Korean J. Animal Reprod. 27(4) : 299~307 (2003)

Effects of Changes in Glycosylation Sites on Secretion of Recombinant Human Erythropoietin in Cultured CHO Cells

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ABSTRACT

The effects of additions/deletions in glycosylated residues of recombinant human EPO (rhEPO) produced in CHO-K1 on their secretion were examined. hEPO cDNA was amplified from human liver mRNA and cloned into the pCR2.1 TOPO. Using overlapping-extension site-directed mutagenesis method, glycosylation sites at 24th, 38th, 83rd, and 126th were respectively or accumulatively removed by substituting its asparagine (or serine) with glutamine. To add novel glycosylation sites, 69 and 105th leucine was mutated to asparagine. Mutant and wild type rhEPO constructs were cloned into the pcDNA3 expression vector with CMV promoter and transfected into CHO cell line, CHO-K1, to produce mutant rhEPO mutant rhEPO proteins. Enzyme-linked immunosorbant assay (ELISA) and Western analysis with monoclonal anti-EPO antibody were performed using supernatants of the cultures showing transient and stable expressions respectively. Addition of novel glycosylation reduced rhEPO secretion dramatically while deletion mutants had little effect except some double deletion mutants (△24/83 and △38/83) and triple mutant (△24/38/83). This fact suggests that not single but combination of changes in glycosyl groups affect secretion of rhEPO in cell culture, possibly via changes in their conformations.

(Key words : rhEPO, Glycosylation site, Site-directed mutagenesis, CHO cell culture, ELISA)

I. INTRODUCTION

Human erythropoietin (EPO) is a glycoprotein hormone with a molecular mass of ca. 30 kDa that circulating plasma (Fisher, 1997). EPO protein contains three N-glycosylation sites (24, 38 and 83th Asn residues) and one O-glycosylation site (126th Ser residue). EPO is primarily produced in adult kidney and fetal liver cells (Sasaki et al., 1987). The function of EPO is to stimulate the proliferation and differentiation of erythroid ancestor cells (Goldwasser and Kung, 1968). 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; Nagao et al., 1992; Wen et al., 1993; Shoemaker et al., 1986). The overall scope of glycoforms can have a variety of different biophysical and biochemical properties, although details of structure-function relationships are poorly understood (Macmillan et al., 2001). The presence of sialic

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acid on the oligosacharide chains is essential for the full *in vivo*, but not *in vitro*, biological activity of EPO (Takeuchi et al., 1989). Elliott et al. (1997) reported that EPO has the important regions for its biological activity : amino acids 11 to 15, 44 to 51, 100 to 108, and 147 to 151. The N-linked carbohydrates chain at position 38 is critical for the polarized secretion (Kitagawa et al., 1994). The carbohydrate of glycosyl groups are different because of their variety monosaccharide compositions, sequences, branching sites, linkages, and modifications involving phosphate and sulfate groups (Kawasaki et al., 2001). Nowadays, only one type of recombinant human EPO (rhEPO), produced in CHO cell line, is vastly used in therapy to cure severe anemia.

To understand the function of glycosylation in rhEPO protein, many researchers employed mutation analysis, especially site-directed mutagenesis. The transient expression levels varied among mutants, being highest for mutants Asn24Gln followed by wild-type rhEPO but Asn38Gln, Asn83Gln, Ser126Thr, and Ser126Gly were secreted at lower levels than rhEPO of wild-type (Fibi et al., 1995). The expression system was used to study three mutants in which the N-glycosylation sites were changed to cysteins (Asn24Cys, Asn38Cys and Asn 83Cys). Consequently, specific activities of these cysteine mutants were significant, but they were reduced (60, 22 and 70%, respectively), compared to wild type (Bill et al., 1997).

EPO play an important role therapeutic glycoprotein with three sites of N-glycosylation that are essential for *in vivo* biological activity (Macmillan et al., 2001). The elimination of all three N-glycosylation sites decrease in EPO production to 10% of the wild-type EPO. Also, the fraction of cellular extracts demonstrate that the mutant proteins are lacking glycosylation at each of these three sites (38, 83 and 126), and they are mainly associated with membrane components and others are degraded rapidly (Dube et al., 1988). The N-linked glycosylation sites are necessary for EPO to express its full biological activity *in vitro* (Takeuchi et al., 1990). The mean terminal half-life for intravenous novel erythropoiesis stimulation protein (NESP) was three fold longer than that for intravenous EPO (Macdougall et al., 1999).

This study examined the role of glycosylation in rhEPO protein in its secretion by producing glycosylation sites mutants of rhEPO protein. Many studies have suggested the important role of glycosylations on secretion and activities of the protein. This experiment attempts to review their claims. Following these results, the glycosylation sites were added where their claims are credible in the rhEPO, and their secretion in cultured cells were investigated.

Ⅱ. MATERIALS AND METHODS

1. Preparation of PCR Primers and EPO cDNA

The hEPO cDNA was amplified by polymerase chain reaction (PCR) using oligonucleotide primers described in Table 1. Primer for the 5' and 3' ends of hEPO cDNA contained unique restriction sites with *Eco*R Ⅰ and *Sal* Ⅰ. 582 bp of hEPO cDNA was amplified from human liver cDNA library

2. Site-directed Mutagenesis

hEPO cDNA clone by PCR was designed to replace the codons for asparagine at amino acid positions 24, 38, and 83 with glutamine codons, and to replace the codon for serine at position 126 with a glutamine codon. And oligonucleotides of 27~31 base also were designed to replace the codons for leucine at amino acid positions 69, 105 with asparagine codons. The former are changed glycosylation site into asparagine, the latter are substitute Leu for asparagine. Wild type EPO

Table 1. PCR primers for hEPO mutant constructs

Primer	Sense(F)	Antisense(R)	Codon mutation
EPO	tgaattcaccatgggggtgcacgaatgtcct	gagtcgacctcatctgtcccctgtcctgca	
24	aaggaggccgag**cag**atcacgacg	gcccgtcgtgat**ctg**ctcggcctc	Asn → Gln
38	agcttgaatgag**cag**atcactgtc	tgggacagtgat**ctg**ctcattcaa	Asn → Gln
83	gccctgttggtc**cag**tcttcccag	cggctggaaaga**ctg**gaccaacag	Asn → Gln
126	ccagatgcggcc**cag**gctgctcca	gagtggagcagc**ctg**ggccgcatc	Ser → Gln
69	cagggcctggcc**aac**ctgtcggaaact	gacagcttccgacag**gtt**caggcc	Leu → Asn
105	ggccttcgcagc**aac**accactctgctt	aagcagagtggt**gtt**gctgcgaaggcc	Leu → Asn

(rhEPO) was produced by EPO cDNA using primers EPO forward primer (EPO F) and EPO reverse primer (EPO R), and then EPO mutants were constructed with rhEPO template. For instance 24EPO mutant was constructed by amplifying rhEPO using EPO F and 24 R primer, and 24 F and EPO R primers. Gel-purified two different template constructs were produced amplifying with EPO F and EPO R. Other mutants were constructed with the same method, resulting in Asn24Gln, Asn38Gln, Asn83Gln and Ser126Gln.

3. Polymerase Chain Reaction

The reaction (25 μl) of PCR contained 30 pM sense or anti sense primer; 10 ng of EPO cDNA; 0.4 mM dNTP; 10×Buffer; 1 unit rTaq DNA polymerase (Toyobo, Japan). They were subjected to 1 cycles of 2 min pre-denaturation at 94℃, 30 cycle (1 min denaturation at 94℃, 1 min annealing at 55℃ and 1 min extension at 74℃).

4. Cloning Vector

Gel-purified rhEPO and mutant EPO were ligated into the cloning vector pCR2.1 (Invitrogen). Ampicillin-resistant clones (50 μg/ml) were plucked, and positive clones were identified by restriction digest analysis using *Eco*RⅠ. The DNA was transfected into *E. coli* stain XL1-MRF. The constructs were fully sequenced the variants hEPO genes to check for PCR errors and to confirm the correct reading frame for protein expression. The presence of the mutations was confirmed by sequencing.

5. Expression Vector Construction

rhEPO and mutant EPO were digested with *Eco*RⅠ and *Sal*Ⅰ. The variant fragments were ligated into pcDNA3 expression vector (Invitrogen). This vector is 5.4 kb vector contained CMV (human cytomegalovirus) promoter and bovine growth hormone (bGH) polyadenylation singnal for polyadenylation of transcribed mRNAs. Also this vector involved SV40 origin for transient episomal replication in cells expression SV40 large T antigen and neomycin resistance marker (Fig. 1).

6. Transfection

Transfections were performed according to published protocols (Gibco, BRL). Cells were seeded at 6×10^5 per well in a 60 mm dishes to obtain 50~80% confluence the following day. Complexes were prepared with 6 μg (50 μl) DNA and lipid

solution 10 μl in 600 μl serum free medium. The two solutions were mixed gently and incubated at room temperature for 15 to 45 min to allow DNA-lipid complexes to form. While complexes form, the dishes were rinsed once with 4 ml of serum-free medium that added the complexes (1.8 ml) directly to each dish and mixed gently. The transformants were incubated at 37℃ in 5% CO_2 for 5 to 24 hrs. Following incubation, 2.4 ml of the twice normal concentration of serum were added that without removing the transfection mixture. And the medium changed with fresh, complete medium at 24 hrs following the start of transfection. The supernatants were collected after 72 hrs and stored in −20℃ until analysis.

7. Erythropoietin ELISA

EPO protein was measured by sandwich type ELISA (Medac, Germany). For ELISA, two monoclonal antibodies that recognized different epitopes of EPO were used. The supernatant samples were diluted with cold DPBS (1:200). The optical absorbance at 405 nm was measured by a microplate reader (BIO-RAD, Model 550).

8. SDS-PAGE and Western Blot Analysis

SDS-PAGE and Western blot was performed with the 12.5% polyacrylamide gel and Western kit (BIO-RAD, cat. 170-8235 to 8240). Briefly, rhEPO in culture supernatant was separated by 12.5% polyacrylamide gel. Samples of desalted and concentrated culture supernatant were denatured by boiling for 4 min with 5×sample buffer (0.6 ml 1 M Tris-HCl-pH 6.8, 5 ml 50% glycerol, 2 ml 10% SDS, 0.5 ml 2-mercapoethanol 1 ml 1% bromphenol blue 0.9 ml H_2O. After SDS-PAGE, the gel was ready for Western blot analysis. The protein was transferred to Hybond P extra membrane (Amersham) in methanol-containing buffer, and detected by monoclonal mouse anti-human EPO antibody (R&D, Cat. MAB287). The blot was washed to remove unbound antibody, incubated with a secondary antibody linked to horseradish peroxidase (HRP), and then washed again to remove unbound secondary antibody. The blot was incubated in the Opti-4CN substrate for up to 30 min. Afterward, It was incubated in the Bio-Rad amplification reagent, washed incubated in streptavidin-HRP, and It washed again before being incubated in the Opti-4CN substrate.

Ⅲ. RESULTS

1. The Secretion of rhEPO

Secretion of the rhEPO from CHO cell supernatant was measured by ELISA assay and Western assay. The secretion levels were 45~50 IU/ml. A protein for rhEPO was detected one band with molecular weight of 30 kDa (Fig. 1).

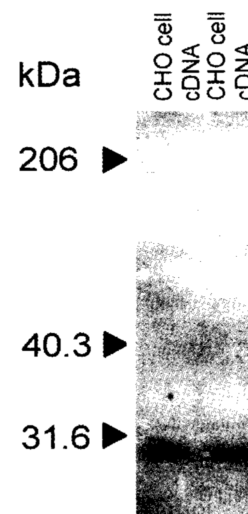

Fig. 1. SDS-PAGE with subsequent immunoblotting of CHO cell supernatant. Samples were stained with a biotinylated mouse monoclonal antibody against hEPO using horseradish peroxidase-labeled streptavidin and a chemiluminescent substrate. Lane 1: CHO cell cDNA supernatant-1 Lane 2: CHO cell cDNA supernatant-2.

2. Constructs of Erythropoietin Mutants

The EPO has three possible N-glycosylation sites at position 24, 38 and 83, according to the presence of Asn - X (except proline) - Ser/Thr in erythropoietin. As illustrated on Fig. 2, eight deleted mutants and three additional mutants were generated by site-directed mutagenesis. The deleted mutants were constructed that replaced Asn with Gln (Asn 24Gln, Asn38Gln, Asn83Gln, Ser126Gln, Asn24, 38Gln, Asn24,83Gln, Asn38,83Gln, Asn24,38,83 Gln) and additional mutants that replaced Leu with Asn (Leu69Asn, Leu105Asn, Leu69, 105Asn). Eleven EPO cDNA mutants were transfected into CHO cells (Fig. 2).

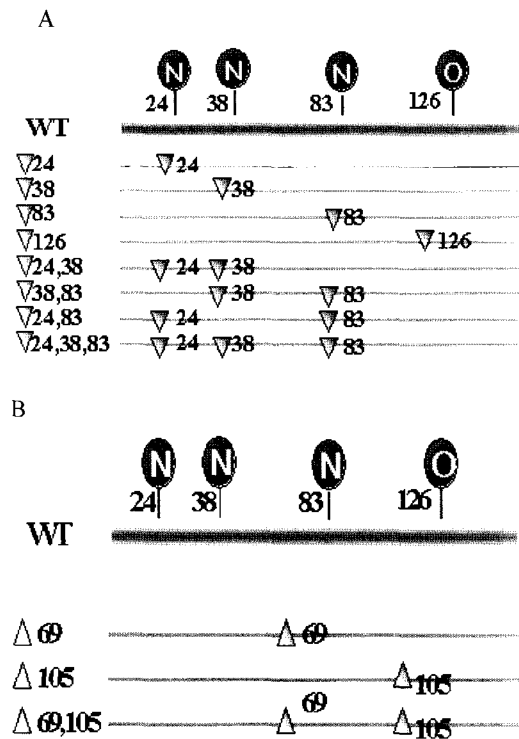

Fig. 2. Diagram of elimination and addition of glycosylation sites. A: The diagram describe constructs of single, double and triple mutants. B: Constructs of additional mutants were constructed by site-directed mutagenesis.

3. Transient and Stable Expression of Deletion Mutants

This experiment was performed with eight different mutants that lacks the N-glycosylation sites. Its transfected into the CHO cell line. The supernatants were harvested and the concentrations of EPOs were measured by ELISA. In the single mutants, 24, 38 and 83 were secreted normally

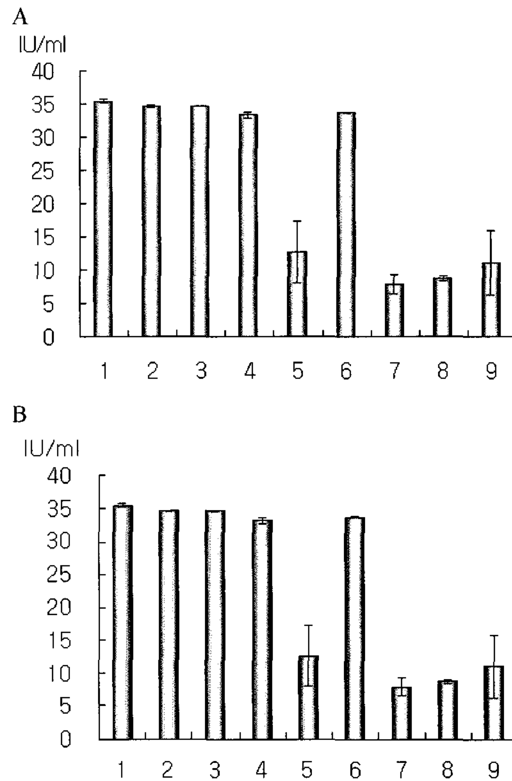

Fig. 3. The concentrations of rhEPO according to transient or stale expression (Eliminated glycosylation). CHO cells were transfected with the plasmids bearing the mutated rhEPO genes. At 72 hrs after transfection, the concentrations of rhEPO in the culture medium were assayed by ELISA. A: Transient expression, B : Stable expression, 1 : WT, 2 : 24 mutants, 3 : 38 mutants, 4 : 83 mutants, 5 : 126 mutants, 6 : 2438 mutants, 7 : 2483 mutants, 8 : 3883 mutants, 9 : 243883 mutants.

rhEPO, but 126 was inhibited the secretion. In the double and triple mutants, only Asn24,38Gln was secreted normally rhEPO, but remaining mutants were slightly secreted. Asn83Gln combination such as Asn24,83Gln, Asn38,83Gln and Asn24,38,83Gln mutants prevented the mutant secretion. This result suggests that interaction of 83 glycosylation site and other glycosylation sites significantly affected the secretion. Various concentrations of protein indicated that each sample differed from secretion of

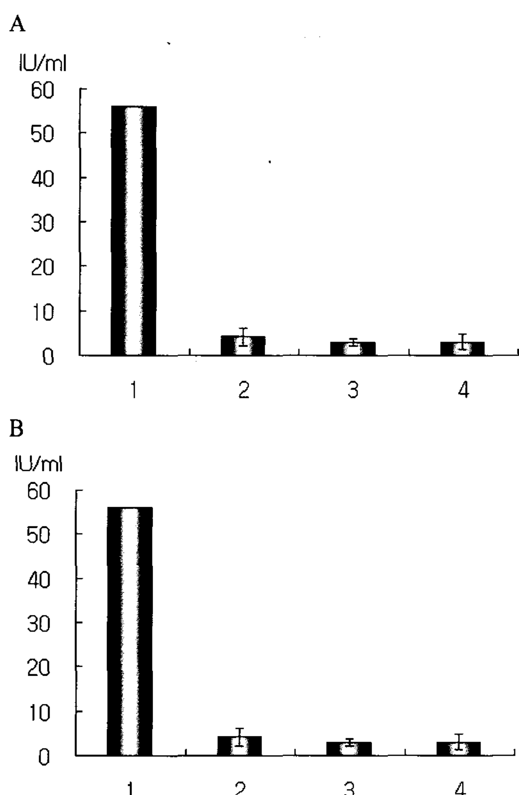

Fig. 4. The concentrations of rhEPO according to transient or stable expression (additional glycosylation). CHO cells were transfected with the plasmids bearing the mutated rhEPO genes At 72 hrs after transfection, the amounts of rhEPO in the culture medium were assayed by ELISA. A: transient expression, B: stable expression, 1 : WT, 2 : 69 mutants, 3 : 105 mutants, 4 : 69105 mutants.

ability or transfection efficiency (Fig. 3A). Due to the differences in transfection efficiency of each sample, stable transfection study was required. After neomycin selection, supernatants were measured by ELISA. The results of the mutants of rhEPO, Asn24Gln, and Asn24,38Gln were not influenced the secretion of rhEPO. In contrast, other mutants inhibited the secretion of rhEPO (Fig. 3B).

4. Transient and Stable Expression of Additional Mutants

To examine whether additional N-linked glycosylation sites could be affect the secretion of rhEPO, one or two of the nonglycosylated leucins amino acid (69, 105) were replaced with asparagines. Our results showed that all of additional mutants poorly secrete rhEPO (Fig. 4).

Ⅴ. DISCUSSION

Individual glycosylation sites of human erythropoietin were removed or added by site-directed mutagenesis to assess effects of the changes in the glycosylation sites on secretion. According to previous reports, prevention of the addition of N-linked carbohydrate at positions 38, 83, 126 have a significant role in secretion of EPO protein (Dube et al., 1988). Fibi et al. (1995) reported mutants of glycosylation, including Asn38Gln, Asn83Gln, Ser 126Thr, Ser126Gly, were secreted at lower levels than rhEPO. The study also showed that single mutant Asn126Gln, double mutants Asn24,83Gln and Asn38,83Gln and triple mutant (Asn24,38,83 Gln) were secreted slightly. A double mutant (Asn38,83Gln) and a triple mutant (Asn24,38,83 Gln) were poorly secreted (Delorme et al., 1992). Wasley et al. (1991) reported that rhEPO normally secreted without O-linked carbohydrate. However, in this experiment, the EPO protein level in Asn

126Gln supernatant was slight. Asn24Gln mutant is also considered to have biological activities that are superior to rhEPO (Fibi et al., 1995). Takeuchi et al. (1989) suggested that higher branching of the N-linked oligosacharide chains were essential for effective expression of *in vivo* biological activity of EPO. Thus, tetraantennary glycans affected secretion in the case of N-glycosylation. Cointe et al. (2000) also indicated that unusual glycan structures of EPO-RPMI (produced by human lyphoblastoid cell line) did not modify the *in vitro* and *in vivo* biological activities measured in animals. Our result suggest that Asn126Gln, Asn38,83Gln, and triple mutation (Asn24,38,83Gln) play an important role in secretion. 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. Thus, improvement of the secretion and bioactivity rhEPO was expected when added glycosylation sites. In this study, all additional mutants poorly secreted, but rhEPO normally secreted not only stable expression also transient expression. Ellioitt et al. (1997) reported that 105th 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. The carbohydrate appeared to play the critical role in stabilizing the EPO molecule to denaturing conditions, and this increased stability does not depend on the presence of sialic acid (Narhi et al., 1991). Fukuda et al. (1989) reported that asialoEPO was produced by treatment of rhEPO with sialidase, and it found to be cleared rapidly from circulation within ten minutes. These results suggested that the galactose binding protein of hepatic cells was involved in the clearance of asialoEPO. Also, probably deglycosylated rhEPO rapidly degraded in supernatants. Taken Together, the result from study indicate that rhEPO mutants differ transient expression from stable expression because of transfection efficiency or reduced factor. Also, additional mutants reduce secretion of rhEPO against the expectation. It affects the protein folding that change of the 69 and 105th amino acid in the rhEPO.

Our experiments were designed to obtain rhEPO protein by transfected CHO cell supernatant after deleted or added glycosylation. Removal of N-glycosylation sites were reported as not impairing receptor binding of EPO, and some mutations are reported to increase binding affinity to the receptor (Yamaguchi et al., 1991). In this experiment, we observed that the Asn24Gln, Asn24,38Gln and rhEPO proteins were secreted in transfected CHO cell line. Egrie and Browne (2001) reported that Novel erythropoiesis stimulating protein (NESP, darbepoetin alfa) has a longer serum half-life and greater *in vivo* biological activity compared to rhEPO. However, in this study, the proteins with added glycosylation was not detected by ELISA possibly due to the change of protein folding. ELISA used in this study consists of two mouse monoclonal antibodies which recognize different epitopes of hEPO. As a result of ELISA assay, it was noticed that secretion levels in the transient expression differed from stable expression after elimination of glycosylation.

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(Received September 16, 2003;
Accepted October 21, 2003)