

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

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Human erythropoietin (EPO) is a glycoprotein hormone secreted from the kidney and controls red blood cell production. EPO has a wide clinical use in the treatment of anemia associated with renal disease, certain chronic diseases, and anemia related to chemotherapy and radiotherapy. One major issue regarding the clinical use of EPO is its relatively short half-life due to its clearance by glomerular filtration. Thus, the therapeutic protocol used in the treatment of patient-required frequent injections of EPO. To address this issue, we constructed a chimeric gene that contains the sequence of the carboxyl-terminal peptide (CTP) of human chorionic gonadotropin- β subunit bearing four O-linked oligosaccharide recognition sites and the coding sequence of human EPO cDNA. Fusing the CTP to the carboxyl-terminal of EPO did not affect

secretion, receptor binding affinity, or *in vitro* bioactivity. However, both *in vivo* potency and half-life of EPO-CTP were significantly enhanced. A single injection dose (660 IU/kg) of EPO wild-type administered once a week had no significant effect on haematocrit levels. However, EPO-CTP administered as 660 IU/kg once a week was effective as well as the same total dose of EPO wild-type administered as 220 IU/kg three times a week. This may emphasize the importance of sustained blood levels rather than total dose of administration for *in vivo* bioactivity. These data established the rationale for using this chimera as a long-acting EPO analog. The therapeutic efficacy of EPO-CTP analog needs to be established in higher animals and human clinical trials. (*Endocrinology* 148: 5081–5087, 2007)

ERYTHROPOIETIN (EPO) is a glycoprotein hormone produced primarily by cells of the peritubular capillary endothelium of the kidney (1, 2). EPO is a member of an extensive cytokine family that also includes GH, prolactin, IL-2 through IL-7, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, oncostatin-M, leukemia inhibitory factor, and ciliary neurotrophic factor (3–5). EPO production is stimulated by reduced oxygen content in the renal arterial circulation. Circulating EPO binds to EPO receptors on the surface of erythroid progenitors, resulting in replication and maturation to functional erythrocytes by an incompletely understood mechanism (6, 7).

The gene encoding human erythropoietin was cloned in 1985 leading to the production of recombinant human EPO (rhuEPO) (8, 9). The rhuEPO has been used successfully in a variety of clinical situations to increase production of red blood cells (10–14). Currently this agent is licensed for use in the treatment of the anemia of renal failure, the anemia associated with HIV infection in zidovudine-treated patients, and anemia associated with cancer chemotherapy (15–17).

Administration of rhuEPO has become routine in the treatment of anemia secondary to renal insufficiency in which doses of 50–75 U/kg are given three times per week to gradually restore haematocrit and eliminate transfusion dependency (18).

One major issue regarding the clinical use of EPO is its relatively short half-life *in vivo* due to its rapid clearance (~5 h) from the circulation when it is injected iv (19). Thus, the therapeutic protocol used in the treatment of patients requires frequent injections of EPO. The recommended therapy with rhuEPO is two to three times per week by sc or iv injections. Therefore, it can be anticipated that enhancing the *in vivo* half-life of EPO would reduce the number of injections per week.

Previous studies indicated that fusing the carboxyl-terminal peptide (CTP) of human chorionic gonadotropin (hCG) β -subunit (hCG β) to human follitropin (FSH β) (20, 21), hCG α subunit (22), or thyrotropin (TSH β) (24) did not affect assembly, secretion, receptor binding affinity, or *in vitro* bioactivity. However, the *in vivo* potency and circulatory half-life of the proteins containing CTP were substantially increased. Other reports indicated that addition of N-linked oligosaccharides to human (h) FSH prolonged its half-life and increased bioactivity *in vivo* (23). In the present study, the CTP of hCG β subunit that contains four O-linked oligosaccharide recognition sites was fused to human EPO coding sequence. Our results indicate that ligation of CTP to the coding sequence of EPO did not affect secretion of the chimeric protein into the medium, receptor binding affinity, and

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Abbreviations: BFU-E, Erythroid burst-forming; CHO, Chinese hamster ovary cells; CTP, carboxyl-terminal peptide; EPO, erythropoietin; EPO-WT, EPO wild type; FCS, fetal calf serum; h, human; hCG, human chorionic gonadotropin; rhuEPO, recombinant human EPO.

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in vitro bioactivity, compared with EPO wild type (EPO-WT). However, both the *in vivo* potency and half-life in the circulation of EPO bearing the CTP were significantly enhanced.

Materials and Methods

Enzymes used in the construction of DNA vectors and constructs were purchased from New England BioLabs (Beverly, MA). Oligonucleotides used for chimeric construction were purchased from (Invitrogen, Paisley, UK). Cell culture media and reagents were obtained from Biological Industries (Beit Haimic, Israel). Rabbit antisera against EPO were purchased from Fitzgerald (Concord, MA). Bovine ^{125}I -EPO (2500 Ci/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). The eukaryotic expression vector (pGT-EPO) and zeocin were purchased from InvivoGene (San Diego, CA). PM²-hCG β plasmid was kindly supplied by Professor Irving Boime (Washington University, St. Louis, MO).

Construction of chimeric genes and expression vectors

A cassette gene containing the CTP of hCG β was fused in tandem to the coding sequence of human EPO. This chimeric gene was constructed using overlapping mutagenesis PCR technique as described before (25). Using this technique, the cassette gene containing the recognition sites of the O-linked oligosaccharides on CTP was ligated in tandem to the coding sequence of EPO as a single peptide chain (Fig. 1). The following oligonucleotides were used for the chimeric construction: primer 1, 5'-ACCAGATCTACCGTCATCATG GG-3'; primer 2, 5'-ACCTCCA-GAGTGC GGATCCAGAAG-3'; primer 3, 5'-CAGGACAGGGGACA-GATCCTCTTCTCAAAGGC-3'; primer 4, 5'-GCCTTTGAGGAA-GAGGATC TGCCCCTGTCTCG-3'. pGT-EPO plasmid was used as a template for primers 1 and 2. Primer 1 contains the EPO 5'-end sequence, which includes a newly formed *AgeI* site. Primer 2 contains the first four codons of the CTP and the last four codons of the 3'-end of EPO sequence. Therefore, the newly synthesized fragment contains the entire EPO coding sequence and a part of the CTP sequence. PM²-hCG β plasmid was used as a template for primers 3 and 4 to generate a product containing the 3'-end of EPO and the CTP fragment. Primer 3 contained the sequence corresponding to the last four C-terminal codons of EPO and the first five codons of the CTP. Primer 4 contains some of the flanking sequence of the 3'-end of hCG β -CTP that also included a newly created *Bam*HI site. These fragments were used as overlapping templates to synthesize the single EPO-CTP gene using primers 1 and 4.

The eukaryotic expression vector pGT contains the ampicillin, zeocin resistance genes, and a strong promoter of the cytomegalovirus. The *AgeI*/*Bam*HI fragment containing the EPO-CTP chimeric gene was inserted in the *AgeI*/*Bam*HI cloning site of pGT and used for transfection. The PCR-generated constructs were completely sequenced to ensure that no errors were introduced during the PCR.

DNA transfection and clone selection

Chinese hamster ovary cells (CHO) were transfected with expression pGT vectors containing EPO-WT or EPO-CTP using the calcium phosphate method as described before (26). Cells were selected for insertion

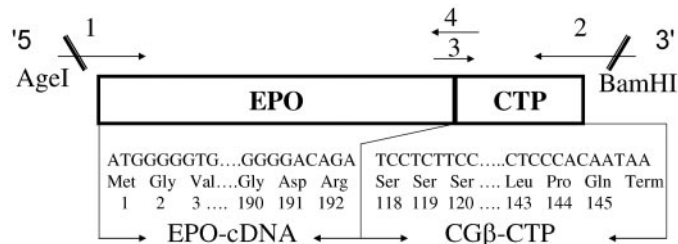


FIG. 1. Construction of EPO-CTP chimeric gene. The chimeric gene containing the cDNA of human EPO and the hCG β carboxyl-terminal peptide was prepared using overlapping PCR mutagenesis. Primers are indicated in arrows (see text for details).

of the plasmid DNA by growth in culture medium containing 100 ng/ml of zeocin. Transfected colonies resistant to zeocin were harvested and screened for the expression of EPO by Western blot analysis and EPO immunoradiometrics assay using a double-antibody RIA according to the method described in the manual accompanying the kit (Diagnostic Products Corp., Los Angeles, CA).

Cell culture

CHO cells were maintained in medium I (Ham's F-12 medium) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), and glutamine (2 mM) containing 5% fetal calf serum (FCS) at 37 C in a humidified 5% CO₂ incubator. Transfected clones were maintained in the above culture medium supplemented with 100 ng/ml of zeocin (medium II). For hormone collection, cells secreting EPO-WT or EPO-CTP were plated and grown to confluency in T-75 flasks. Cells were washed twice with serum-free medium and 20 ml of medium III (medium I without FCS) were added. Medium was collected every 24 h, clarified by centrifugation, and concentrated using centriprep concentrators (Amicon Corp., Danvers, MA). Concentrations of EPO were determined by EPO immunoradiometric assay and a double-antibody RIA (Diagnostic Products).

Western blotting

Samples were electrophoresed on denaturing 15% sodium dodecyl sulfate-polyacrylamide gels as described before (27). Gels were allowed to equilibrate for 10 min in 25 mM Tris and 192 mM glycine in 20% (vol/vol) methanol (28). Proteins were transferred to a 0.2 μm pore size nitrocellulose membrane (Sigma, St. Louis, MO) at 250 mA for 3 h using a minitrans-blot electrophoresis cell (Bio-Rad Laboratories, Richmond, CA) according to the method described in the manual accompanying the unit. The nitrocellulose membrane was incubated in 5% nonfat dry milk for 2 h at room temperature. The membrane was incubated with EPO antiserum (1:1000 titer) overnight at 4 C followed by three consecutive washes in PBS containing 0.1% Tween 20 (10 min/wash). Then the membrane was incubated with secondary antibody conjugated to horseradish peroxidase (Zymed, San Francisco, CA) for 2 h at room temperature followed by three washes. Finally, the nitrocellulose paper was reacted with enhanced chemiluminescent substrate (Pierce, Rockford, IL) for 5 min, dried with Whatman sheet, and exposed to x-ray film.

Receptor binding

The erythroleukemia cells (TF-1) were obtained through the American Type Culture Collection (Manassas, VA) and used for the radio-receptor assay. Cells (400,000) were plated into 6 \times 35-mm dishes on d 0 and fed on d 2 with RPMI 1640 medium supplemented with 5 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10% fetal bovine serum, and 1 ng/ml granulocyte-macrophage colony-stimulating factor. On d 3 the cells were gently washed twice with cold assay medium (Waymouth MB752/1, 1 mg/ml BSA, 40 μg /ml gentamicin) and incubated for 16 h at 4 C with 1 million cpm [^{125}I]EPO with or without different concentrations of EPO-WT or EPO-CTP in a final volume of 1 ml assay medium. The next day the cells were washed twice with 2 ml cold assay medium and solubilized with 0.5 ml buffer [50 mM Tris (pH 7.8), 150 mM NaCl, 5 mM EDTA, and 0.6% (wt/vol) sodium dodecyl sulfate] and transferred to plastic tubes for γ -counting. The displacement of [^{125}I]EPO binding to receptors by the chimera was plotted semilogarithmically. The IC₅₀, in which the variants displace 50% of the [^{125}I]EPO binding, was detected from the plots.

In vitro bioassay

The biological activity of EPO analogs was demonstrated by measuring their ability to stimulate the proliferation of erythroid burst-forming colonies (BFU-E) from human peripheral blood. BFU-E colonies were grown from blood of healthy donors using a microwell modification of the methylcellulose technique (29). Low-density mononuclear cells (4×10^5 /ml) were suspended in DMEM containing 0.8% methylcellulose (Dow Chemical Co., Midland, MI), 10% FCS, 0.01% 5-thioglycerol, penicillin, and streptomycin. Cells were incubated in triplicate with different concentrations of EPO variants (0.1–2 IU/ml) and exam-

ined on an inverted microscope and scored after 20 d of incubation in a fully humidified 5% CO₂ atmosphere. Colonies with at least three clusters of 50 cells were defined as BFU-E (30).

In vivo bioassay

ICR mice (7 wk) were obtained from Harlan (Jerusalem, Israel) and housed in air-conditioned quarters with a 12-h light, 12-h dark schedule. Standard food and water were available *ad libitum*. Institute ethical committee approved the *in vivo* protocols.

EPO-WT, EPO-CTP, or rhuEPO were injected to anesthetized animals as described in Table 1. The animals were weighed, and each received an identical amount (in international units) of EPO variants by iv injection (0.2 ml/animal). Haematocrit was determined using blood samples obtained by filling two heparinized microhaematocrit tubes from the inferior caval vein under anesthesia. The frequency of treatment was either thrice weekly (d 1, 3, and 5) or once only (d 1). The level of haematocrit was determined three times a week and the experiment was stopped after 3 wk.

Metabolic clearance rate

The metabolic clearance of EPO-WT and EPO-CTP was determined after iv injection of 20 IU/animal into male mice. At selected intervals after injection, blood samples were collected every 24 h, clarified by centrifugation, and concentrated using centrprep concentrators (Amicon). Concentrations of EPO were determined by EPO immunoradiometric assay and a double-antibody RIA according to the method described in the manual accompanying the kit (Diagnostic Products).

Statistical analysis

Data were expressed as the mean \pm SEM. Statistical analysis of the data were performed using Student's *t* test and one-way multivariate ANOVA to calculate *P* value. *P* < 0.05 was considered statistically significant.

Results

Secretion of EPO from transfected CHO cells was assessed by Western blot analysis under denaturing conditions using human EPO-specific antiserum. The EPO-WT migrated faster than EPO-CTP (Fig. 2). EPO-CTP exhibited high molecular mass (~48 kDa), compared with EPO-WT (~36 kDa), due to the addition of 28 amino acids and the O-linked oligosaccharides linked to CTP. These data show that the O-linked glycosylation recognition site of the C-terminal region is preserved, even though the sequence is fused to different proteins.

The *in vitro* biological activity of the EPO analogs was demonstrated by measuring their ability to stimulate the proliferation of BFU-E colonies from human peripheral blood. BFU-E colonies were grown from blood of healthy donors using a microwell modification of the methylcellulose technique as described in *Materials and Methods*. The

TABLE 1. Constitution of test groups and dose levels of EPO derivatives for the *in vivo* experiments

Group no.	No. of animals	Treatment		Dosing regimen
		Compound	Dose level ($\mu\text{g}/\text{kg}$)	
1	7	Control	0	1 time weekly
2		EPO-CTP	2.5	
3			5	
4			15	
5		EPO-WT	5	3 times weekly
6			15	
7		Commercial rhu-EPO	15	
8			5	

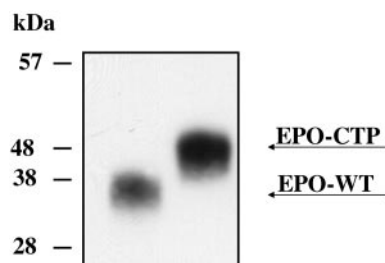


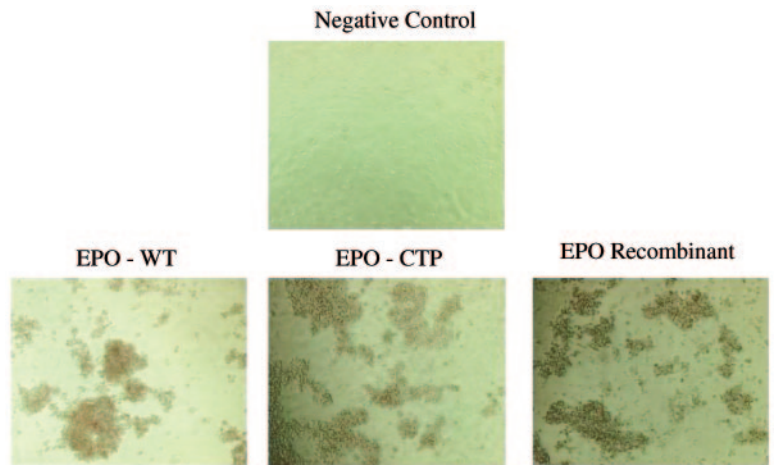
FIG. 2. Expression of EPO-WT and EPO-CTP from transfected CHO cells. Conditioned media from transfected cells were prepared for SDS-PAGE and proteins were detected by Western blot as described in *Materials and Methods*.

optimal formation of BFU-E colonies achieved by EPO-CTP was similar to that achieved by EPO-WT and rhuEPO using 1 U/ml of the protein (Fig. 3). The affinities of human EPO variants to the receptor were determined by radioreceptor assay using CHO cells expressing human EPO receptor. hTSH-WT as well as EPO-CTP displaced [¹²⁵I]bTSH binding in a dose-dependent manner (Fig. 4). The IC₅₀ detected for EPO-WT and EPO-CTP was 90 and 110 $\mu\text{U}/\text{ml}$, respectively. The results indicated that addition of the CTP sequence to the coding region of EPO had no significant effect on receptor binding affinity or *in vitro* bioactivity. For further pharmacological evaluation of EPO-CTP, comparative pharmacodynamic studies of EPO-CTP and commercial rhuEPO were performed in male C57BL mice (*n* = 7/group) using different frequencies and a wide dose range as described in Table 1. The *in vivo* efficacy was obtained by measuring the mean values of haematocrit percentage in the blood. The results indicated that EPO-CTP is significantly (*P* < 0.05) more efficient than EPO-WT when administered iv once a week with a dose of 5 $\mu\text{g}/\text{kg}$ (Fig. 5A). EPO-CTP can successfully increase the haematocrit when administered once a week with a dose of 15 $\mu\text{g}/\text{kg}$ (Fig. 5B). Once weekly dosing with the same concentration of commercial rhuEPO or EPO-WT was significantly (*P* < 0.001) less efficient than once weekly dosing of EPO-CTP.

An interesting observation from the present study was the ability of a single injection once a week of EPO-CTP (15 $\mu\text{g}/\text{kg}$) to increase the levels of haematocrit, whereas the same effect was achieved by administration of the same total dose of rhuEPO administered three times a week as 5 $\mu\text{g}/\text{kg}$ per injection (Fig. 6). These results indicated the importance of sustained blood levels, rather than total dose of EPO. These findings are consistent with the hypothesis that the ability of a single injection of EPO-CTP to increase haematocrit results from its increased stability in the circulation.

The increased biopotency of the chimera may reflect a change in their metabolic clearance *in vivo*. Therefore, the circulatory half-lives of the hormones were determined. EPO-WT or EPO-CTP chimera were injected iv into immature male mice and RIA monitored the plasma half-lives. The results indicated that a higher level of the chimera is still detectable in serum after 24 h. The half-life of EPO CTP is increased 2- to 3-fold, compared with EPO-WT (Fig. 7). These data suggest that the mechanism of EPO metabolic clearance is affected by the presence of CTP.

FIG. 3. *In vitro* biological activity of rhuEPO derivatives. Purified cells from human peripheral blood were incubated for 20 d with or without EPO analogs. EPO-WT and EPO-CTP were produced in CHO cells. EPO-recombinant was commercially obtained. BFU-E colonies were identified by microscopic examination and photographed.



Discussion

The present study demonstrated that addition of hCG-CTP extension to the coding region of EPO cDNA using overlapping PCR technique, significantly increases the *in vivo* bioactivity and prolonged its circulating half-life, but it, at most, minimally alters EPO secretion, receptor binding affinity, or *in vitro* bioactivity. These results are consistent with earlier studies using human glycoprotein hormones (FSH, TSH, and hCG), which indicated that fusing the CTP sequence to hFSH β (20, 21), hCG α subunit (22), or hTSH β (24) subunits resulted in a significant increase in the *in vivo* potency and half-life of the hormones in the circulation. On the other hand, deletion of the CTP from the hCG β subunit caused a significant reduction in the *in vivo* bioactivity of hCG without effect on receptor binding affinity and *in vitro* biological activity (31).

hCG is a member of the glycoprotein hormone family that

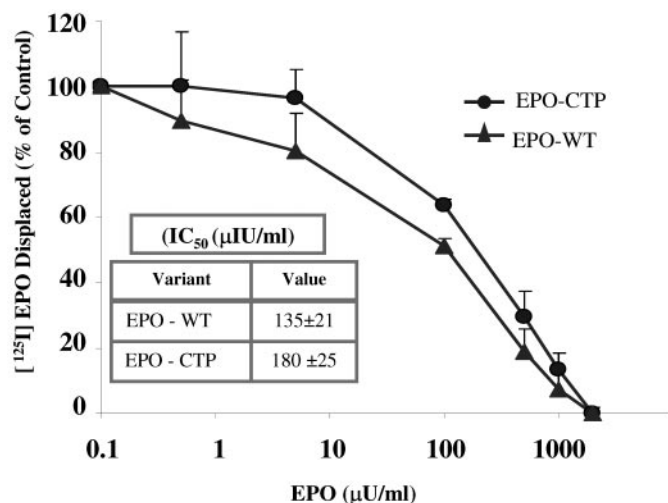


FIG. 4. Radioreceptor assay of recombinant EPO analogs. Binding activity was measured by displacement of ^{125}I -hEPO binding from TF-1 cells. Cells (400,000) were incubated in 1 ml of assay medium containing ^{125}I -hEPO (1 million cpm/ml) for 16 h at 4 C with or without different concentrations of EPO-WT or EPO-CTP. The amount of ^{125}I -hEPO bound to the cells was determined and is reported as percentage of control bound. Data shown are the mean \pm of three different experiments.

are heterodimers containing two subunits, hCG α and hCG β . The hCG β subunit is distinguished among the other β -subunits because of the presence of the CTP. This extension contains 28 amino acids with several proline and serine residues and four O-linked oligosaccharides. Previous studies indicated that the CTP sequence can be shuttled into different proteins and still be an acceptor for the O-linked oligosaccharides (20–22, 24). It was postulated that the O-linked oligosaccharides add flexibility and hydrophilicity to the protein (32). This may explain the disinterference of CTP on the protein conformation and thus on receptor binding and bioactivity *in vitro*. On the other hand, it was suggested that the four O-linked oligosaccharides play an important role in preventing plasma clearance and thus increasing the half-life of the protein in the circulation (20, 31–34). These roles have been postulated because the O-linked oligosaccharides are ended with sialic acid, which is negatively charged. It is known that negatively charged forms of the hormones are less cleared through the glomerular filtration (35). Thus, addition of four O-linked oligosaccharide chains to the backbone of the protein decreased the renal clearance in which the kidney is the main site of clearance for glycoprotein hormones (36) and thus prolonged its half-life in the circulation.

Human erythropoietin has a wide clinical use in the treatment of anemia associated with a renal failure, HIV, and chemotherapy (10–17). rhuEPO was produced in 1985 and it was commercially available. One major issue regarding the clinical use of EPO is its relatively short half-life due to clearance mechanisms. To address this issue, it was hypothesized that increasing the sialic acid containing carbohydrate of EPO would increase its serum half-life and thereby the *in vivo* biological activity. Increased sialic acid content will be result in producing a more negatively charged form of EPO with a longer half-life due to decreased glomerular filtration.

Recently long-acting hyperglycosylated EPO analog was designed by addition of N-linked oligosaccharides to the backbone of the protein (37). To introduce new carbohydrate attachment sites into the EPO backbone, the DNA sequence of the cloned human EPO gene was modified by site-directed mutagenesis (37). The amino acid sequence of this analog is differs from that of human EPO at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr). This analog

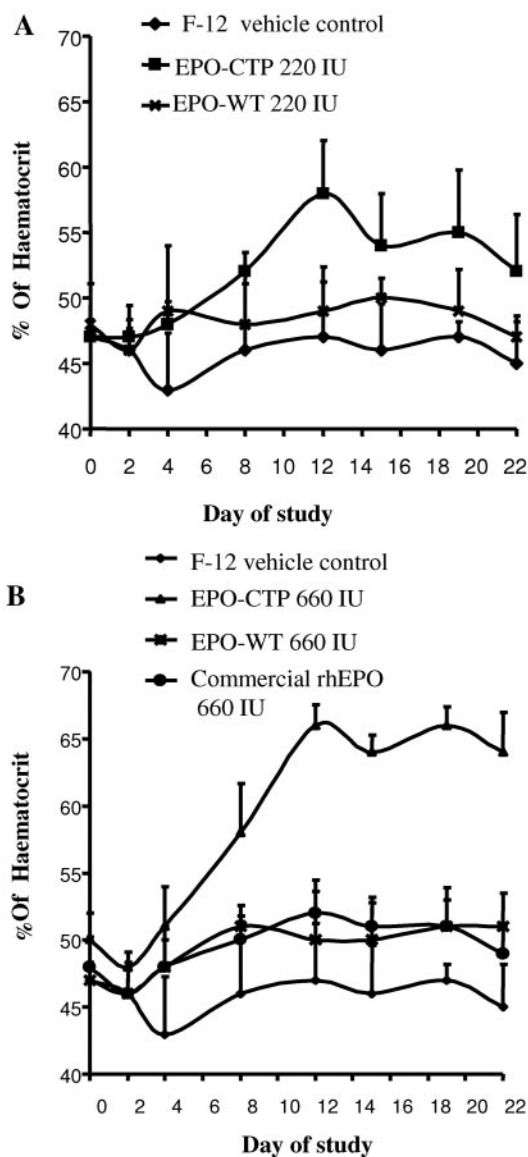


FIG. 5. *In vivo* bioactivity of rhuEPO derivatives. A, ICR mice (n = 7/group) received a single iv injection per week (220 IU/kg) for 3 wk of EPO-CTP or EPO-WT. B, EPO-WT, EPO-CTP, and commercial rhuEPO were injected iv (660 IU/kg) once a week for 3 wk. Control animals were injected iv with F-12 medium free of serum. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (percent) ± SE.

had a 3-fold longer serum half-life and created *in vivo* potency, compared with rhuEPO-WT. However, its relative affinity for the EPO receptor was approximately 4-fold lower than that of rhuEPO. Moreover, the immunogenicity of this isoform is still not clear. On the other hand, it was found that addition of CTP to the coding sequence of hFSH does not affect secretion or receptor binding affinity or bioactivity of the hormone *in vitro* (20–22). Human exposure to FSH-CTP in phase I studies was safe. No antibodies against FSH-CTP were detected and measurements of local tolerance demonstrated that sc administration of FSH-CTP was well tolerated, and no increase in intensity of injection-site responses was

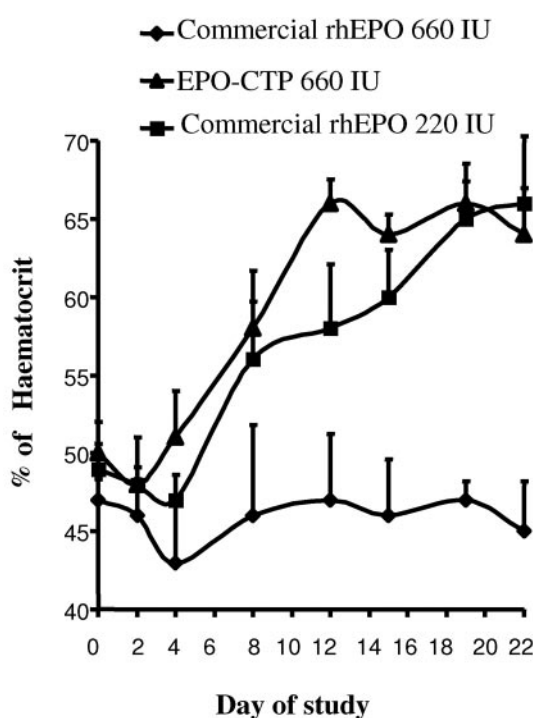


FIG. 6. Ability of a single iv injection of EPO-CTP vs. three iv injections of rhuEPO to increase haematocrit levels in mice. ICR mice (n = 7/group) received a single iv injection per week for 3 wk of EPO-CTP (660 IU/kg) or commercial rhuEPO (220 IU/kg) that were injected iv three times a week for 3 wk. Control animals were injected iv with F-12 medium free of serum. Blood samples were collected three times a week and haematocrit levels were detected. Each point represents the group average of haematocrit (percent) ± SE.

observed after repeated exposure to FSH-CTP (38). Pharmacokinetic studies in humans indicated that the elimination half-life of FSH-CTP was approximately 2 times longer than recombinant FSH (39). In addition, FSH-CTP appeared to be a potent inducer of multiple follicular growth (40), and the first pregnancy and live birth were achieved after ovarian

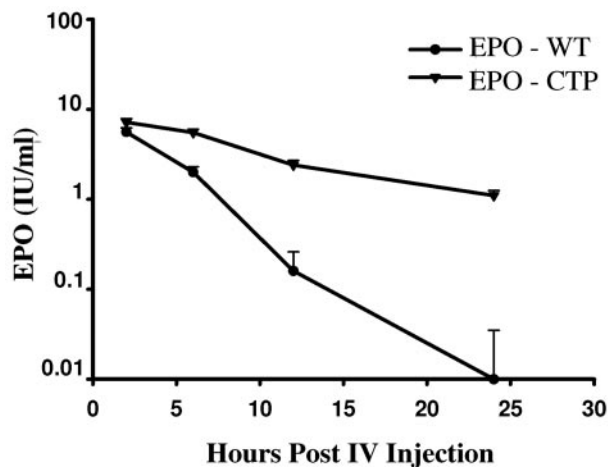


FIG. 7. Metabolic clearance of EPO-WT and EPO-CTP *in vivo*. Mice were injected iv with 20 IU of EPO-WT or EPO-CTP and blood samples were drawn at the indicated times. Serum EPO levels were determined by RIA as described in *Materials and Methods*. Mean ± SE of five determinations. Basal EPO levels before treatment were unmeasured.

stimulation by a single sc injection of FSH-CTP (41). These studies may emphasize the rationale for using the CTP in designing long-acting recombinant proteins for clinical use. However, the immunogenicity of EPO-CTP should be tested in human clinical trials. The addition of CTP to the coding sequence of EPO could elicit an immune reaction. However, the hCG subunit is normally secreted in both men and women; the immune system may not recognize the EPO-CTP chimera as a foreign protein. In addition, other studies have demonstrated that the CTP is weakly immunogenic (42). These observations may indicate that addition of CTP to the protein backbone will not be immunogenic in human.

An interesting observation from the present study was the ability of a single injection of EPO-CTP to be sufficient for stimulation of haematocrit production in mice. A single injection of 15 IU/wk of EPO-WT was ineffective in increasing haematocrit production, whereas the same total dose of EPO-WT administered as three times a week of 5 IU/kg per injection was required to produce an increase as effective as one injection of 15 IU/kg EPO-CTP. These results indicated the importance of sustained blood levels, rather than total dose of EPO, in stimulating haematocrit production. These findings are consistent with our hypothesis that the ability of a single injection of EPO-CTP per week to induce bioactivity results from its half-life increase in the circulation.

The present study describes a novel long-acting recombinant erythropoietin agonist designed by fusion of the CTP sequence to the coding sequence of EPO. This did not interfere with secretion, receptor binding affinity, or *in vitro* bioactivity. In contrast, CTP sequence significantly increased the *in vivo* potency and half-life of EPO. The results indicate that administration of a single dose of EPO-CTP per week is sufficient for haematocrit production. These data establish a rationale for using this chimera as a long-acting EPO analog. The therapeutic efficacy of this analog needs to be established in higher animals and in human clinical trials.

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