Biological Activity of Human Dimeric Hyperglycosylated Erythropoietin (dHGEPO) Fusion Proteins

Purevjargal Naidansuren and Kwan-Sik Min†

Animal Biotechnology, Graduate School of Bio. & Information Technology, Institute of Genetic Engineering, Hankyong National University, Ansung 456-749, Korea

ABSTRACT

Erythropoietin (EPO) is a glycoprotein hormone secreted from primarily cells of the peritubular capillary endothelium of the kidney, and is responsible for the regulation of red blood cell production. We constructed and expressed dimeric cDNAs in Chinease hamster ovary (CHO) cells encoding a fusion protein consisting of 2 complete human EPO domains linked by a 2-amino acid linker (Ile-Asp). We described the activity of dimeric hyperglycosylated EPO (dHGEPO) mutants containing additional oligosaccharide chains and characterized the function of glycosylation. No dimeric proteins with mutation at the 105th amino acid were found in the cell medium. Growth and differentiation of the human EPO-dependent leukemiae cell line (F36E) were used to measure cytokine dependency and *in vitro* bioactivity of dHGEPO proteins. MTT assay at 24 h increased due to the survival of F36E cells. The dHGEPO protein migrated as a broad band with an average molecular mass of 75 kDa. The mutant, dHGEPO, was slightly higher than the wild-type (WT) dimeri-EPO band. Enzymatic N-deglycosylation resulted in the formation of a narrow band with a molecular mass twice of that of of monomeric EPO digested with an N-glycosylation enzyme. Hematocrit values were remarkably increased in all treatment groups. Pharmacokinetic analysis was also affected when 2.5 IU of dHGEPO were intravenously injected into the tails of the mice. The biological activity and half-life of dHGEPO mutants were enhanced as compared to the corresponding items associated the WT dimeric EPO. These results suggest that recombinant dHGEPO may be attractive biological and therapeutic targets.

(Key words : Dimeric erythropoietin, Hyperglycosylation, Pharmacokinetic)

INTRODUCTION

Erythropoietin (EPO) is a 34-kD glycoprotein produced mainly by kidney paratubular cells in response to reduced oxygen delivery. Low levels of EPO are synthesized by the liver and possibly by macrophages in the bone marrow (Lacombe *et al.*, 1988; Benjamin and Franklin, 1999). Recombinant human EPO (rhEPO) hormone is widely used to compensate for reduced endogenous EPO production in renal failure and to correct the associated anemia (Dalle *et al.*, 2001). Active hEPO consists of a single 166-amino acid polypeptide chain with 3 N-glycosylation sites at Asn^{24}, Asn^{38} and Asn^{83} and 1 O-glycosylation site at Ser^{126} (Broudy *et al.*, 1987; Egrie *et al.*, 1986; Sasaki *et al.*, 1987; Takeuchi *et al.*, 1989).

Many approaches to extend the half-life of EPO have been developed through genetic or chemical modification of native EPO (Bunn, 2007; Macdougall *et al.*, 1999; Dalle *et al.*, 2001). One major issue regarding the clinical use of EPO is its relatively *in vivo* short half-life due to its rapid clearance (~5 h) from circulation when it is injected intravenously (i.v) (Macdougall, 2002). These strategies have some effects in extending the half-life and enhancing the activities of rhEPO. Homodimerization, in particular, restores the biological activity of inactive EPO mutants, and the EPO receptor can be dimerized by various molecules (Qiu *et al.*, 1998). The recombinant fusion protein, consisting of 2 complete human EPO molecules in tandem separated by a 17-amino acid linker, has substantially enhanced potency and efficacy both *in vitro* and *in vivo* as compared to the conventional rhEPO after a single subcutaneous injection (Sytkowski *et al.*, 1999). Dalle *et al.* (2001) reported that 2 human EPO molecules linked by a peptide linker of 9 amino acids enhanced erythropoietic activity *in vivo* and *in vitro*.

Glycosylation of EPO is required for its *in vivo* bioactivity, although not for *in vitro* receptor binding or stimulation of EPO-dependent cell lines.

Two additional N-linked carbohydrate sites were introduced into the primary EPO sequence to create a novel erythropoietin stimulating protein (NESP), which has a longer half-life than endogenous EPO. NESP is likely to confer a clinical advantage rhEPO by allowing less

† Corresponding author : Phone: +82-31-670-5421, E-mail: ksmin@hknu.ac.kr

frequent dosing in anemia patient (Macdougall *et al.*, 1999). NESP, a hyperglycosylated analog of EPO, has a lower affinity for the EPO receptor than that shown by EPO. rhEPO was degraded faster than NESP at the cellular level of intracellular trafficking and receptor binding and degradation (Gross and Lodish, 2006). The EPO chimeric protein which contains the carboxyl-terminal peptide of the human chorionic gonadotropin-β subunit bearing 4 O-linked oligosaccharide recognition sites does not affect secretion, receptor binding affinity, or *in vitro* bioactivity, but both *in vivo* potency and half-life are significantly enhanced (Fares *et al.*, 2007).

In previous studies, we used recombinant glycoproteins equine chorionic gonadotorpin (eCG), follicle-stimulating hormone (FSH) and thrombopoieitn (TPO), and found that deglycosylation affectes the expression and biological activity of these recombinant derivatives. Thus, glycosylation sites play a pivotal role in functioning and signal transduction in these glycoproteins (Min, 2000, 2001; Min *et al.*, 1996, 1997, 2004; Saneyoshi *et al.*, 2001; Kim *et al.*, 2007). We also studied the rhEPO production in mammalian cells, and observed that hyperglycosylated rhEPO (HGEPO) has enhanced erythropoietic activity compared to wild-type rhEPO both *in vitro* and *in vivo* (Lee *et al.*, 2003; Park *et al.*, 2005; JarGal and Min, 2009).

In this study, we produced recombinant dimeric hyperglycosylated EPO (dHGEPO) fusion protein mutants linked by a 2-amino acid linker (Ile-Asp). The dHGEPO protein was characterized *in vivo* and *in vitro*. Nortably, we observed substantially enhanced potency and efficacy over conventional recombinant dimeric-hEPO.

MATERIALS AND METHODS

Materials

The expression vector pcDNA3 was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). CHO-K1 cells and human leukemiae cell line F-36E were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The general PCR reaction cocktail (Top-Taq™ Polymerase, 10× buffer, dNTP mixture) was purchased from Core Bio System (Seoul, Korea). Restriction enzymes 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 purchased from Hyclone Laboratories (UT, USA). Centriplus® Centrifugal Filter Devices were purchased Amicon Bio separations (MA, USA). The EPO ELISA assay kit was purchased from R&D Systems Inc. (MN, USA). MTT cell growth kit was purchased from Chemicon International (USA). ICR (Institute of Cancer Research) mice were purchased from Korea Animal Technology (Koatech) (Seoul, Korea). All the other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

Construction of EPO-EPO Fusion Protein cDNA and Site-directed Mutagenesis

hEPO cDNA was amplified performing PCR by using the following oligonucleotide primers: [sense: 5'-ggT ACCATgggggTgCACggTgAgTACTCg; antisense: 5'-Tgg-AATTCACCATgggggTgcACgAATgTCCT]. Primers were added to the *Eco*RI and Kozak sites to at the 5'-end and to XhoI at the 3'-end of EPO cDNA. The 582bp cDNA fragment predicted for hEPO was amplified from human liver cDNA library. The fragments were ligated into *Eco*RI and *Xho*I digested sites of the pcDNA3 mammalian expression vector. Site-directed mutagenesis at amino acids 69 and 105 of EPO cDNA was performed using 4 primers as described previously (JarGal and Min, 2009). To substitute Leu (CTG) at positions 69 and 105 on the EPO cDNA by Asn (AAC), PCR was performed with pcDNA3-hEPO as the template by using EPO F-▲69 R, and ▲69 F-EPO R primers. Finally, the 2 fragments from the first PCR step were used for the second PCR step. Other mutants were constructed using the same method, resulting in Δ105 and Δ69, 105. To obtain dimeric EPO, cDNA encoding full-length EPO was fused with the mature EPO without a signal sequence using overlapping PCR mutagenesis (Min *et al.*, 2004; Park *et al.*, 2010). Site-directed mutagenesis was performed by using 4 primer [primer 1: 5'-TggAATTCACCATgggggTgCACg-AATgTCCT-3'; primer 2: 5'-AggATATCTCTgTCCCCTgT-CCTgCAggC-3'; primer 3: 5'-ATgATATCgCCCCACCA-CgCCTCATC-3'; primer 4: 5'-TACTCgAgTTCATCTgTC-CCCTgTCCTgCA-3'] with 2-step overlapping PCR. All mutants were sequenced to confirm the Kozak site and to identify PCR errors.

Expression Vector Construction

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

The wild-type EPO-EPO dimer PCR fragments were digested with *Eco*RI and *Xho*I endonucleases, and inserted into the same digested sites of pcDNA3 (pcDNA3-dimer-hEPO). Other mutants were constructed using the same method, resulting in pcDNA3-WT+▲69, pcDNA3-▲69+WT, pcDNA3-▲69+▲69, pcDNA3-WT+▲105, pcDNA3-▲105+WT, pcDNA3-▲69+▲105, pcDNA3-▲105+▲69 and pcDNA3-▲105+▲105.

Cell Culture and Functional Expression

Expression vectors were transfected into CHO-K1 cells

by the liposome formulation (Lipofectamine™2000) transfection method according to the manufacturer's instructions. rec-hEPO protein resulting from transient transfection was collected from the supernatants at 72 h after transfection. Stable cell transfectants were selected by incubation in a growth medium supplemented with G418 (800 μg/ml) for several weeks after transfection according to previously previously method (Min *et al.*, 2004). After incubation of selected stable cells (1×10^6) in 10ml CHO-S-SFM-II at 37℃ for 48 h, the culture medium was collected and centrifuged at 3,000 rpm at 4℃ for 5 min to remove cell debris. rec-hEPO protein was quantified using the ELISA method according to the manufacturer's protocol.

Northern Blot Analysis

For Northern blot analysis, RNA was extracted from EPO cell lines expressing each mutant. Briefly, the RNA was resuspended in electrophoresis buffer containing 100 % formamide and 37% formaldehyde, heated at 65℃ for 5 min, electrophoresed on a 1% denaturation agarose gel and transferred to a nylon membrane, Hybond-N. The membrane was hybridized at 68℃ overnight with Dig-labeled hEPO cDNA in hybridization buffer. After washing with washing buffer, 5 μl anti-Dig antibody (Ab) was added to blocking reagent and incubated at room temperature (RT) for 1 h. Next, the membrane was washed with detecting buffer at RT for 5 min. Finally, RNA was detected using CDP-star.

Cell Proliferation Assay

Short-term cell proliferation was examined using a colorimetric assay. F-36E cell line was incubated at a density of 1×10^4 cells per 100 μl 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 in 96-well plates. Following 4 h incubation at 37℃, isopropylalcohol containing 0.04 N HCl was added to each well. Within 1 h, cell proliferation was measured at 630 nm.

SDS-PAGE and Western Blot Analysis

Samples were concentrated 5~10 times using Centricon filter and applied to 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μm) using a semidry electroblotter apparatus. After blotting, the membrane was blocked with 1% blocking reagent for 1 h and bound by monoclonal anti-human EPO antibody (10 μg/10 ml of blocking solution) for 1~3 h. The blot was washed to remove unbound antibody, incubated with a secondary antibody linked to anti-mouse IgG-POD (37.5 μl/15 ml of blocking solution) for 30 min, then washed. The membrane was incubated for 5 min in 2 ml Lumi-Light substrate solution. The membrane was exposed on X-ray film for 1 to 10 min.

Enzymatic Release of N-Linked Oligosaccharides

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

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

Two groups of ICR-mice (7 weeks old) were used. To analyze *in vivo* biological activity, mice were injected subcutaneously with 10 IU on days 0 and 2. Hematocrit values were measured at 6 days after the first injection. Analysis was conducted using blood obtained by filling heparinized microhematocrit tubes from a retro-optical venous plexus. Before rec-hEPO administration, 100~150 μl of blood was 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 each protein by intravenous injection. Blood samples were taken at 2, 4, 8, and 24 h after dosing. Pharmacokinetic parameters for intravenous administration were estimated.

RESULTS

rec-hEPO Production

The rec-hEPO was assayed using an ELISA kit. Recombinant proteins of EPOWT, WT+WTEPO (dimeric-EPO), WT+▲69, ▲69+WT and ▲69+▲69 were secreted over 160 mIU/ml. Thus, dimeric-EPO was efficiently secreted into CHO cell medium. However, ▲105 mutant proteins were not secreted in the culture medium supernatant. Thus, it was not detected in the culture medium of WT+▲105, ▲69+▲105, ▲105+▲69 and ▲105+▲105 mutants (Fig. 2).

Northern Blot Analysis

For Northern blot analysis, total RNA was extracted from the CHO-K1 cell line selected by G418 using Trizol reagent according to the manufacturer's instruction. After electrophoresis, RNA was visualized under UV light. Gel contents were transferred onto the membrane and hybridized with hEPO cDNA (582 bp). The northern blot showed a main band in each lane. Dimeric EPO samples were detected slightly higher than WT or ▲69. All cell lines selected by G418 expressed EPO mRNA (Fig. 3).

Cell Proliferation Assay

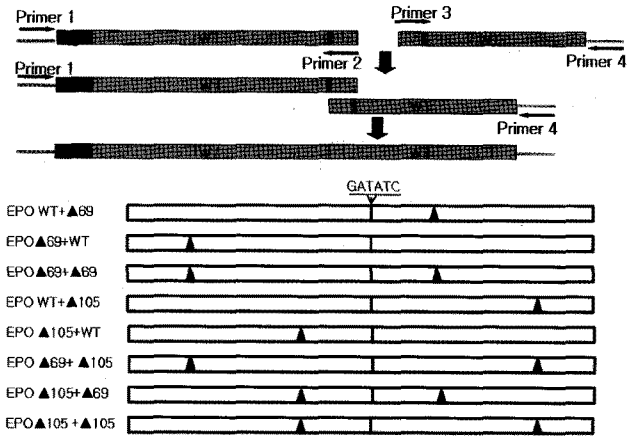

Fig. 1. Schematic diagrams of recombinant hyperglycosylated human erythropoietins. Recombinant hEPOs, dimeric hEPO (WT+WT) and 8 glycosylated dimeric hEPO mutants (WT+69, 69+WT, 69+69, WT+105, 105+WT, 69+105, 105+69, and 105+105) contained added glycosylation sites at amino acids 69 and 105. A black triangle "▲" denotes an N-linked oligosaccharide addition. Dimeric-hEPO genes were constructed by overlapping PCR mutagenesis. The first PCR was performed using 4 primers (1 and 2; 3 and 4). Fragments were subjected to the second PCR using primers 1 and 4 to generate dimeric-hEPO (WT+WT). Following Eco RI/Xho I digestion, fragments encoding dimeric-hEPO were inserted into the pcDNA3 expression vector.

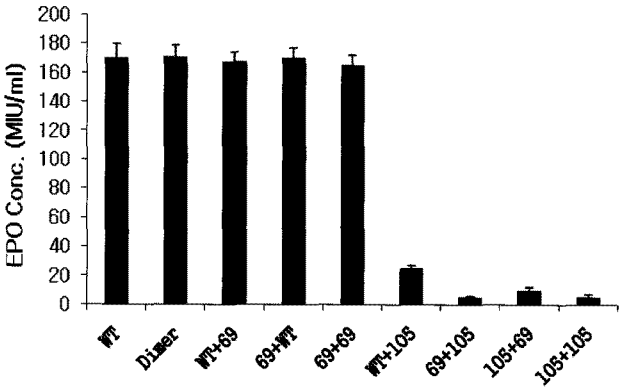

Fig. 2. Quantification of transient expression in rec-hEPO mutants. ELISA results of the standard curve are not shown. The amount EPO mutant expression was measured by ELISA. Experiments were performed in triplicated. Note that there is no significant difference between dimeric-hEPO (WT+WT) and glycosylated mutants at amino acid 69 (WT+69, 69+WT and 69+69). However, we did not observe potein in the culture medium of glycosylated mutants at amino acid 105 (WT+105, 69+105, 105+69, 105+105).

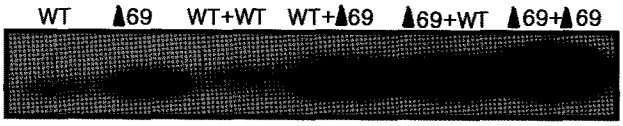

Fig. 3. Northern blot analysis of hEPO mRNA from cell lines expressing hEPO proteins. Total RNA (each 5 μg) was prepared from cultured cell lines. After electrophoresis, RNA was transferred to the membrane and hybridized with a582-bp fragment from the cloned hEPO as described. The blot was washed followed by detection on X-ray film.

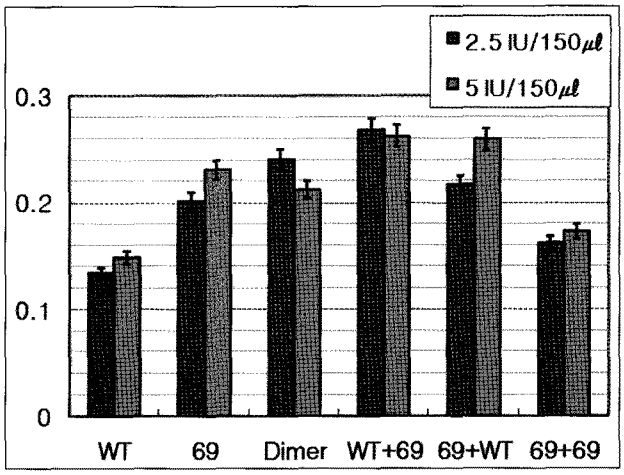

Fig. 4. Responsiveness forF-36E cell lines of rec-hEPOs. EPO-dependent human leukemia cells (F-36E) were incubated at a density of 1×10^4 cells/100 μl in 96-well plates in growth medium in the presence of 2.5 and 5 IU of rec-hEPO protein. Following 4 h incubation, protein levels were measured at 630 nm. Results are shown as the mean of triplicate experiments.

The *in vitro* biological activity of EPO derivatives was determined using the EPO-dependent, human leukemia F-36E cell lines. EPO is the most potent cytokine for F-36E cell lines. Growth and proliferation of F-36E cells by EPO molecules are shown in Fig. 4. Cell proliferation is increased by EPO proteins (2.5 IU and 5 IU) in 24 h. Specially, the maximal stimulation by EPO was nearly equal in a short-term assay with 2.5 IU treatment. By comparison, dimeric-EPO protein appears to have nearly the same activity *in vitro*, although it was a slightly reduced in the ▲69+▲69 group (Fig. 4).

Western Blot and Treatment of N-linked Oligosaccharide

Efficient EPO WT translation was detected at approximately 37 kDa in previous studies. However, dimeric-EPO (WT+WT) proteins were detected at approximately 68~80 kDa. The most prominent band appeared at 75 kDa and a smaller band was detected at 68 kDa. Other mutant proteins appeared slightly higher (approximately 80 kDa) (Fig. 5A, 5B). N-linked digestion remarkably decreased sizes dimeric EPOs to 38 kDa. This suggests that oligosaccharides of approximately 40 kDa are added to dimeric-EPO proteins.

In Vivo Biological Activity

Four groups of mice were injected subcutaneously with 10 IU of rec-hEPO on days 0 and 2. Hematocrit values were measured 6 days after the first injection. Hematocrit values were remarkably increased in all treatment groups (Fig. 6). Hematocrit values of dimeric-EPO were increased from 37.4% to 42.6% (about 5.2%). Other mutants (WT+▲69, ▲69+WT and ▲69+▲69) were increased (44.6~

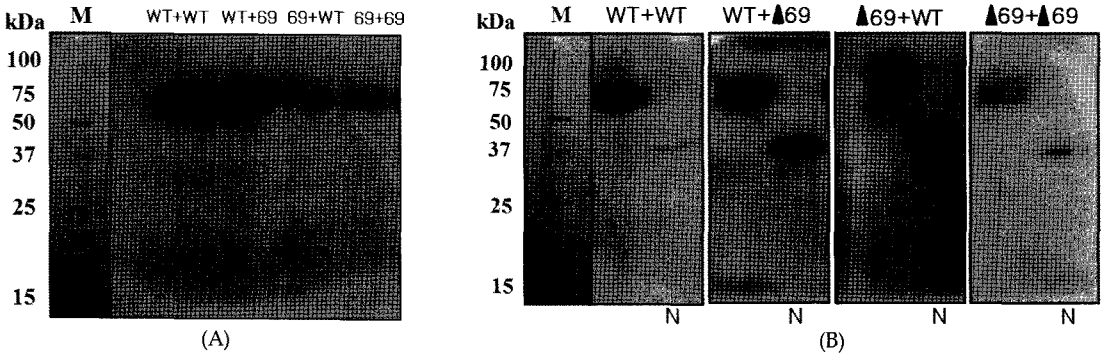

Fig. 5. Western blotting results by N-linked glycosylation digestion. Dimeric-hEPO protein produced in CHO cell lines. Dimeric-hEPO proteins (A) (*WT+WT, WT+69, 69+WT* and *69+69*) and proteins digested with N-linked glycosylation enzyme (B) were submitted to a 12 % SDS-PAGE and transferred to a PVDF membrane. After blotting, the membrane was bound by monoclonal anti-human EPO antibody (10 μg/10 ml of blocking solution) for 1 h. The blot was incubated with a secondary antibody linked to anti-mouse IgG-POD (37.5 μl/15 ml of blocking solution) for 30 min. then washed. Next, the membrane was incubated with Lumi-Light substrate solution and exposed on X-ray film for 1~10 min.

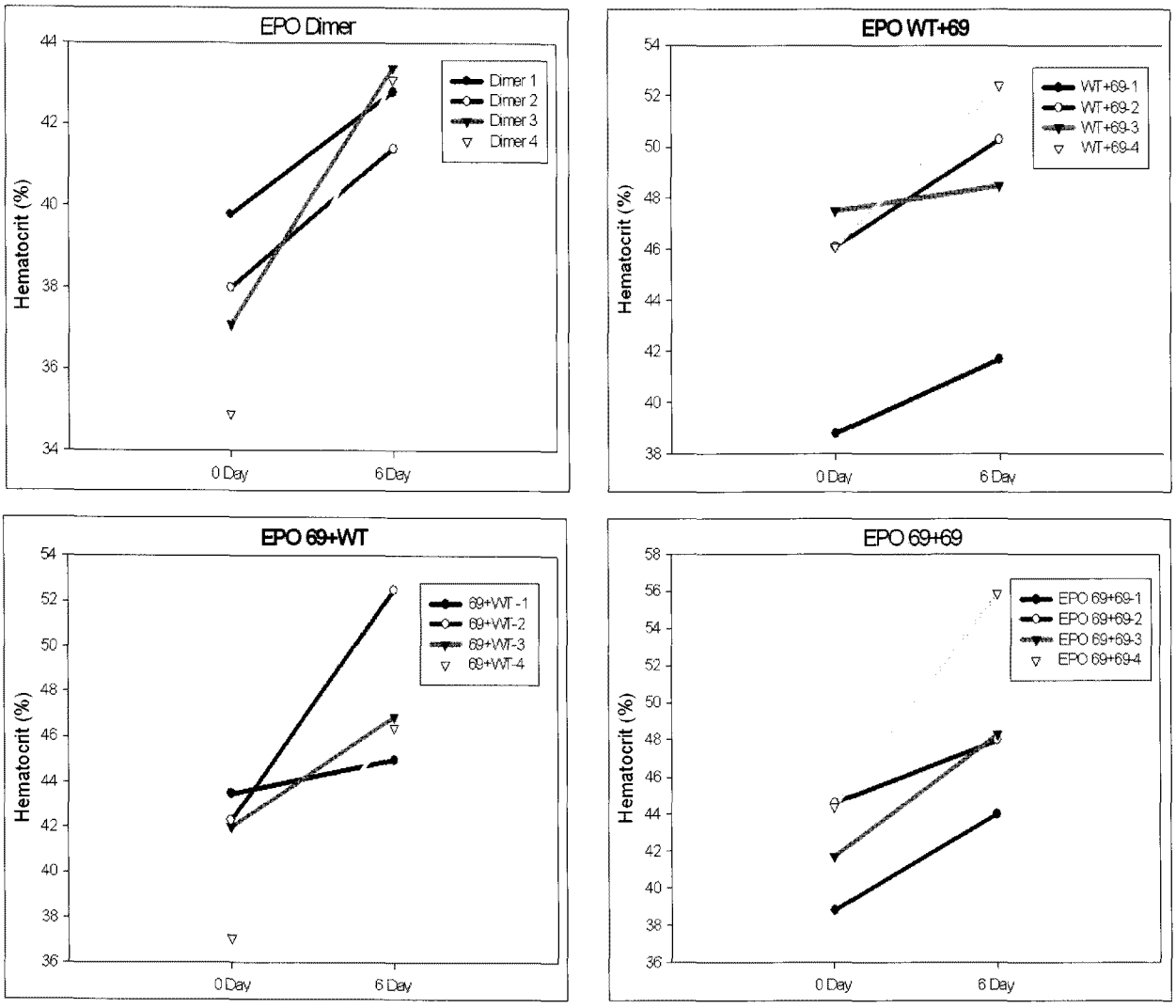

Fig. 6. EPO induced hematocrit increase by rec-hEPO mutants. Four groups of ICR mice (7 weeks old) were injected subcutaneously with 10 IU of rec-hEPOs on days 0 and 2. Hematocrit values were determined at pretreatment (day 0) and 6 days after injection. Values are given for each mouse.

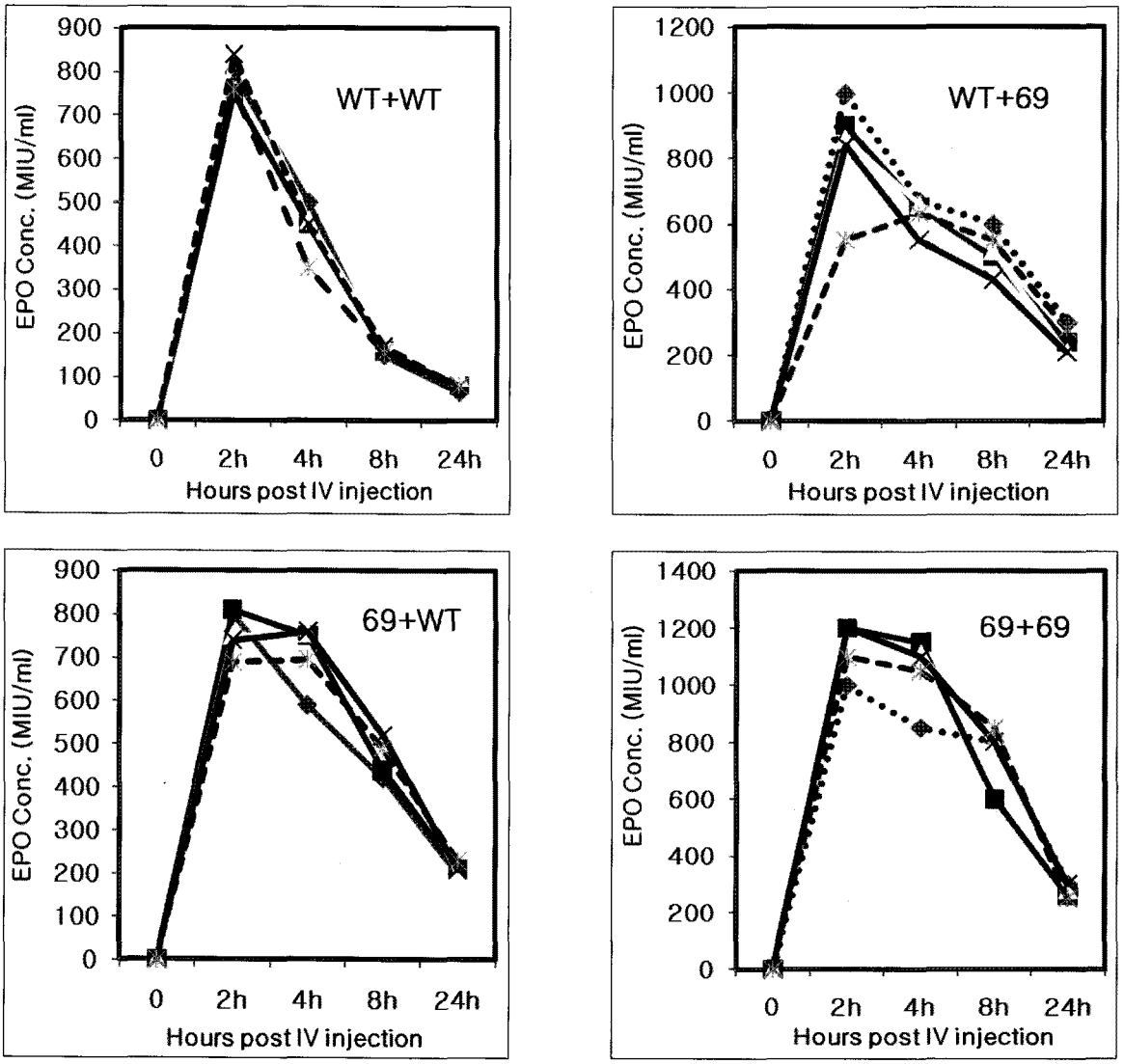

Fig. 7. Pharmacokinetics of rec-hEPOs (WT+WT, WT+69, 69+WT and 69+69) *in vivo*. Mice were injected iv with 2.5 IU of recombinant EPOs, and blood samples were drawn at the indicated times. Serum EPO levels were determined by ELISA as described in material and methods.

48.2%, an approximately 3.6% increase; 41.2~47.7%, approximately 6.5% increase; 42.3~49.0%, approximately 6.7 %). Thus, 2 mutant (▲69+WT and ▲69+▲69) groups particularly enhanced hematocrit values when subcutaneous injection was used.

Pharmacokinetic Analysis

We also analyzed the pharmacokinetic behavior of dimeric-EPOs. As shown in Fig. 7, highest levels occurred 2 h after injected in the dimeric-EPO group and the other 3 groups. The level was remarkably decreased in the dimeric-EPO group. However, the pattern was slightly different in 3 mutants (WT+▲69, ▲69+WT and ▲69+▲69). EPO protein was detected 8 h after injection in 3 mutants and was longer than *in vivo* dimeric-EPO. These proteins could exist in the blood more than 8 h after injection. Thus, these mutants could potentially be use new therapeutic agents instead of wtEPO and dimeric-EPO.

DISCUSSION

In this study, we demonstrated that dimeric-EPO mutants produced in CHO cell lines have enhanced *in vitro* and *in vivo* biological activity. EPO▲69 contained an added glycosylation site at 69 Leu. Thus, activity of ▲69+WT, WT+▲69 and ▲69+▲69 *in vivo* was remarkably enhanced compared to dimeric-EPO. Hematocrit values were also enhanced in these 3 mutants. EPO▲105 was not secreted into the culture medium; this is likely due to the folding of amino acid at position 105. It is also suggests

that amino acid 105 of hEPO is very important for secretion.

Despite the enormous success of rhEPO therapy, there is concern among patients and physicians regarding the financial cost (often >$10,000 per year). The emergence of a less expensive, generic rhEPO has been delayed by extended and sequential patents. Additionally, many patients object to the frequent parenteral administration, occurring as often as 3 times per week (Bunn, 2007). The small molecule EPO has biological activity both *in vitro* and *in vivo* (Wright *et al.*, 1996). After multiple rounds of screening, Wright *et al.* (1996) isolated and identified a 20-amino acid peptide (GGTYSCHFGPLTWVCKPQGG) that binds to EpoR with a Kd of 200 nM.

EPO is a heavily glycosylated protein with a carbohydrate contentof approximately 40%. Three N-linked (Asn-24, 38, and 83) and one O-linked (Ser-126) acidic oligosaccharide side chains are present 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 produced primarily in adult kidney and fetal liver cells (Sasaki *et al.*, 1987). The addition of these extra-carbohydrate chains results in greater metabolic stability and a half-life 3.6 times longer than rhEPO glycosylation sites (Egrie *et al.*, 2003; Macdogall *et al.*, 1999).

In the early days of treating hemodialysis patients with rhEPO, one of the uncommon complications was thrombosis, particularly in arteriovenous shunts or fistulas. This problem was thought to be due to excessive dosing, with hematocrit levels rising to the mid- or high-40s (Bunn, 2007). Administration of rhEPO to dogs resulted in a decline in platelet count but enhanced platelet reactivity (Wolf *et al.*, 1997a). In healthy human volunteers, intravenous rhEPO administration (100 U/kg or 500 U/kg) resulted in a 10~20% increase in platelet count as well as activation of both platelet and the endothelium (Wolf *et al.*, 1997b). However, platelet rise may be due in part to induction resulting from iron deficiency due to the increase in red-cell mass (Loo and Beguin, 1999).

Darbepoetin alfa, a hyperglycosylated rec-hEPO analogue with 2 extra carbohydrate chains, is approximately 13- to 14-fold more potent than WT rec-hEPO (Egrie and Browne. 2001, 2002; Egrie *et al.*, 2003). Macdougall *et al.* (1999) reported a similar result that NESP, a hyperglycosylated analogue of rec-hEPO, has an increased terminal half-life in animal models. In this study EPO▲105+▲105 was not secreted into the culture medium. Ellioitt *et al.* (1997) reported that the position 105 residue of EPO protein is important for folding, which is required for biological activity. Thus, a glycosylation site addition and mutation at site 105 alters the protein folding pattern of mutant EPO and likely inhibits its secretion and possibly its function.

However, wild type monomeric EPO is significantly more active than the wild-type dimer and the R103A dimer (Qiu *et al.*, 1998). This suggests EPOR can be dimerized by different forms and sizes of molecules, providing that 2 recognition motifs are present in the same molecule. Subcutaneous administration of a single EPO-EPO dose to mice results in a significant increase in red blood cell production within 7 days (Sytkowski *et al.*, 1999). In contrast, no measureable effects were seen with administration of an equivalent dose of conventional recombinant EPO. The pharmacokinetic behavior of EPO-EPO differs significantly from that of EPO. Dalle *et al.* (2001) reported the design and characterization of a recombinant fusion protein comprised of 2 human EPO molecules linked by a peptide linker of 9 amino acids. This dimer has enhanced erythropoietic activity, both *in vitro* on primary human erythroid progenitors and *in vivo* in normal mouse compared with its monomer counterpart. Another group reported a chimeric gene containing a carboxyl-terminal peptide (CTP) sequence from the human chorionic gonadotropin β-subunit constructed and produced into CHO cells. This fusion protein did not affect secretion, receptor binding affinity, or *in vitro* bioactivity. However, both *in vivo* potency and the half-life of EPO-CTP were significantly enhanced (Fares *et al.*, 2007). However, our results produced mutant dimeric EPOs, glycosylated at 67 amino acids, that were shown to have higher activity than that of dimeric EPO. Thus, these results can be used to produce new EPO molecules with increased activity. We are attempting to produce transgenic mice expressing dimeric mutant (EP▲69+ EPO▲69) protein into the milk.

We observed an increase in the biological specific activity of dimeric-EPO mutants relative to dimeric-EPO activity. These studies present a newly developed EPO. EPO glycosylation sites appear to play an important role in its biological activities. These data suggest that EPO▲69+EPO▲69 increase the biological specific activity in comparison with the activity of dimeric-EPO. Thus, glycosylated dimeric mutants (EPO▲69+EPOWT, EPOWT+EPO▲69 and EPO▲69+EPO▲69) can reduce the amount of therapeutic EPO required for treatment of various chronic anemia. Additionally, the availability of a more active EPO molecule will be useful for the development of EPO-based gene therapy approaches.

REFERENCES

1. Benjamin LE, Franklin B (1999): Regulation of the erythropoietin gene. Blood 94:1864-1877.
2. Broudy VC, Tait JF, Powell JS (1988): Recombinant human erythropoietin: Purification and analysis of carbohydrate linkage. Arch Biochem Biophys 265:329-336.
3. Bunn HF (2007): New agents that stimulate erythropoiesis. Blood 109:868-872.
4. Dalle B, Henri A, Rouyer-Fessard P, Bettan M, Scher-

man D, Beuzard Y, Payen E (2001): Dimeric erythropoietin fusion protein with enhanced erythropoietic activity *in vitro* and *in vivo*. Blood 15:3776-3782.
5. Egrie JC, Brown JK (2001): Development and characterization of novel erythropoiesis stimulating protein (NESP). Nephrol Dial Transplant 16:3-13.
6. Egrie JC, Brown JK (2002): Development and characterization of darbepoetin alfa. Oncology 16:13-22.
7. Egrie JC, Dwer E, Browne JK, Hitz A, Lykos MA (2003): Darbepoitin alfa has a longer circulating half-life and greater *in vivo* potency than recombinant human erythropoietin. Exp Hematol 31:290-299.
8. Egrie JC, Strickand TW, Lane J, Aoki K, Cohen AM, Samlling R, Trail G, Lin FK, Browne JK, Hines DK (1986): Characterization and biological effects of recombinant human erythropoietin. Immunology 172: 213-244.
9. Ellioitt S, Lorenzini T, Chang D, Barzilay J, Delorme E (1997): Mapping of the active site of recombinant human erythropoietin. Blood 89:493-502.
10. Fares F, Ganem S, Hajouj T, Agai E (2007): Development of a long-acting erythropoietin by fusing the carboxyl-terminal peptide of human chorionic gonadotropin β-subunit to the coding sequence of human erythropoietin. Endocrinology 148:5081-5087.
11. Gross AW, Lodish HF (2006): Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP). J Biol Chem 281:2024-2032.
12. JarGal N, Min KS (2009): Development and characterization of hyperglycosylated recombinant human erythropoietin (HGEPO). Reprod Dev Biol 33:77-83.
13. Kim BS, JarGal P, Min KS (2007): Biological activity of recombinant human thrombopoietin. J of Life Science 17:1497-1504.
14. Lacombe C, Da Silva JL, Bruneval P (1988): Peritubular cells are the site of erythropoietin synthesis in the murine hypoxic kidney. J Clin Invest 81:620-623.
15. Lai PH, Everett R, Wang FF, Arakawa T, Goldwasseer E (1986): Structural characterization of human erythropoietin. J Biol Chem 261:3116-3121.
16. Lee HG, Lee PY, Kee YJ, Kim SJ, Chung HK, Seo MK, Park JK, Min KS, Chang WK (2003): Effects of changes in glycosylation sites on secretion of recombinant human erythropoietin in cultured CHO cells. Korean J Animal Reprod 27:299-307.
17. Loo M, Beguin Y (1999): The effect of recombinant human erythropoietin on platelet counts is strongly modulated by the adequacy of iron supply. Blood 93:3286-3293.
18. Macdougall IC (2002): Optimizing the use of erythropoietic agents-pharmacokinetic and pharmacodynamic considerations. Nephrol Dial Transplant 17:66-70.
19. Macdougall IC, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, Egrie J (1999): Pharmacokinetics of novel erythropoiesis stimulating protein compared with epoetin alfa in dialysis patients. J Am Soc Mephol 10: 2392-2395.
20. Min KS (2000): Biological functions of N- and O-linked oligisaccharides of equine chorionic gonadotropin and lutropin/choriogonadotropin receptor. Korean J Animal Reprod 24:357-364.
21. Min KS (2001): Biosynthesis of a biological active single chain equine chorionic gonadotropin. J Life Science 11:103-107.
22. Min KS, Hattori N, Aikawa K, Shiota K, Ogawa T (1996): Site-directed mutagenesis of recombinant equine chorionic gonadotropin/luteinizing hormone: differential role of oligosaccharides in luteinizing hormone and follicle-stimulating hormone-like activities. Endocrine J 43:585-593.
23. Min KS, Hiyama T, Seong HH, Hattori S, Tanaka S, Shiota K (2004): Biological activities of tethered chorionic gonadotropin (eCG) and its deglycosylated mutants. J Reprod Dev 50:297-304.
24. Min KS, Shiota K, Saneyoshi T, Hirosawa M, Ogawa T (1997): Differential role of oligosaccharides in equine chorionic gonadotropin (eCG)/luteinizing hormone (LH) to express follicle stimulating hormone (FSH)-like and LH-like activities. J Reprod Dev 43: 177-179.
25. Park JJ, JarGal N, Yoon JT, Min KS (2010): β-subunit 94~96 residues of tethered recombinant equine chorionic gonadotropin are important sites for luteinizing hormone and follicle stimulating hormone like activities. Reprod Dev Biol 34:33-40.
26. Park JJ, Lee HG, Nam IS, Park HJ, Kim MS, Chung YH, JarGal N, Kang HY, Lee PY, Park JG, Seong HH, Chang WK, Min KS (2005): Biological activity of recombinant human erythropoietin (EPO) *in vivo* and *in vitro*. Reprod Dev Biol 29:69-79.
27. Qiu H, Belanger A, Yoon HWP, Bunn HF (1998): Homodimerization restores biological activity to an inactive erythropoietin mutant. J Biol Chem 273: 11173-11176.
28. Sakaki H, Bothner B, Dell A, Fukuda M (1987): Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Bio Chem 27:24773-24778.
29. Saneyoshi T, Min KS, Ma XJ, Nambo Y, Hiyama T, Tanaka S, Shiota K (2001): Equine follicle-stimulating hormone: Molecular cloning of β subunit and biological role of the asparagines-linked oligosaccharide at asparagine^{56} of α subunit. Biol Reprod 65:1686-1690.
30. Sytkowski AJ, Lunn ED, Risinger MA, Davis KL (1999): An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties. J Biol Chem 274:24773-24778.
31. Takeuchi M, Inoue N, Strickand TW, Kobata M, Wada M, Shimizu R, Hoshi S, Kozutsumi H, Takasaki S, Kokbata A (1989): Relationship between sugar chain structure and biological activity of recombinant hu-

man erythropoietin produced in Chinese hamster ovary cells. Proc Natl Acad Sci USA 86:7819-7822.
32. Wolf RF, Peng J, Friese P, Gilmore LS, Burstein SA, Dale GL (1997a): Erythropoietin administration increases production and reactivity of platelets in dogs. Thromb Haemost 78:1505-1509.
33. Wolf RF, Gilmore LS, Friese P, Downs T Burstein SA, Dale GL (1997b): Erythropoietin potentiates thrombus development in a canine arterio-venous shunt model. Thromb Haemost 77:1020-1024.
34. Wright NC, Farrell FX, Chang R (1996): Small peptides as potent mimetics of the protein hormone erythropoietin. Science 273:458-463.
(Received: 19 November 2010 / Accepted: 3 December 2010)