Dimeric erythropoietin fusion protein with enhanced erythropoietic activity in vitro and in vivo

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High doses of recombinant human erythropoietin (rhEpo) are required for the treatment of chronic anemia. Thus, it is clear that therapy for chronic anemia would greatly benefit from an erythropoietin derivative with increased erythropoietic activity rather than the native endogenous hormone. In this report, the activity of a human Epo-Epo dimer protein, obtained by recombinant technology, is described and compared with its Epo monomer counterpart produced under identical conditions. Although monomer Epo and dimer Epo-Epo had similar pharmacokinetics in normal mice, the increase in hematocrit value was greater with the dimer than with the monomer. Moreover, in clonogenic assays using CD34+ human hematopoietic cells, the human dimer induced a 3- to 4-fold-greater proliferation of erythroid cells than the monomer. Controlled secretion of dimeric erythropoietin in mice by intramuscular electrotransfer of a mouse Epo-Epo plasmid containing the tetO element and of a plasmid encoding the tetracycline controlled transactivator TTA. Administration of tetracycline completely inhibited the expression of the mEpo dimer. On tetracycline withdrawal, expression of the Epo-Epo dimer resumed, thereby resulting in a large and sustained hematocrit increase in β-thalassemic mice. No immunologic response against the dimer was apparent in mice because the duration of the hematocrit increase was similar to that observed with the monomeric form of mouse erythropoietin. (Blood. 2001;97:3776-3782)
TCCCCTCTCTGTGAT-3'; Epo3, 5'-AACGGGCCCGTCTCCCAAGGCTC-5'-G; Epo4, 5'-CGGAATTCGCGGCTGCTGGTACC-5'-G; Epo6, 5'-CGGATCTGGTGTCTCCCTGCTGCA-5'; Epo7, 5'-CGGATTTCTGGACACAACGTCATC-3'; Epo8, 5'-CGGAATTCGAGATGGGGGTGCAGAATG-3'; and Epo9, 5'-CATGCAGCTTGTCGCCCTGCTTGGCA-3'.

**Dimeric and monomeric mouse erythropoietin-expressing plasmids**

The N-terminal Epo domain of the mouse Epo dimer-encoding construct was amplified by polymerase chain reaction (PCR) from a plasmid containing the mouse Epo cDNA29 with primers Epo1 and Epo2. With the exception of the stop codon, this domain contains the complete erythropoietin open-reading frame. The C-terminal Epo domain was prepared using primers Epo3 and Epo4. It contains the complete open-reading frame except the signal sequence. A plasmid was constructed by ligation of the peptide linker fragment encoding 9 amino acid residues (Figure 1) into the BamHI- and EcoRI-digested BS-KS II+ phagemid (Strатеген, Saint-Quentin en Yvelines, France). After the insertion of the mEpo N-terminal fragment between the Xbal and BamHI sites of this plasmid, the C-terminal fragment was introduced at the NarI and EcoRI sites, giving the pBS-mEpoD plasmid. Epo sequences were verified by sequencing and were found to be in agreement with the expected sequence. The final construct, ptt-mEpoD, was obtained by subcloning the ClaI-HindIII mEpoD fragment from pBS-mEpoD in the ptet-splice plasmid (Gibco, Cergy Pontoise, France). This plasmid encodes the mouse Epo dimer under the control of the tetO promoter.26 The ptt-mEpoD plasmid (encoding the mouse erythropoietin native molecule) was obtained by removing the internal BsrGI fragment from ptt-mEpoD.

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**Hemagglutinin tag-containing constructs**

Human Epo monomer (hEpoM)- and dimer (hEpoD)-encoding cDNAs were amplified by PCR from ptt-hEpoD with primers Epo8 and Epo9. After EcoRI and Pmel digestion of the purified PCR products, both fragments were introduced into the mammalian epoio tag expression vector pMH (Roche Diagnostics), in-frame with a C-terminal hemagglutinin (HA) tag-encoding sequence, giving pMHhEpoM-HA and pMHhEpoD-HA plasmids.

Polymerase chain reactions were performed on 100 ng template, with 300 nM primers, 200 μM each dNTP, 1.5 mM MgCl₂, and 1 U Expand high-fidelity PCR enzyme mix (Roche Diagnostics) for 30 cycles of 30-second denaturation at 94°C, 30-second annealing at 55°C, and 90-second extension at 72°C.

**Erythropoietin-derived proteins**

Murine C57BL/6 myoblasts were derived from the skeletal leg muscle of an adult C3H mouse (CERDIC; Sophia Antipolis, France). Nontagged proteins were obtained from culture medium of C57BL/6 cells cotransfected with the transactivator-encoding plasmid, ptt-tTk (Gibco), and either ptt-hEpoM or ptt-hEpoD. Transfections were performed in 24-well plates with 2.5 μL polyethylenimine (Exgen 500; Euromedex, Souffleweyersheim, France), 100 ng ptt-tTk, and 400 ng ptt-hEpo per well, in 200 μL Optimem (Gibco). Two hours after transfection, cells were cultured for 24 hours in Dulbecco modified Eagle medium containing 10% fetal calf serum and were grown in serum-free medium for another 24 hours. Secreted proteins were concentrated 100-fold with Centricon Plus 80 Centrifugal filter devices (Amicon, Beverly, MA). hEpoM-HA– and hEpoD-HA–tagged proteins were obtained as for the nontagged molecules after the transfection of C57BL/6 cells with the pMHhEpoM-HA and pMHhEpoD-HA plasmids, respectively.

**EPO assays**

Epo-derived molecules were assayed either biologically or by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Oxon, United Kingdom; or Medac Diagnostika, Wedel, Germany). For the bio-assay, 2 types of cells were used—the Epo-dependent mouse DA7 cells, derived from the nonerythroid hematopoietic DA-1(c1.14) cell line,26 and spleen cells from phenylhydrazine-treated mice.26 For the DA7 assay, 3000 cells were seeded into wells of a microtiter plate and incubated for 3 days with several dilutions of samples or rhEpo (epoetin β; Roche Pharmaceuticals). Cell survival assays were performed 3 days later using the WST-1 reagent as indicated by the manufacturer (Roche Diagnostics). Concerning the spleen cells, Epo assays were based on 3H-thymidine incorporation, as described by Krystal.26

**Western blot analysis**

Samples were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond C extra membrane (Amersham Pharmacia, France) in methanol-containing buffer. Western blots were probed either with a goat anti-hEpo polyclonal antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, 1/500) or with an anti-HA tag antibody (BAbCO, 1/1000). Horseradish peroxidase–conjugated anti-IgG antibodies were used as secondary antibody. The secondary antibody was either goat IgG–POD (1/2000; Sigma, Saint-Quentin Fallavier, France) to detect goat anti-Epo or antinouse IgG–POD (1/1000; Roche Diagnostics) to detect mouse anti-HA. Blotted antigens were detected by chemiluminescence using the Lumi-light Plus Western blotting substrate (Roche Diagnostics).

**Clonogenic assay**

Normal human bone marrow cells were subjected to Ficoll gradient (Seromed Biochrom KG, Berlin, Germany). Cells with densities lower than 1.077 g/cm³ were harvested and purified with the Dynal (Compiegne, France) CD34 progenitor cell selection system as specified by the manufacturer.
For BFU-e assays, CD34⁺ hematopoietic cells were plated in methylcellulose-based medium (Methocult H4230; Stem Cell Technologies, Meylan, France) containing 1000 U/mL rhu-Epo (TEBU; Le Perray en Yvelines, France) and 20 ng/mL rhuSCF (TEBU) at a density of $2.5 \times 10^5$ cells/mL per 35-mm Petri dish. Cells were incubated in a fully humidified atmosphere with $5\%$ CO₂ in air at $37 \degree C$ for 14 days in the presence of different amounts of monomeric or dimeric erythropoietin. At day 14, colonies were counted and cells were harvested and cytocentrifuged. Percentages of erythroid cells were ascertained after May-Grünwald-Giemsa staining. Assays were performed in triplicate, and statistical analysis was performed using the 2-tailed Student $t$ test.

For late erythroid progenitor assays, $2.5 \times 10^5$ to $5 \times 10^5$ CD34⁺ cells were initially grown in 25-cm² flasks in the presence of BIT 9500 (Stem Cell Technologies), IL-3, SCF, and IL-6 (TEBU) as described. Six days later, cells (late erythroid progenitors) were incubated with $1 \mu g$ monoclonal CD36 IgG1 antibody (clone FA6-152; Immunotech, Marseilles, France) per 10⁶ cells, and CD36⁺ cells were purified with Dynabeads M-450 goat antimouse IgG (Dynal) as specified by the manufacturer. Five thousand CD36⁺ cells were plated in semisolid medium (Methocult H4230; Stem Cell Technologies) containing rhuSCF (20 ng/mL) and incubated in a fully humidified atmosphere with $5\%$ CO₂ in air at $37 \degree C$ for 7 days in the presence of different amounts of monomeric (hEpoM) or dimeric (hEpoD) erythropoietin. At day 7, colonies were counted, and cells were harvested and cytocentrifuged. Percentages of erythroid cells were ascertained after May-Grünwald-Giemsa staining. Assays were performed in triplicate, and statistical analysis was performed using the 2-tailed Student $t$ test.

In vivo bioactivity
Three days before injection, groups of 6 C57Bl/6 mice (Ifa Credo, L’Arbresle, France) were ether-anesthetized for identification. Blood was withdrawn for hematocrit determination. Mice were injected intraperitoneally on days 1, 3, and 5 with human EpoM or EpoD. Resultant hematocrit was determined on day 8 by a standard micro-hematocrit method.

In vivo pharmacokinetics
Groups of 3 C57Bl/6 mice were injected subcutaneously, intraperitoneally, or intravenously with the same amounts of hEpoM and hEpoD. Plasma Epo levels were determined over a 24-hour period by ELISA (R&D Systems).

Long-term and controlled secretion of dimeric erythropoietin in β-thalassemic mice
β-Thalassemic mice (Hbb-thal1) were kept in pathogen-free animal facilities. A mixture of $2 \mu g$ ptet-Off (Clontech, Basel, Switzerland) and $20 \mu g$ ptet-mEpoD was injected into the leg tibial cranial muscle of 5 β-thalassemic mice. Electric pulses were delivered through external plate electrodes placed on each side of the leg as described. Blood samples were obtained every 2 weeks by retro-orbital puncture under ether anesthesia. Plasma Epo concentrations were measured by ELISA (R&D Systems). When specified, tetracycline (Research Organics, Cleveland, OH) was added to the drinking water at a final concentration of $1 mg/mL$ in $2.5\%$ sucrose.

Statistical analysis
Hematologic data are expressed as the mean ± SD. For each mouse group, discrete variables were compared by using a 2-tailed Student $t$ test. Results were considered significant at $P < .05$.

Results
Characterization and activities of Epo dimer and monomer
C₂C₁₂ myoblasts were transfected with ptet-hEpoM (monomer) or ptet-hEpoD (dimer), together with ptet-tTAk. Two days after transfection, serum-free culture medium was harvested and concentrated. Human monomer and dimer were analyzed by SDS-PAGE and characterized with a polyclonal anti-Epo antibody raised against an N-terminal human Epo fragment. As expected, 2 distinct Epo immunoreactive species could be distinguished, with molecular weights of approximately 35 kd for the monomer and 70 kd for the dimer (not shown), which correspond to the glycosylated forms of erythropoietin. Epo bioactivity of the 2 molecules was initially assayed on the Epo-responding cell line DAE7 and on mouse spleen cells and was compared to rhEpo standards. No difference was found between the 2 in vitro bioassays. Based on these results, the Epo activities were measured by ELISA using tests from 2 different manufacturers (R&D Systems; Medac). Intrinsic activities of monomer and dimer were equivalent to those observed by proliferation assay performed with the Medac ELISA. With the R&D ELISA, the intrinsic activity of the human dimer was 5 times greater than that observed for its monomer counterpart. The affinity of the anti-Epo antibodies might have been different for the monomer and the dimer because of the presence of 1 or 2 epitopes; hence, ELISA values might not have reflected the relative number of Epo domains in the 2 molecules.

To determine the concentration of human monomer and dimer in cell culture supernatants more accurately, C-terminal HA-tagged human Epo dimer and monomer were evaluated. Indeed, in these molecules, only one immunoreactive epitope was detected. Epo values were determined by DAE7 proliferation assay and by ELISA calibrated with rhEpo. As for the nontagged molecules, R&D ELISA overestimated the tagged dimer by a factor of 5. Different concentrations of HA-tagged monomer and dimer were subsequently run on SDS-PAGE, submitted to Western blot analysis, and revealed by a monoclonal anti-HA antibody. As shown in Figure 2, similar intensities could be detected when 5 times more dimer than monomer (as determined by R&D ELISA) were transferred to the Western blot. These results confirmed that the R&D ELISA overestimated the dimer by a factor of 5 compared to the monomer. This means that the dimer is as effective as the monomer in inducing DAE7 and spleen cell proliferation. Because of these results, subsequent experiments (in vitro and in vivo) were performed using equal amounts of monomer and dimer, as determined by proliferation bioactivity.

In vivo erythropoietic activity of monomeric and dimeric hEpo
Two groups of mice were injected intraperitoneally with $300 U/kg$ (as determined by proliferation assays) of human Epo-derived molecules on days 1, 3, and 5. Hematocrit values measured 8 days after the first injection were compared with values obtained 3 days before injection (Figure 3A-B). Mean hematocrit values were slightly raised, from $49.6\% ± 1.1\%$ and $49.4\% ± 2.9\%$ to $51.6\% ± 1.5\%$ and $54.4\% ± 2.4\%$ for the monomer and the dimer, respectively. The mean increase was significantly higher ($P < .05$) for the dimer ($5.0\% ± 2.2\%) than for the monomer ($2.0\% ± 1.9\%)$. To compare the relative in vivo efficacy of the 2 molecules, 5...
groups of mice were injected with 300, 900, and 1800 U/kg monomer or 300 and 600 U/kg dimer (Figure 4). The mean hematocrit increase was significantly higher with 300 U/kg dimer (3.6% ± 1.4%) than with 300 U/kg monomer (1.1% ± 1.4%; \( P = .01 \)) and 900 U/kg monomer (1.6% ± 1.5%; \( P = .04 \)) but was not significantly different between 1800 U/kg monomer and 300 U/kg dimer. The mean hematocrit increase was significantly higher with 600 U/kg dimer (5.5% ± 1.1%) than with all other groups (\( P = .000004 \) for NaCl; \( P = .00001 \) for 300 U/kg monomer; \( P = .0005 \) for 900 U/kg monomer; \( P = .01 \) for 1800 U/kg monomer; \( P = .03 \) for 300 U/kg dimer). Based on these results, it appeared that the dimeric human Epo is approximately 6 times more active than the monomer on a per mole basis and approximately 3 times more active on a per weight basis.

**Pharmacokinetics of monomeric and dimeric hEpo**

Six groups of mice were injected intraperitoneally, intravenously, or subcutaneously with monomeric and dimeric human Epo (200 U/kg as determined by proliferation assay). Blood samples (100 μL) were obtained either 15 minutes or 1, 2, 4, or 8 hours after intraperitoneal or intravenous injection or, alternatively, after 2, 8, and 24 hours after subcutaneous injection. Because the R&D ELISA is not sensitive to mouse erythropoietin, human erythropoietin level was determined by this assay. Noninjected control mice were included in the study to determine whether endogenous mouse erythropoietin induced by the bleedings might have interfered with the ELISA measurement. At 8 hours, endogenous mouse erythropoietin was indeed detectable in mice bled at 15 minutes and at 1, 2, 4, and 8 hours after injection. Therefore, results are given only for the first 4 hours. When mice were bled only 3 times (after 2, 8, and 24 hours), no endogenous erythropoietin was detected. Results are given in Figure 5 as the percentage of the maximal values, observed 15 minutes, 1 hour, or 2 hours after intravenous (Figure 5A), intraperitoneal (Figure 5B), or subcutaneous (Figure 5C) injection, respectively. As shown, the erythropoietin kinetics did not differ between the 2 groups and decreased to half the maximum value in approximately 50 minutes, 3 hours, and 7.5 hours after intravenous, intraperitoneal, or subcutaneous injection, respectively.

**Erythroid activity of monomeric and dimeric hEpo**

Erythropoietic activities of monomer and dimer were evaluated on human CD34+ hematopoietic progenitors. This was performed by...
counting the total number of erythroid cells after 2 weeks of culture in the presence of monomeric and dimeric human Epo molecules, at various concentrations. An example of such an experiment performed in triplicate is shown in Figure 6. Whereas the number of recruited BFU-e was not significantly different between monomer and dimer (Figure 6A), 0.4 to 0.6 U/mL human dimer induced a 3- to 4-fold increase \( (P < 0.02) \) of the total number of erythroid cells (Figure 6B). At higher Epo concentrations (greater than 1 U/mL), the maximum number of erythroid cells was reached by using both monomer and dimer (not shown).

In semisolid medium, a 5-fold increase in the number of colonies from late erythroid progenitors (CFU-e) was observed in the presence of 0.1 U/mL dimer compared to the value observed in the presence of the same concentration of monomer (Figure 7A). At a higher erythropoietin concentration (0.4 U/mL), no difference in the number of colonies could be observed in the presence of either monomer or dimer, suggesting that the Epo receptors were saturated by erythropoietin. In this assay, the increase in the total erythroid cell number (Figure 7B) was similar to the increase in the number of colonies, indicating that the increased effect of the dimer occurred mostly at the CFU-e level.

**Epo dimer and hematocrit increase in \( \beta \)-thalassemic mice**

To determine whether the dimeric Epo molecule could induce a hematocrit increase in \( \beta \)-thalassemic mice and would induce antibodies, dimeric mouse erythropoietin was produced in vivo. The mouse erythropoietin dimer encoding plasmid ptet-mEpoD and the tetracycline-controlled transactivator encoding plasmid pCMV-tTA35 were co-electrotransferred in the tibial cranial skeletal muscle of \( \beta \)-thalassemic mice. Erythropoietin was measured during 26 weeks by R&D ELISA, and hematocrit changes were observed for a period of 38 weeks. Tetracycline was added to the drinking water from weeks 4 to 8, 16 to 20, and 26 to 29.5. Circulating Epo immunoreactivity was high during the first 2 weeks after electrotransfer and then slightly decreased (Figure 8B). This peak and then slight decrease in erythropoietin production has also been observed with a pCMV-mEpo plasmid constitutively expressing erythropoietin in \( \beta \)-thalassemic mice. As expected, tetracycline blocked erythropoietin secretion (Figure 8B) and reduced hematocrit to values similar to those for untreated \( \beta \)-thalassemic mice (Figure 8A). Thirty-eight weeks (9 months) after electrotransfer, tetracycline withdrawal still led to an increase in hematocrit levels. These results suggest that the partial decline of erythropoietin expression observed 1 to 3
weeks after injection was not due to the presence of antibodies raised against the mouse erythropoietin dimer but to a slight variation of expression from the injected plasmid. The long-lasting hematocrit increase observed as late as 9 months in the absence of tetracycline further confirmed the absence of an antibody response against dimeric Epo.

**Discussion**

We have designed and characterized a dimeric Epo-Epo protein obtained by recombinant DNA-mediated fusion of Epo coding regions linked by the Gly-Ser-Gly-Cys-Ser-Gly-Ala peptide. Based on the working hypothesis that Epo-Epo fusion may trigger the conformational change of the Epo receptor to its active state, this study has investigated the intrinsic activity of the Epo-Epo dimer in vitro and in vivo after gene transfer into mice. Dimeric and monomeric forms of human and mouse recombinant Epo and their HA-tagged homologs were produced by C3H12 mouse myoblast cells, known to be efficient for transgenic Epo production both in vitro and in vivo.37,38 The activity of the monoblast cells, known to be efficient for transgenic Epo already been described.39

Similar activities of human monomeric and dimeric Epo have been determined by proliferation assay, R&D ELISA findings overestimated the dimeric Epo by 5-fold. Dimeric Epo most likely had a higher binding affinity for the antiamoebic Epo than monomeric Epo. Similar differences in biologic and immunologic activities of human monomeric and dimeric Epo have already been described.39

DAE7 proliferation assay calibrated with human recombinant Epo determined that the Epo dimer induced a 6-fold-higher increase in hematocrit compared with the monomeric form when injected on days 1, 3, and 5 in normal mice at concentrations of 300 U/kg. A similar effect on hematocrit has been observed with chemically linked Epo dimer injected in rabbits27 and with an Epo-Epo fusion protein injected in mice.28 It has been postulated that this effect is mainly the result of an enhanced blood lifetime of the dimeric form. However, identical pharmacokinetics of monomeric and dimeric Epo injected through different routes—intravenously, intraperitoneally, and subcutaneously—do not support this hypothesis. To rule out any effect of the injected erythropoietin concentration on the measurements, we compared the pharmacokinetics of intraperitoneally injected proteins at low Epo concentration (40 U/kg). As with a higher Epo concentration (200 U/kg; Figure 5), no difference between monomer and dimer pharmacokinetics was observed (data not shown). Discrepancies between our data and previously published results22,23 could be related to (1) the sequence or the length of the linker peptide, which was longer in previous studies (17 amino acid residues vs 9 residues in this study), (2) the producing Epo and Epo-Epo cell line, which may have modified the glycosylation pattern, or (3) the monomeric Epo used as reference for the pharmacokinetic studies. In this study, monomeric and dimeric Epo were produced under identical conditions, by the same cell line, without purification but by concentration of the culture medium using ultrafiltration.

To understand the hematocrit increase induced by the dimeric Epo when compared to its monomeric counterpart, human CD34+ cells were studied with various concentrations of dimeric and monomeric Epo forms. Although a small relative increase in the number of BFU-e induced by the Epo dimer was observed, this trend was not significant, and this contrasted with the severalfold increase in the total erythroid cell number above that was induced by monomeric Epo (Figure 6). This difference in the increase in erythroid cell number was parallel to the increase in the CFU-e (Figure 7), indicating that dimeric Epo stimulated the late progenitors or prevented their apoptosis. Stem cell factor added to the culture medium, which has a major erythropoietin-stimulating activity on the early stages of erythroid differentiation,26,41 might have masked the effect of dimeric Epo, if any, on the recruitment of late BFU-e. One important point is the parallel between the increase in the erythropoietic activity induced by the Epo dimer added in vitro and the increased hematocrit induced by the Epo dimer in normal mice.

Erythropoietin binds to the Epo receptor through 2 binding domains, one with high affinity for the receptor and the other with low affinity; the latter is required for the activation of the receptor.27,28 When the low-affinity binding moiety of Epo is mutated, the Epo receptor is no longer activated. However, when mutated Epo monomers are linked together by a peptide containing 7-glycine residues, the presence of a second high-affinity binding motif in the dimeric Epo molecule restores the erythropoietic transduction activity.39 These results suggest that the presence of 2 molecules of Epo might bring together 2 high-affinity binding sites and facilitate binding to the Epo receptor. A 5-fold increase in the number of erythroid cells was detected when erythroid progenitors were induced by the dimeric Epo form. This contrasted with the similar proliferation activity of the dimeric and monomeric Epo forms in DAE7 cells and phenylhydrazine-induced spleen cells (present results) or UT7 cell lines.39 These differences suggest that the environment of the Epo receptor at the cell surface or that the various components of the culture medium (present in fetal calf serum or added factors) might have modified the binding or the transduction activity of the dimeric Epo in comparison with the monomeric form. The Epo dimer did indeed induce a hematocrit increase in normal mouse, which was 6-fold higher than with the Epo monomer, even though there was no difference in erythropoietin pharmacokinetics. This provides further evidence that the dimer had a specific erythroid activity that was higher (approximately 6 times on a per mole basis and 3 times higher on a per weight basis) than the monomer.

Long-term expression of mouse Epo dimer in β-thalassemic mice strongly suggests that the dimeric form of Epo does not induce any neutralizing anti-Epo antibody production. Epo dimer expression under a tet-off control can be suppressed by the addition of tetracycline in the drinking water. The delay in the reduction in hematocrit with the addition of tetracycline reflects the long (10-day) half-life of erythropoietin-treated thalassemic red blood cells.56 The withdrawal of tetracycline from the drinking water was followed by a novel increase of circulating Epo and a subsequent increase in hematocrit. This would have been impossible in the presence of neutralizing antibodies or cellular immune reactions against the dimeric Epo-producing muscle cells.

In conclusion, we have observed an increase in the biologic
specific activity of an Epo dimer in comparison with the activity of the Epo monomer. This increase in activity was shown in vitro on primary erythroid cells and in vivo in mice. Thus, the presently proposed Epo dimer could reduce the amount of therapeutic Epo required for the treatment of various chronic anemias. In addition, the availability of a more active Epo molecule could be useful for the development of Epo-based gene therapy approaches.

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References

2. Lacome C, Da Silva JL, Brunevail P, et al. Perilu-

3. Bondurant MC, Koury MJ. Anemia induces accu-
4. Koury MJ, Bondurant MC. Control of red cell pro-
duction: the roles of programmed cell death (apo-
ptosis) and erythropoietin. Transfusion. 1990;30:
673-674.

promote erythroid progenitor survival by re-

7. Eschbach JW, Kelly MR, Haley NR, Abels RI, Ad-
amson JW. Treatment of the anemia of progres-
sive renal failure with recombinant human eryth-
8. Eschbach JW. Erythropoietin: the promise and the

9. Cazzola M, Mercuriali F, Brugnara C. Use of re-
combinant human erythropoietin to patients with homozygous
thalassemia. Br J Haematol. 1995;90:341-
345.

Erythropoietin secretion and physiological effect in mouse after intramuscular plasmid DNA elec-
12. Gossen M, Bujard H. Tight control of gene ex-
pression in mammalian cells by tetracycline-
13. Payen E, Bettan M, Rouyer-Fessard P, Beuzard

Y, Scherzer D. Improvement of mouse beta-
thalassemia by electrophoresis of erythropoietin

therapy approach to regulated delivery of erythro-
16. Qiu H, Belanger A, Yoon HW, Bunn HF. Ho-
mobility to mouse erythropoietin receptor is not defined by a linear conformation.
17. Matthews DJ, Topping RS, Cass RT, Giebel LB.

A sequential dimerization mechanism for eryth-
18. Philo JS, Aoki KH, Arakawa T, Narhi LO, Wen J.

Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: one high-
affinity and one low-affinity interaction. Biochem-

DG. A modified tetracycline-regulated system provides autoregulatory, inducible gene expres-
21. Arakawa M, Ishikawa Y, Tsuda S, et al. The expres-
sion of functional erythropoietin receptors on an interleukin-3 dependent cell line. Biochem Biophys Res Commun. 1978;147:7-12.
22. Krystal G. A simple microassay for erythropoietin

based on ‘-Thymidine incorporation into spleen
cells from phenyldihydroxine-treated mice. Exp He-

therapy approach to regulated delivery of erythro-
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