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Biochemical and Biophysical Research Communications 339 (2006) 380–385
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The prolonged half-lives of new erythropoietin derivatives via peptide addition ☆

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Received 2 November 2005
Available online 14 November 2005

Abstract

Erythropoietin, or Epo, is a hematopoietic cytokine that promotes erythropoiesis, and recombinant human Epo has been used in the treatment of anemia in various chronic diseases. Here, we have constructed novel Epo derivatives with prolonged half-lives by adding peptides to the carboxy terminus of Epo without using linkers. The fused peptides were selected from the carboxy terminal region of human chorionic gonadotropin (hCG) or human thrombopoietin (hTpo), which promote the proper folding, secretion, and stabilization of bioactive glycoproteins. Addition of these peptides did not interfere with secretion or receptor binding, and significantly increased the in vivo half-life of human Epo, as measured by intravenous administration in rats. The plasma half-life of the Epo constructs was longest when the carboxy terminal 28 aa of the β subunit of hCG was added (Epo–CGC), a half-life that was slightly longer than NESP (Aranesp), which is the most effective Epo product in current clinical use. The transformation of four Ser glycosylation sites to Ala on the CGC sequence also lengthened the plasma half-life of Epo, indicating that the in vivo stabilizing effect of the hCG peptide was due to both structures within the peptide itself and its O-glycosylations. The application of the carboxy terminal half of hTpo also resulted in remarkably reduced elimination of the Epo chimera (Epo–TpC), possibly due to protection by the TpC sequence. The in vivo hematopoietic activity of Epo derivatives in mice was consistent with their pharmacokinetic profiles. Therefore, these derivatives with prolonged half-lives may provide opportunities for developing new Epo therapeutics with less frequent administration.
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Keywords: Human erythropoietin; Human chorionic gonadotropin; Human thrombopoietin; Fusion protein; Glycosylation; Plasma half-life

Erythropoietin (Epo) is a hematopoietic factor that promotes the generation and differentiation of erythroid precursor cells [1,2]. Recombinant human Epo (rEpo) has been used in the treatment of anemia associated with acute renal failure, cancer chemotherapy, etc., with increasing clinical use in recent years [3–5]. However, the current therapeutic use of rEpo mandates three intravenous injections per week because of its short half-life, and this administration paradigm results in extremely high plasma concentrations followed by rapid decreases. Subcutaneous administration results in slightly better maintenance of plasma concentrations, but the Epo treatment must also be given with the equal frequency a week for therapeutic purposes [6,7]. Thus, increasing the drug half-life would significantly improve its therapeutic potential.

Much of the research to extend the Epo half-life has focused on amino acid substitutions or chemical modifications, including the development of NESP (darbepoetin alfa, Aranesp) [8,9] and the Epo dimer [10]. Active Epo contains three Asn-linked carbohydrate chains and one Ser-linked oligosaccharide [11,12], and the sugar moieties have been thought to be fairly relevant to the in vivo activity of Epo [13,14]. Two additional N-glycosylation sites were added to NESP via point mutations. A recombinant

☆ Protein Accession Nos.: human erythropoietin (Epo), P01588; human chorionic gonadotropin (hCG), AAA96690; human thrombopoietin (hTpo), NP_000451.

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.11.034

dimer protein, consisting of two complete Epo sequences in tandem, separated by a 17-amino acid linker, has also been constructed [10,15]. All of these modified Epo variants manifest longer half-lives in vivo than rEpo and would require only a single injection per week.

In this study, we have developed new Epo derivatives with a prolonged half-life via peptide addition at the carboxy terminus of Epo. These peptides were derived from the carboxy terminal region of human chorionic gonadotropin (hCG) and human thrombopoietin (hTpo). The carboxy terminal peptide of the β subunit of hCG (CGC), which contains four Ser-linked oligosaccharides, has been reported to increase the plasma half-life and consequently to improve the potency of bioactive glycoproteins in vivo. The examples of this are a longer-acting thyrotropin, which harbors a 25 aa CGC peptide, and the hCG dimer, which itself contains the alpha subunit chimera with a CGC at the carboxy terminus [16,17]. The CGC sequence is also essential for N-glycosylation and the proper folding of the β subunit of hCG itself, and promotes assembly of the functional hCG heterodimer [18].

hTpo is a hematopoietic cytokine that stimulates megakaryocyte development and platelet production, acting in a manner analogous to the hematopoietic activity of Epo in the erythropoiesis [19–21]. In addition, the amino terminal half of hTpo is highly homologous to the full-length of Epo, with the two essential Cys residues conserved in similar positions in both proteins. The carboxy terminal half of hTpo (TpC) consists of mostly hydrophilic residues and six potential N-glycosylation sites, and it has been proposed that the heavily glycosylated TpC may be necessary to stabilize hTpo [22,23]. In addition, two independent mutational analyses clearly showed that the TpC sequence was necessary for the effective secretion of hTpo, and that its N-glycosylations, particularly two sugar chains linked to Asn213 and Asn234, were the main factor in improving the secretion of the protein, whereas the carboxy terminal peptide itself interfered with the in vitro hematopoietic activity of hTpo, possibly due to an unfavorable interaction with the Tpo receptor [24,25]. These secretion-promoting and stabilizing properties of the carboxy terminal regions of hCG and hTpo suggest that adding sugar-rich peptide tails to Epo might improve the secretion and plasma half-life.

In fact, the addition of CGC and TpC to human Epo in the present study resulted in a significant increase in in vivo half-life. Therefore, we attempted to determine the glycosylation effects of the fusion Epo, via comparisons of the glycosylated peptides with deglycosylated varieties, in order to determine whether the stabilizing effect of the peptide addition is, in fact, the result of the additional glycosylation, or of the structure of the peptide itself.

Materials and methods

Construction of the Epo fusion expression vector. Epo and hTpo cDNAs were obtained by PCR amplification from a human fetal liver cDNA library (Invitrogen). The sequences for CGC, the carboxy terminal peptide (aa 118–145) of the hCG β subunit, and CGA, a deglycosylated mutant of CGC with four Ser residues mutated to Ala (aa 121, 127, 132, and 138 of hCG), were generated using overlapping PCR. Epo–CGC and Epo–CGA, chimeric forms of Epo ligated to the normal or mutated sequences of hCG, were cloned. Epo–TpC is a fusion protein of Epo and the carboxy terminal half of hTpo (aa 155–332), and was cloned by blunt ligation of PCR-amplified sequences of Epo and TpC. Epo–TpS contains only the last 17 amino acids of the carboxy terminus of hTpo (aa 316–332). Every Epo derivative lacks its carboxy terminal Arg residue, and no linking peptide was used.

Transfection and expression in CHO cells. Epo fusion expression vectors were transfected into CHO DG44 cells using FuGENE6 transfection reagents (Roche). The pLTRdhfr26 (ATCC) vector carrying the dhfr gene was co-transfected [26]. After transfection, the culture was continued for 7–14 days in DMEM (Gibco) containing 10% fetal calf serum and colonies resistant to G418 were selected. These cell lines were pooled and assayed using an ELISA kit (StemCell Technologies). Amplification of the transfected gene was performed using methotrexate to increase the expression of Epo derivatives.

Purification of Epo derivatives. Immunoaffinity resin for purification was prepared by coupling an anti-Epo monoclonal antibody (R&D Systems) to CNBr-activated Sepharose-4B (Pharmacia). The cells were grown to confluence in DMEM containing 10% fetal calf serum, and the medium was replaced with serum free medium, CHO-A SFM (Gibco). The conditioned medium was concentrated 5-fold using Centriprep (Millipore, cutoff 10,000). The concentrate was loaded to the immunoaffinity column, washed with PBS, and eluted with 0.1 M glycine.

Analysis of glycosylation. The purified Epo derivatives were dialyzed and suspended in the reaction buffer (20 mM sodium phosphate, 5 mM EDTA, and 0.1% SDS, pH 7.2). rEpo (Roche) was run in parallel as a control. Samples adjusted to 1000 U/100 μl were boiled for 10 min, and then 0.5 μl Triton X-100 and 1 U *N*-glycosidase F (Roche) were added [27]. After incubating at 37 °C for various intervals (1, 2, 4, and 15 h), the reaction was stopped by boiling for 10 min and analyzed by Western blot using an anti-Epo polyclonal antibody (R&D Systems).

In vivo half-life determination. The purified fusion protein was intravenously administered to male Sprague–Dawley rats (SLC, Japan) at a dosage of 200 U/rat ($n = 5$). Recormon (Roche) and Aranesp (Amgen) were also used. To evaluate the concentration in blood, blood was gathered via the femoral artery using PE-50 polyethylene cannula and treated with heparin at specified times, 10, 30, 60, 180, 300, and 480 min after administration. The plasma samples were prepared by centrifugation from heparinized blood. The plasma concentration was determined using ELISA.

Efficacy study. The in vitro biological activity of Epo derivatives was determined with an Epo-dependent cell line, human leukemia F-36E (Riken Cell Bank RCB0776, Japan) [28]. The MTT assay was performed as described previously [29], and the laboratory standard of Epo used to generate the standard curve was calibrated against the International Standard (Epo ampoule coded 87/684 of NIBSC).

The in vivo activity of Epo derivatives was examined in 8-week-old male ICR mice (SLC, Japan). Each mouse ($n = 7$–8/group) was subcutaneously administered 2000 U/kg Epo derivative weekly. On day 7 and 14, blood was obtained from the retro-orbital venous plexus and hematocrit was determined using a blood cell counter (ABC VET, France).

Results and discussion

Construction of Epo derivatives

The structures and characteristics of our modified Epos (Epo–CGC, Epo–CGA, Epo–TpC, and Epo–TpS) are summarized in Fig. 1A. All of the cloned nucleotides were confirmed via sequencing. Epo–CGC was designed to be a fusion protein composed of Epo and the carboxy-terminal peptide (aa 118–145) of the β subunit of hCG, which con-

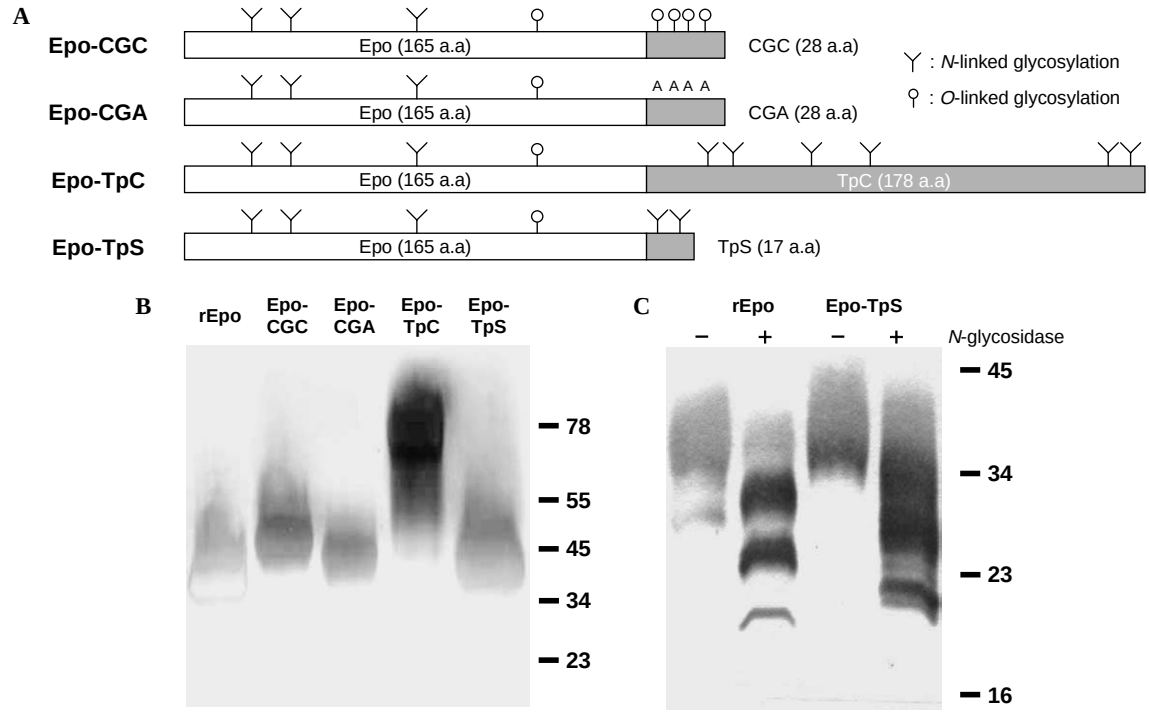

Fig. 1. (A) Schematic diagram of designed Epo derivatives. Full-length Epo, except the terminal Arg (165 aa), was fused to peptides from the beta subunit of hCG and hTpo. Glycosylation sites are denoted by specific symbols. (B) SDS–PAGE of purified Epo derivatives using immunoaffinity chromatography. Each derivative was run on 8–16% gradient SDS–PAGE and visualized with a silver staining. The estimated MW of Epo derivatives, except for Epo–TpS, were acceptable when compared to the size of the Epo control. (C) Western blot analysis of Epo-derivatives after partial *N*-glycosidase F digestion. Purified Epo proteins were subjected to a partial digestion with *N*-glycosidase F for 1 h at 37 °C. Each sample was run on a 4–20% gradient SDS–PAGE. Epo–TpS contains five N-glycosylation sites. However, one or two N-glycosylations were determined not to occur in the production of Epo–TpS.

tains four O-glycosylation sites [30]. Epo–CGA is a deglycosylated mutant of Epo–CGC that contains Ser to Ala substitutions (aa 121, 127, 132, and 138 of hCG β subunit) to determine the effects of glycosylation. Epo–TpC was designed as a fusion protein of Epo and the carboxy-terminal half region of hTpo (aa 155–332). Epo–TpS is composed of Epo and a short carboxy-terminal peptide of hTpo (aa 316–332), which possesses two N-glycosylation sites, Asn319 and Asn327, of hTpo.

The crystal structure of Epo–EpoR indicates that Epo binds to two extracellular binding domains of the Epo receptor, and that the residues Asn147, Arg150, and Gly151, all of which are proximal to the carboxy terminus of Epo, are involved in principal interactions with the receptor [31]. The addition of peptides to the carboxy terminus of Epo might serve to modulate the ligand-receptor interaction and, hence, either abolish or reduce hematopoietic activity [32,33]. This possibility is particularly likely for the hTpo fusion construct, since the added hTpo fragment is larger than Epo itself. For this reason, Sytkowski et al.[10] constructed an Epo-dimer consisting of two complete Epo sequences in tandem, separated by a 17-amino acid linker. However, the three-dimensional structure of Epo indicates that the carboxy terminus of Epo is at least 14 amino acid residues away from the receptor binding site [31]. We therefore prepared constructs without linker sequences, and expected that these additions would not interfere with receptor binding or hematopoietic activity.

Expression of Epo derivatives and in vitro efficacies

After transfection, we acquired a set of transfected cells expressing the Epo derivatives. Addition of the peptides did not affect Epo expression or secretion, with at least 95% of the Epo derivatives secreted from cells (data not shown). Although the added TpC sequence (178 aa) is larger than the secretable region of Epo (165 aa), the fusion protein (Epo–TpC) was successfully expressed and secreted, possibly because the size and amino acid sequence of Epo is highly homologous to the amino terminal half of hTpo [19]. Therefore, the structure of Epo–TpC may be similar to that of hTpo. The TpC sequence has been known to be necessary for the effective secretion of hTpo, as was revealed by the systematic truncational analysis conducted by Muto et al. [24].

We were able to obtain cell lines that highly expressed the Epo derivatives via methotrexate selection procedures. After 24 h of culturing in serum-free medium, we collected the supernatants and filter-purified them to approximately 95% purity via single immunoaffinity column chromatography, and then analyzed them via PAGE. The relative sizes of Epo–CGC and Epo–CGA were acceptable when compared to that of Epo control, whereas Epo–TpC and Epo–TpS were detected at smaller MW or as a hyper-degraded pattern (the predicted MW of each of the Epo proteins, including putative glycosylations of the proteins: rEpo, Epo–CGC, Epo–CGA, Epo–TpC, and Epo–TpS

were 32, 37, 34, 80, and 41 kDa, respectively; Fig 1B). The dimeric form of the Epo derivatives was not detected, or only at negligible level. As for the Epo–TpS, we predicted that the MW of the Epo derivative with the TpS sequence would be larger than that of Epo–CGC, due to the N-glycosylations in the short carboxy terminal peptide of hTpo. However, the estimated size of Epo–TpS in the gel was determined to be slightly smaller than Epo–CGC. Five N-glycosylation sites have been supposed to exist in Epo–TpS: three in Epo and two in TpS, respectively. However, on the basis of partial *N*-glycosidase F treatment (1 h), one or two of the five N-glycosylation sites in Epo–TpS was, in fact, not glycosylated (Fig. 1C). The smeared band pattern of Epo–TpC, which possesses nine putative N-glycosylations, would be also due to incomplete glycosylation of the protein.

We confirmed the hematopoietic activity of the Epo derivatives using an Epo-dependent cell line, human leukemia F-36E [28]. We conducted an MTT assay to assess the viability, proliferation, and activation of the Epo-dependent cells. All of the Epo derivatives exhibited functional hematopoietic activity, whereas roughly estimated efficacy was less than that of rEpo (Recormon, Roche), possibly due to insufficient purification of the fully glycosylated and active Epo derivatives (data not shown). These results indicate that our Epo fusion proteins with no linker sequence could bind and signal through the Epo receptor to trigger the erythropoietic proliferation.

In vivo half-lives and efficacies of Epo derivatives

We compared the pharmacokinetics of our chimeric Epo derivatives and the current Epo products, rEpo (Recormon, Roche) and NESP (Aranesp, Amgen), in SD rats. We measured plasma concentrations of the Epo derivatives via ELISA, which was conducted twice at several dilutions to exclude the possibility of a high dose hook effect from non-specific binding to plasma proteins.

The Epo derivatives showed better pharmacokinetic profiles than Epo itself (Fig. 2A). The half-life of the control rEpo was 141 ± 15 min. The half-life of each derivative was: Epo–CGC, Epo–CGA, Epo–TpC, Epo–TpS, and NESP had half-lives of 440 ± 52, 257 ± 28, 388 ± 96, 218 ± 24, and 382 ± 140 min, respectively. Particularly, Epo–CGC had a longer half-life within the error range than NESP, which is the most effective erythropoiesis stimulator in clinical use [2].

We also assessed in vivo hematopoietic activity in mice for 2 weeks and evaluated changes in the hematocrit levels (Fig. 2B). Epo derivatives were subcutaneously administered once a week (at 0 and 7th day). As was compared with vehicle administration, rEpo scarcely affected hematocrit levels, possibly due to the short half-life of rEpo. However, NESP and our Epo derivatives apparently showed better efficacy than the control Epo. It is also important to note that the pattern of the potency results was concordant with the acquired pharmacokinetic profiles.

Other strategies for improving Epo pharmacokinetics assumed that a linker sequence between Epo and the fusion partners would be unnecessary for in vivo biological activity of the Epo derivatives, apart from the employment of a 17 amino acid linker to construct the Epo dimer [10,32]. As was expected, our Epo derivatives exhibited significant hematopoietic activity, suggesting that adding peptides without a linker sequence did not interfere with receptor binding or hematopoietic activities of the Epo derivatives,

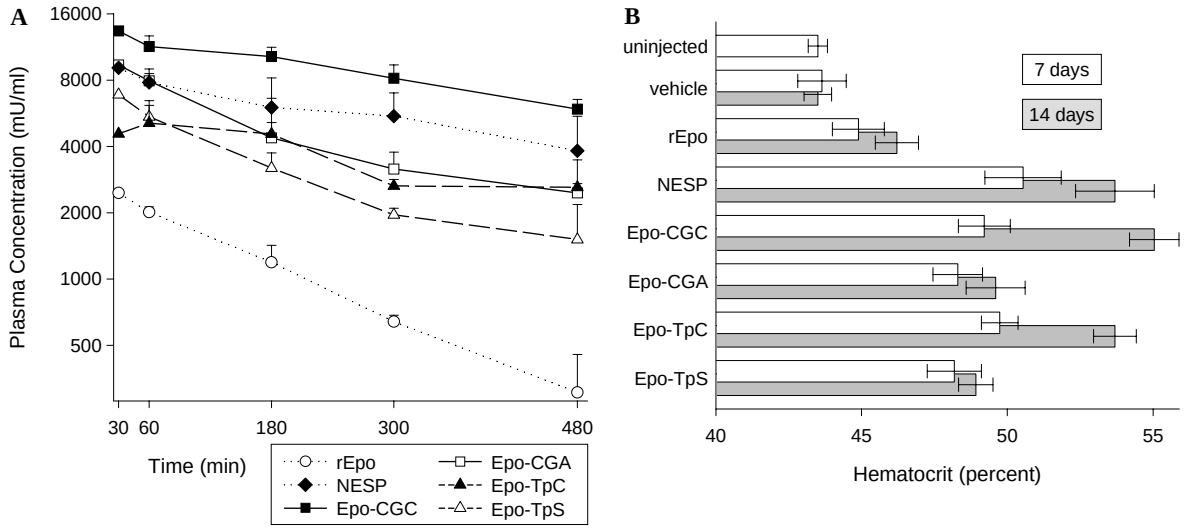

Fig. 2. (A) Pharmacokinetic profiles of Epo derivatives. Male SD rats ($n = 5$/group) were administered Epo derivatives at 200 U/rat via the femoral vein. Blood was collected at relevant time points and plasma concentrations were determined by ELISA. The half-lives of rEpo (Recormon, open circle), NESP (Aranesp, dark diamond), Epo–CGC (dark box), Epo–CGA (open box), Epo–TpC (dark triangle), and Epo–TpS (open triangle) were 141 ± 15, 382 ± 140, 440 ± 52, 257 ± 28, 388 ± 96, and 218 ± 24 min, respectively. Each point represents mean ± SD. (B) In vivo efficacy of Epo derivatives in mice. Each mouse ($n = 7$–8/group) was subcutaneously administered 2000 U/kg of Epo derivatives once a week (0 and 7th day). Then mice were sacrificed and the hematocrit change was measured at day 7 and 14. Values are expressed as means ± SD.

and hence that no linker interpolation would be necessary to construct bioactive Epo derivatives. Therefore, our Epo derivatives may be useful tools for evaluating the in vivo activity of different peptide and glycosylation species.

The addition of the normal carboxy-terminal peptide of the beta subunit of hCG resulted in the highest enhancement of the in vivo half-life of human Epo (Fig. 2A, dark square), even slightly better than NESP (dark diamond). Epo–CGC also induced the highest hematocrit level of all Epo recombinants (Fig. 2B). As described previously, the CGC sequence contains four O-glycosylation sites, and CGC chimeras showed longer half-lives for other glycoproteins, such as FSH, thyrotropin, and the alpha subunit of hCG itself [16,17]. And, it is well known that the in vivo stability of Epo depends on its glycosylation, and a deglycosylated Epo variant exhibited no in vivo activity due to its inherent instability [14]. However, it was unclear whether this extension of half-life required additional glycosylation at the CGC fusion sequence, which possesses four O-glycosylation sites. The Epo–CGA derivative, a deglycosylated mutant of CGC, also had a longer half-life than rEpo (Fig. 2A, open box compared with open circle). Although this increase in half-life was lower than Epo–CGC and NESP, the result indicated that the stabilizing effect of CGC resulted not only from the O-glycosylated constitution of the sequence, but also from the structure of the CGC peptide itself.

The delayed elimination of Epo–TpC (Fig. 2A, dark triangle) could result from protection by the carboxy-terminal region of hTpo, suggesting that the TpC sequence mediates functions besides the secretion of cytokines [24]. The amino terminal half region of hTpo retains its functional activity and is susceptible to proteolytic processing [34]. Therefore, it has been proposed that the carboxy terminal half of hTpo, which is profoundly glycosylated, may be responsible for the stability of hTpo [22,23]. Contrary to this idea, however, mutational analysis of the murine Tpo showed that truncation of the carboxy terminal half did not affect the in vitro stability of the protein in MTT proliferation assays [25]. Our data suggest that the TpC sequence can stabilize a circulatory protein in vivo, and therefore that the sequence may actually account for the natural stabilization of hTpo itself. In addition, it is also noteworthy that the addition of the TpC sequence increased the hematopoietic activity of Epo (Fig. 2B), in contrast to the inhibitory effect of the sequence on the in vitro hematopoietic activity of hTpo [24]. These results suggest that the carboxy terminal sequence of hTpo may be a useful tool for improving plasma stability and potency of proteins in vivo.

Epo–TpS contains only a small portion of the carboxy terminal region of hTpo (17 aa) and two N-glycosylation sites. Because sequence truncation and elimination of glycosylation did not dramatically affect hTpo secretion, the TpS peptide has been regarded not to be required for proper folding or secretion of hTpo [24]. Epo–TpS exhibited a longer half-life than rEpo (Fig. 2A open triangle), but a much shorter one than other derivatives, probably because of its shorter sequence and loss of at least one glycosylation (Fig. 1C).

The current rEpo should ideally be administered approximately three times a week, even with subcutaneous injection, because of its short half-life [6]. Aranesp (NESP), the recently approved erythropoietic product, requires only once-weekly treatment, is more convenient, and has lower overall clinical costs [2]. Our Epo derivatives, Epo–CGC and Epo–TpC, which were constructed by fusion with the carboxy terminal peptides of hCG or hTpo, exhibited similar or enhanced half-lives compared to Aranesp. These Epo derivatives may therefore be worth developing for clinical use.

References

[1] J.L. Spivak, Recombinant erythropoietin, Annu. Rev. Med. 44 (1993) 243–253.
[2] J.W. Fisher, Erythropoietin: physiology and pharmacology update, Exp. Biol. Med. (Maywood) 228 (2003) 1–14.
[3] D.H. Henry, G.N. Beall, C.A. Benson, J. Carey, L.A. Cone, L.J. Eron, M. Fiala, M.A. Fischl, S.J. Gabin, M.S. Gottlieb, et al., Recombinant human erythropoietin in the treatment of anemia associated with human immunodeficiency virus (HIV) infection and zidovudine therapy. Overview of four clinical trials, Ann. Intern. Med. 117 (1992) 739–748.
[4] M. Buemi, C. Aloisi, E. Cavallaro, F. Corica, F. Floccari, G. Grasso, A. Lasco, G. Pettinato, A. Ruello, A. Sturiale, N. Frisina, Recombinant human erythropoietin (rHuEPO): more than just the correction of uremic anemia, J. Nephrol. 15 (2002) 97–103.
[5] T. Ng, G. Marx, T. Littlewood, I. Macdougall, Recombinant erythropoietin in clinical practice, Postgrad. Med. J. 79 (2003) 367–376.
[6] W. Fried, Erythropoietin, Annu. Rev. Nutr. 15 (1995) 353–377.
[7] F.G. McMahon, R. Vargas, M. Ryan, A.K. Jain, R.I. Abels, B. Perry, I.L. Smith, Pharmacokinetics and effects of recombinant human erythropoietin after intravenous and subcutaneous injections in healthy volunteers, Blood 76 (1990) 1718–1722.
[8] J.C. Egrie, J.K. Browne, Development and characterization of novel erythropoiesis stimulating protein (NESP), Br. J. Cancer 84 (Suppl. 1) (2001) 3–10.
[9] C. Chmielewski, Aranesp (darbepoetin alfa): a new erythropoiesis-stimulating protein, Nephrol. Nurs. J. 29 (2002) 67–68.
[10] A.J. Sytkowski, E.D. Lunn, M.A. Risinger, K.L. Davis, An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties, J. Biol. Chem. 274 (1999) 24773–24778.
[11] K. Jacobs, C. Shoemaker, R. Rudersdorf, S.D. Neill, R.J. Kaufman, A. Mufson, J. Seehra, S.S. Jones, R. Hewick, E.F. Fritsch, et al., Isolation and characterization of genomic and cDNA clones of human erythropoietin, Nature 313 (1985) 806–810.
[12] H. Sasaki, B. Bothner, A. Dell, M. Fukuda, Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA, J. Biol. Chem. 262 (1987) 12059–12076.
[13] L.C. Wasley, G. Timony, P. Murtha, J. Stoudemire, A.J. Dorner, J. Caro, M. Krieger, R.J. Kaufman, The importance of N- and O-linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin, Blood 77 (1991) 2624–2632.
[14] K. Yamaguchi, K. Akai, G. Kawanishi, M. Ueda, S. Masuda, R. Sasaki, Effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological properties, J. Biol. Chem. 266 (1991) 20434–20439.
[15] A.J. Sytkowski, E.D. Lunn, K.L. Davis, L. Feldman, S. Siekman, Human erythropoietin dimers with markedly enhanced in vivo activity, Proc. Natl. Acad. Sci. USA 95 (1998) 1184–1188.

[16] M. Furuhashi, T. Shikone, F.A. Fares, T. Sugahara, A.J. Hsueh, I. Boime, Fusing the carboxy-terminal peptide of the chorionic gonadotropin (CG) beta-subunit to the common alpha-subunit: retention of O-linked glycosylation and enhanced in vivo bioactivity of chimeric human CG, Mol. Endocrinol. 9 (1995) 54–63.
[17] L. Joshi, Y. Murata, F.E. Wondisford, M.W. Szkudlinski, R. Desai, B.D. Weintraub, Recombinant thyrotropin containing a beta-subunit chimera with the human chorionic gonadotropin-beta carboxy-terminus is biologically active, with a prolonged plasma half-life: role of carbohydrate in bioactivity and metabolic clearance, Endocrinology 136 (1995) 3839–3848.
[18] M. Muyan, I. Boime, The carboxyl-terminal region is a determinant for the intracellular behavior of the chorionic gonadotropin beta subunit: effects on the processing of the Asn-linked oligosaccharides, Mol. Endocrinol. 12 (1998) 766–772.
[19] F.J. de Sauvage, P.E. Hass, S.D. Spencer, B.E. Malloy, A.L. Gurney, S.A. Spencer, W.C. Darbonne, W.J. Henzel, S.C. Wong, W.J. Kuang, et al., Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand, Nature 369 (1994) 533–538.
[20] S. Lok, K. Kaushansky, R.D. Holly, J.L. Kuijper, C.E. Lofton-Day, P.J. Oort, F.J. Grant, M.D. Heipel, S.K. Burkhead, J.M. Kramer, et al., Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo, Nature 369 (1994) 565–568.
[21] T.D. Bartley, J. Bogenberger, P. Hunt, Y.S. Li, H.S. Lu, F. Martin, M.S. Chang, B. Samal, J.L. Nichol, S. Swift, et al., Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpla, Cell 77 (1994) 1117–1124.
[22] K. Kaushansky, Thrombopoietin: the primary regulator of platelet production, Blood 86 (1995) 419–431.
[23] S. Lok, D.C. Foster, The structure, biology and potential therapeutic applications of recombinant thrombopoietin, Stem Cells 12 (1994) 586–598.
[24] T. Muto, M.D. Feese, Y. Shimada, Y. Kudou, T. Okamoto, T. Ozawa, T. Tahara, H. Ohashi, K. Ogami, T. Kato, H. Miyazaki, R. Kuroki, Functional analysis of the C-terminal region of recombinant human thrombopoietin. C-terminal region of thrombopoietin is a "shuttle" peptide to help secretion, J. Biol. Chem. 275 (2000) 12090–12094.
[25] H.M. Linden, K. Kaushansky, The glycan domain of thrombopoietin enhances its secretion, Biochemistry 39 (2000) 3044–3051.
[26] G.R. Stark, G.M. Wahl, Gene amplification, Annu. Rev. Biochem. 53 (1984) 447–491.
[27] K.G. Rice, N. Takahashi, Y. Namiki, A.D. Tran, P.J. Lisi, Y.C. Lee, Quantitative mapping of the N-linked sialyloligosaccharides of recombinant erythropoietin: combination of direct high-performance anion-exchange chromatography and 2-aminopyridine derivatization, Anal. Biochem. 206 (1992) 278–287.
[28] S. Chiba, F. Takaku, T. Tange, K. Shibuya, C. Misawa, K. Sasaki, K. Miyagawa, Y. Yazaki, H. Hirai, Establishment and erythroid differentiation of a cytokine-dependent human leukemic cell line F-36: a parental line requiring granulocyte-macrophage colony-stimulating factor or interleukin-3, and a subline requiring erythropoietin, Blood 78 (1991) 2261–2268.
[29] Y.S. Choi, D.Y. Lee, I.Y. Kim, S. Kang, K. Ahn, H.J. Kim, Y.H. Jeong, G.T. Chun, J.K. Park, I.H. Kim, Ammonia removal using hepatoma cells in mammalian cell cultures, Biotechnol. Prog. 16 (2000) 760–768.
[30] T. Sugahara, M.R. Pixley, F. Fares, I. Boime, Characterization of the O-glycosylation sites in the chorionic gonadotropin beta subunit in vivo using site-directed mutagenesis and gene transfer, J. Biol. Chem. 271 (1996) 20797–20804.
[31] R.S. Syed, S.W. Reid, C. Li, J.C. Cheetham, K.H. Aoki, B. Liu, H. Zhan, T.D. Osslund, A.J. Chirino, J. Zhang, J. Finer-Moore, S. Elliott, K. Sitney, B.A. Katz, D.J. Matthews, J.J. Wendoloski, J. Egrie, R.M. Stroud, Efficiency of signalling through cytokine receptors depends critically on receptor orientation, Nature 395 (1998) 511–516.
[32] H. Qiu, A. Belanger, H.W. Yoon, H.F. Bunn, Homodimerization restores biological activity to an inactive erythropoietin mutant, J. Biol. Chem. 273 (1998) 11173–11176.
[33] D.J. Matthews, R.S. Topping, R.T. Cass, L.B. Giebel, A sequential dimerization mechanism for erythropoietin receptor activation, Proc. Natl. Acad. Sci. USA 93 (1996) 9471–9476.
[34] T. Wada, Y. Nagata, H. Nagahisa, K. Okutomi, S.H. Ha, T. Ohnuki, T. Kanaya, M. Matsumura, K. Todokoro, Characterization of the truncated thrombopoietin variants, Biochem. Biophys. Res. Commun. 213 (1995) 1091–1098.