and *Xho* I digested sites of pcDNA3 mammalian expression vector.

Site-directed mutagenesis was performed by the four primers [Δ69 sense: 5'-CAgggCCTggCC**AAC**CTATC-ggAAACT-3'; Δ69 antisense: 5'-gACAgCTTCCgACAg**g-TT**ggCCAggCC-3'; Δ105 sense: 5'-ggCCTTCgCAgC**AA-C**ACCACTCTgCTT-3'; Δ105 antisense: 5'-AAgCAgAgT-ggT**gTT**gCTgCg AAggCC-3'] method of a two-step overlapping PCR. In order to substitute the Leu (CTG) at position 69 and 105 on the EPO cDNA by Asn (AAC), the PCR was carried out with pcDNA3-hEPO as the template using the EPO F, Δ69 R and Δ69 F, EPO R primers. Finally, two fragments of the first-step PCR were used at the second-step PCR. Other mutant was constructed with the same method, resulting in Δ105 and Δ69,105 as shown in Fig. 1. All mutants were sequenced completely to confirm the Kozak site and PCR errors.

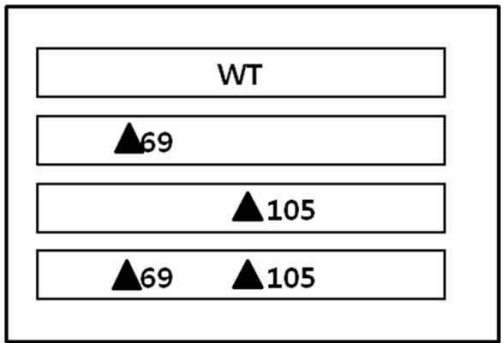


Fig. 1. Modeling of hyperglycosylated human erythropoietin cDNAs.

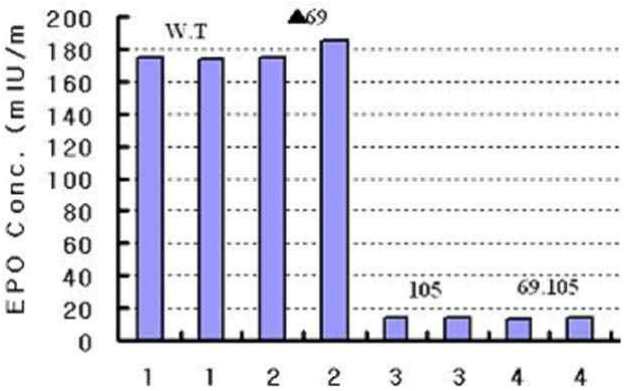


Fig. 2. Quantification of transient expression in rec-hEPO mutants. ELISA result of standard curve wasn't shown. The amount of expression of EPO mutants was measured by ELISA. Experiments were performed in duplicated (1: wild type; 2: Δ69; 3: Δ105; 4: Δ69, 105).

Expression Vector Construction

The expression vector is 5.4 kb vector contained CMV (human cytomegalovirus) promoter and bovine growth hormone (bGH) polyadenylation signal for polyadenylation of transcribed mRNAs. Also this vector involves SV40 origin for transient episomal replication in cells expression SV40 large T antigen and neomycin resistance marker (G418).

The PCR fragments of wild type EPO were digested with *EcoR* I and *Xho* I endonucleases and inserted into the same digested sites of pcDNA3 (pcDNA3-hEPO). Other mutants were constructed with the same method, resulting in Leu69Asn (pcDNA3-hEPOΔ69), Leu105Asn (pcDNA3-hEPOΔ105) and Leu69,105Asn (pcDNA3-hEPOΔ69,105).

Cell Culture and Functional Expression

The expression vectors (pcDNA3-hEPO mutants) were transfected into CHO-K1 cells by the liposome formulation (Lipofectamine™2000) transfection method according to the supplier's instruction. rec-hEPO protein by transient transfection was collected the supernatants

at 72h after transfection. Stably cell transfectants were selected by incubation in growth medium supplemented with G418 (800ug/ml) for several weeks posttransfection according to the method reported previously (Min *et al.*, 2004). After incubation of selected stable cells (1×10^6) in 10ml CHO-S-SFM-II at 37℃ for 48h, the culture medium were collected and centrifuged at 3,000 rpm, 4℃ for 5 min to remove the cell debris. Rec-hEPO protein was quantified using ELISA method according to the supplier's protocol (R&D systems Inc. MN, USA).

Assay for Cell Proliferation

Short-term cell proliferation was examined by a colorimetric assay. EPO-dependent human leukemic cell lines (F-36E) were incubated at a density of 1×10^4 cells/100 ul in 96-well plates in growth medium [DMEM contained penicillin (50 U/ml), streptomycin (50 mg/ml) and 10% FCS] in the presence of 2.5 and 5 IU of rec-hEPO. Next, MTT reagent was added at a final concentration to 0.5 mg/ml. Following four hour incubation at 37℃, the isopropylalcohol with 0.04 N HCI was added was added to each well. Within one hour, the 96-well plates were measured at 630 nm.

***In Vivo* Biological Activity and Pharmacokinetics Analysis**

Two groups of ICR-mice (7 weeks old) were used. To analysis *in vivo* biological activity, mice were injected subcutaneously with 10 IU (EPOWT and EPOΔ69) per mice on days 0 and 2. Red blood cell and hematocrit values were measured at 6 days after the first injection. The analysis was conducted by using blood obtained by filling heparinized microhematocrit tubes from the retro-optical venous plexus.

Before the administration of rec-hEPOs, 100~150 ul of blood were collected from the eyes into heparinized microhematocrit tubes. The tubes were centrifuged, and serum was collected and frozen at −20℃. Each animal received 2.5 IU of EPO WT and EPOΔ69 by intravenous injection. Blood samples were taken at 2, 4, 8 and 24 h after dosing. Pharmacokinetic parameters for intravenous administration were estimated.

SDS-PAGE and Western Blot Analysis

Samples were concentrated 5~10 times by Centricon and submitted to 12% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (0.2 um) via the semidry electroblotter apparatus. After blotting, the membrane was blocked with 1% blocking reagent for 1 hours and bound by monoclonal anti-human EPO antibody (10 ug/10ml of blocking solution) for 1~3 hours. The blot was washed to remove unbound antibody, incubated with a secondary antibody linked to anti-mouse IgG-POD (37.5 ul/15ml of blocking solution) for 30 min. and then washed. After then, the membrane was incubated for 5 min with 2 ml Lumi-Light substrate solution, removed the membrane from the Lumi-light substrate solution and placed it the protein side of membrane up on Saran Wrap. The membrane was covered with a second Saran Wrap and exposed on X-ray film for 1 min~10 min.

Enzymatic Release of N-Linked Oligosaccharides

The rec-hEPO protein was analyzed to remove the added glycans by *N*-glycosylation enzyme. For removal of all N-linked glycans, rec-hEPO was incubated 24 hours at 37℃ with PNase F [2ul enzyme (2.5 U/ml) /30 ul sample+8 ul of 5 x reaction buffers]. The reaction was stopped by boiling for 10 min. And then, it was electrophoresis by SDS-PAGE and analyzed by Western blot.

RESULTS

Production of Transient and Stably expressing rec-hEPOs

The EPO WT and mutant vectors were transfected into CHO-K1 cells. Transient expression was collected the supernatants at 72h after transfection. To establish the stably cell lines, the culture was continued for 21~30 days in F-12 Nutrient Mixture containing 10% fetal calf serum and between six and eight clones were selected for G41. After incubation with 10 ml CHO-S-SFM II for 48h, the culture medium were collected and centrifuged for 5 min to remove the cell debris. The rec-hEPO was assayed using an ELISA kit. But, it wasn't detected any protein in culture medium of EPOΔ105 and EPOΔ69,105 mutants. The EPOΔ69 expression was the same in compared with wild type hEPO. This reason seems to be due to the folding of amino acid at 105. It is also suggest that 105 amino acid of hEPO is very important for secretion.

Northern Blot Analysis

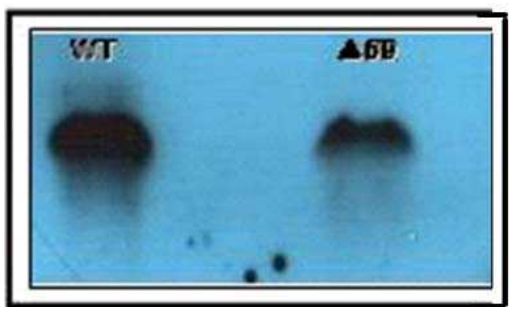


Fig. 3. Northern botting result.

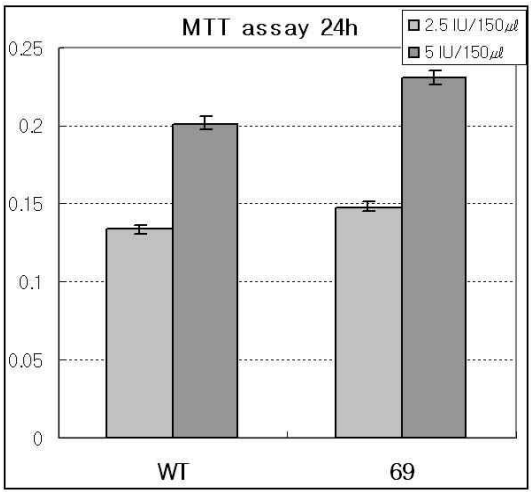


Fig. 4. Responsiveness of F-36E cell lines for rec-hEPO. The results were shown as a mean in triplicate data.

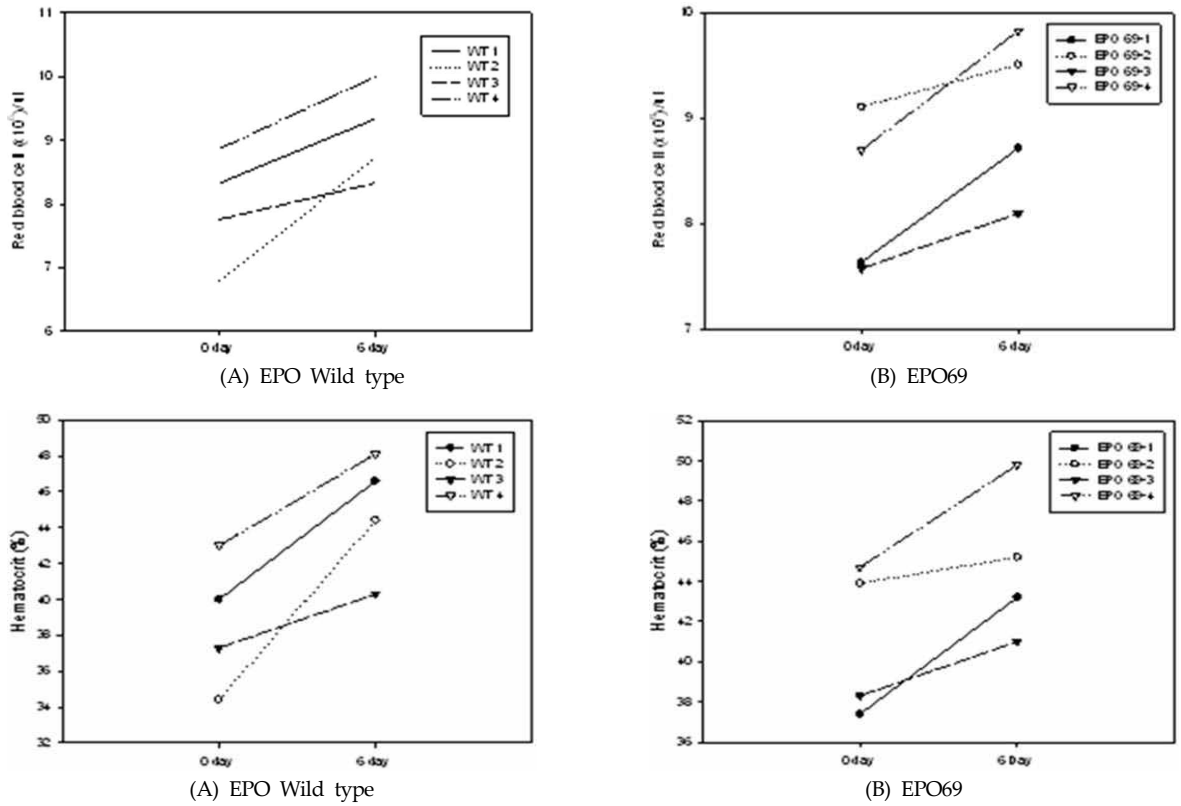
For Northern blot analysis, total RNA was extracted from CHO-K1 cell line selected by G418 by using the Trizol reagent according to the manufacturer's instruction. After electrophoresis, RNA was shown in UV light. And then, the gel was transferred into the membrane and hybridized with the hEPO cDNA (582bp) labeled. A mRNA expression was detected by the X-ray film. The result of northern blot was shown the main band in each lane. As shown in Fig. 3, it was effectively expressed in the wt and Δ69 cell lines.

Assay for Cell Proliferation

The *in vitro* biological activity of EPO derivatives was determined with an EPO-dependent cell line, human leukemia F-36E cells. EPO is the most potent cytokine for the F-36E cell lines. The growth and proliferation of the F-36E cells by EPO levels were shown in Fig. 4. The proliferation of the cells was increased by the EPO concentration in 24 hrs. Specially, the maximal stimulation by EPO was almost equal in short-term assay with 2.5 IU treatment. But, it was a little different in the 5 IU treatment group.

In Vivo Biological Activity

Two groups of mice were injected subcutaneously with 10 IU of rec-hEPO on days 0 and 2. Hematocrit and RBC measured on 6 days after the first injection. The hematocrit values were remarkably increased in all treatment groups. And RBC values were also dramatically increased at the all groups. These results were almost same in the RBC and hematocrit values (Fig. 5).



(A) EPO Wild type (B) EPO69

(A) EPO Wild type (B) EPO69

Fig. 5. EPO induced high RBC and hematocrit increasement by EPOWT and EPOΔ69. Two groups of ICR mice were injected subcutaneously with 10 IU of rec-hEPOs on day 0 and 2. RBC and hematocrit values were determined pretreatment (0) and 6 days after injection. Values are given for each mouse.

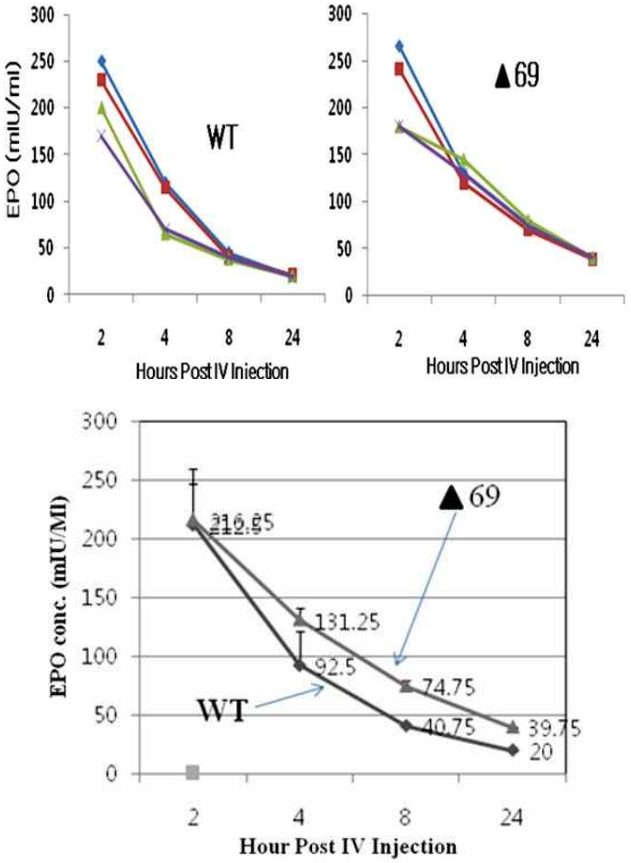


Fig. 6. Pharmacokinetics of EPOWT and EPOΔ69 in *in vivo*. Mice were injected iv with 2.5 IU of recombinant EPOs, and blood samples were drawn at the indicated times. Serum EPO levels were determined by ELISA as described in Material and Methods (under panel represents the average EPO levels of 4 mice.

Pharmacokinetic Analysis

We showed that the pharmacokinetic behavior of EPOΔ69 is markedly different from that of WT EPO (Fig. 6). We injected two groups of mice intravenously either 2.5 IU of rec-hEPO. At specified times, plasma samples were prepared by centrifugation from heparinized blood. The plasma concentration was determined by ELISA. All four animals injected were the highest peak at 2h after injection. The plasma levels of all four animals remained high for many hours after injection (Fig. 6). EPOΔ69 was detectable in the plasma even after 24 h.

Western Blot and Treatment of N-linked Oligosaccharide

Efficient translation of the EPO WT was detected about 37 kDa. However, EPO WT was detected the broad band from 30-38 kDa and EPO Δ69 was a little higher than that of EPO WT and detected a several bands of different size (Fig. 7). By the N-linked digestion, molecular sizes of EPO WT and EPO Δ69 were remarkably decreased to 17~18 kDa. It suggested that

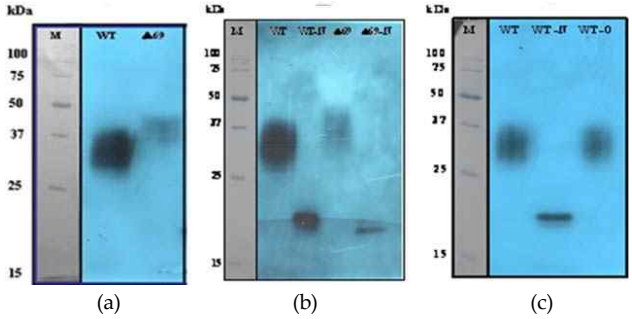


Fig. 7. Western blotting result for N-linked and O-linked glycosylation digestion. (a): EPOWT and EPOΔ69. (b): Digestion with N-linked glycosylation enzyme. (c): Digestion with N-linked and O-linked glycan enzyme.

the oligosaccharides of about 20 kDa were added to rec-EPO. By the O-linked digestion, molecular size was the same with EPO WT. Thus, O-linked oligosaccharides were not modification in the CHO-K1 cell lines.

DISCUSSION

In the present study, we have shown that rec-EPOΔ69 produced into the CHO cell lines has an enhanced biological activity *in vitro* and *in vivo*. EPOΔ69 was added glycosylation site at 69 leu. Thus, activity of EPOΔ69 *in vivo* was remarkably enhanced. Hematocrit values were also enhanced in WT and EPOΔ69. EPOΔ105 was not secreted into the culture medium. This reason seems to be due to the folding of amino acid at 105.

EPO is heavily glycosylated protein with a carbohydrate moiety of approximately 40%. Three N-linked (Asn-24, 38 and 83) and one O-linked (Ser-126) acidic oligosaccharide side chains are contained in the molecule (Lai *et al*. 1986; Broudy *et al*., 1987; Egrie *et al*., 1986; Sasaki *et al*., 1987; Takeuchi *et al*., 1989). EPO is primarily produced in adult kidney and fetal liver cells (Sasaki *et al*., 1987). Human EPO contains 4 cysteine residues that form 2 disulfide bridges between cysteine 7 and 161 and between cysteine 29 and 33 (Lai *et al*., 1986). Urena (2002) reported that the addition of these extra-carbohydrate chains gives greater metabolic stability and a half-life 3.6 times longer than rhEPO glycosylation sites.

Darbepoetin alfa, a hyperglycosylated rec-hEPO analogue with two extra carbohydrate chain, has approximately 13-fold to 14-fold more poetent than rec-hEPO WT (Egrie and Browne. 2001; Egrie *et al*., 2003). Macdougall *et al*. (1999) reported a similar result that NESP (novel erythropoietin stimulating protein), a hyperglycosylated analogue of rec-hEPO (Epoetin), has an increased terminal half-life in animal models. In this study

EPOΔ105+Δ105 was not secreted into the culture medium. Ellioitt *et al*. (1997) reported that 105 th residue of EPO protein is important for folding which required for biological activity. Thus, the addition of glycosylation site, mutation in site 105 changed protein folding pattern of mutant EPO and probably inhibited its secretion and possibly its function.

Taken together, the studies presented above demonstrated that new analogue EPO was developed and glycosylation site of EPO seems to play an important role for its biological activities. This data suggest that EPOΔ69 have observed an increase in the biological specific activity in comparison with the activity of EPO WT. Thus, the EPOΔ69 could reduce the amount of therapeutic EPO required for the treatment of various chronic anemia. In addition, the availability of a more active EPO molecule could be useful for the development of EPO-based gene therapy approaches.

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