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Efficacy estimation of erythropoiesis-stimulating agents using erythropoietin-deficiency anemic mice

Norio Suzuki1*, Yusuke Sasaki2, Koichiro Kato1, Shun Yamazaki1,3, Mitsue Kurasawa2, Keigo Yorozu2, Yasushi Shimonaka2, and Masayuki Yamamoto3*

1 Division of Oxygen Biology, Tohoku University Graduate School of Medicine, Sendai, Japan;
2 Product Research Department, Chugai Pharmaceutical Co., Ltd., Kamakura, Japan;
3 Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan

*Corresponding author:
Norio Suzuki and Masayuki Yamamoto
Division of Oxygen Biology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
E-mail: sunorio@med.tohoku.ac.jp
Tel: +81-22-717-8206 / Fax: +81-22-717-8090

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Erythropoietin (EPO) is an essential growth factor for red blood cell (RBC) production, and it is mainly produced by renal EPO-producing (REP) cells in the kidneys in an anemia/hypoxia-inducible manner.\textsuperscript{1,2} Erythropoiesis-stimulating agents (ESAs), including recombinant human EPO (rHuEPO), have been used to treat EPO-deficiency anemia in kidney disease patients for a quarter century.\textsuperscript{3,4} Due to the short plasma half-life of rHuEPO (approximately 1 day after subcutaneous injection), renal anemia patients require rHuEPO injections every 2 or 3 days to maintain their RBC count at non-anemic levels.\textsuperscript{3} Recently, long-acting ESAs, Darbepoetin alpha (DA, genetically modified EPO) and continuous EPO receptor (EPOR) activator (C.E.R.A., chemically modified rHuEPO), have been developed, with plasma half-lives of approximately 2 days and 5 days, respectively, after subcutaneous injection.\textsuperscript{3,4} Because of the lack of suitable animal models of EPO-deficiency anemia, it has been difficult to elucidate the detailed profiles of ESA-induced erythropoiesis \textit{in vivo}. We recently generated a genetically modified mouse model of EPO-deficiency anemia, inherited super anemia mouse/mice (ISAM, $Epo^{GFP/GFP}:Tg^{3.3K-EpoE3}$ genotype).\textsuperscript{5} Using ISAM, we were able to obtain comparable measurements of the efficacies of 3 ESAs and demonstrated that the efficacies of these agents on erythropoiesis and iron metabolism depend on their plasma half-lives.

ISAM exhibit severe normocytic-normochromic anemia due to the loss of renal EPO production.\textsuperscript{5} The EPO-deficiency anemia in ISAM begins approximately 2 weeks after birth, when the major site of EPO production switches from the liver to the kidneys.\textsuperscript{5,6} We first confirmed that the hematocrit values and hemoglobin concentrations
in the peripheral blood of ISAM were decreased to half of those in control mice at 4 weeks of age (Online Supplementary Figure S1A). Iron concentrations in the serum and liver of ISAM were higher than those in control mice, suggesting that iron usage for erythropoiesis was suppressed due to anemia in ISAM. Consistently, the unsaturated iron binding capacity of transferrin (UIBC) in the peripheral blood was decreased, and the serum level of hepcidin, a peptide hormone that inhibits iron entry into circulation, was increased in ISAM. Additionally, we found that EPO deficiency seems to be indirectly related to both systemic hypoxia and cardiomegaly through chronic severe anemia in mature ISAM (Online Supplementary Figure S1B, C).

Three representative ESAs (rHuEPO, DA and C.E.R.A.) were subcutaneously injected into ISAM at 3.0 µg of EPO-peptide weight per 1-kg body weight (BW), a comparable dose as in clinical use. Three days after single injections of each ESA (day-3), the hemoglobin and RBC concentrations in the peripheral blood of ISAM were increased, as were the mean corpuscular volume (MCV) of RBCs and the weight of the spleen, the major site of EPO-inducible erythropoiesis in mice (Figure 1A). These data indicate that these 3 ESAs similarly induce erythropoiesis in ISAM on day-3. Although C.E.R.A. further increased the levels of hemoglobin and RBC during the last 4 days of observation, the erythropoietic effects of rHuEPO or DA were eliminated on day-7 (Figure 1A). Thus, the profiles of erythropoietic induction by ESAs largely depend on their plasma half-lives.

The elevated concentration of serum hepcidin in ISAM was strongly decreased to levels less than those in normal mice on day-3 (Figure 1B). At day-7,
C.E.R.A. continued to suppress hepcidin levels, whereas hepcidin levels re-increased in ISAM injected with DA or rHuEPO. The circulating hepcidin concentration is fundamentally regulated at the gene (Hamp) transcription level in hepatocytes. Each ESA significantly suppressed Hamp mRNA expression in ISAM livers 48 hours after administration (Figure 1C). At 6 hours after administration, C.E.R.A. dramatically decreased Hamp mRNA levels, whereas rHuEPO or DA administration did not change the induced Hamp levels. Hepatic Hamp expression is strongly suppressed by erythroferrone, which is secreted by erythroblasts immediately after EPO stimulation. In the hematopoietic organs of ISAM, erythroferrone (Fam132b) gene expression was induced in 6 hours after C.E.R.A. administration, and the induced levels were maintained 48 hours after administration (Figure 1C). DA administration induced Fam132b mRNA expression in the bone marrow of ISAM 6 and 48 hours after administration, suggesting different organ distributions of ESAs. In fact, rHuEPO induced Fam132b expression in the bone marrow of ISAM 6 hours after administration but not in the bone marrow and spleen 48 hours after administration (Figure 1C). These data suggest that EPO gradually and persistently suppresses hepatic hepcidin production through the quick and transient induction of erythroblastic erythroferrone production because rHuEPO decreases the elevated hepcidin levels in ISAM on day-2 and day-3 but not at 6 hours after administration (see Figure 1C). Although the mechanism of erythroferrone-mediated hepcidin suppression is unknown, the efficacies of shorter half-life ESAs on hepcidin suppression are weaker than that of C.E.R.A. by the single-dose injection.
The hemoglobin levels of ISAM was continuously increased for 7 days after administration, and the increased level was maintained for an additional 7 days (Figure 2A). Indeed, fixed-point images of the ISAM back skin showed that the thin blood vessels of ISAM were filled with RBCs on day-7 (Figure 2B). Because both the MCV and spleen weights returned to the basal levels of the untreated ISAM at day-10 (Figure 2A), we concluded that C.E.R.A. continuously stimulates erythropoiesis for one week after administration.

C.E.R.A. dramatically reduced serum iron concentrations to the level of normal mice at day-3 (Figure 2A). Because of the rapid reduction in serum iron concentrations, the UIBC of ISAM was higher than that of control mice on day-3 and day-7. The altered levels of both serum iron and UIBC returned to untreated-ISAM levels on day-7. The accumulated iron in the spleens and livers of ISAM were decreased on day-3 and day-7, respectively (Figure 2A). Berlin blue staining also revealed decreased iron deposits in the ISAM spleens on day-7 (Figure 2C). Because the splenic iron was used before the hepatic iron was used, local splenic iron storage may be predominantly utilized for erythropoiesis instead of hepatic iron. The reduced serum hepcidin concentrations and hepatic Hamp mRNA expression in ISAM treated with C.E.R.A. were comparable to those of control mice between day-3 and day-7 and re-increased to their original levels at day-14 (Figure 2A).

We then investigated the cardiomegaly and systemic hypoxia in ISAM after C.E.R.A. administration. ISAM hearts were enlarged due to severe anemia at 12 weeks of age, and C.E.R.A. administration decreased the size and weight within 7 days (Figure
3A,B). The anemia phenotype was reversed 28 days after a single dose of C.E.R.A. (Online Supplementary Figure S2A), and cardiomegaly developed again in ISAM (Figure 3C). These data indicate that severe anemia reversibly causes cardiomegaly in mice. A systemic hypoxic milieu emerged as a result of the severe anemia in ISAM, and the expression levels of the hypoxia-inducible genes *Egln3* and *Slc2a3* were significantly higher in the hearts and kidneys, respectively, of vehicle-treated ISAM than in those of the control mice (Figure 3D). The induced gene expression levels were reduced to the levels of control mice on day-10 (Figure 3D). These therapeutic effects were similarly observed in rHuEPO-treated ISAM when the hemoglobin level was increased to the normal range by 4-time injections for 7 days (Online Supplementary Figure S3).

In the ISAM-REC mice (*Epo*\(^{GFP/GFP}\);\(\text{Tg}^{3.3\text{K}-\text{Epo}E3}\);\(\text{Rosa}26^{\text{LSL-tdTomato}:Tg^EpoCre}\) genotype), tdTomato expression permanently labels all REP cells, and EpoGFP expression is a marker for REP cells in which the transcription of the *Epo* allele is activated.\(^5\) To characterize REP cells under stable non-anemic conditions, C.E.R.A. was injected into ISAM every week for 4 weeks. The hematocrit values of ISAM remained in the normal range after the second injection (Online Supplementary Figure S2B), and *EpoGFP* mRNA expression was dramatically decreased, whereas the expression of REP-cell markers (*tdTomato* and *Pdgfrb*) were unaffected on day-28 (Figure 3E).\(^{13}\) The expression of the hypoxia-inducible *Adm* (Adrenomedullin) gene in ISAM kidneys was significantly reduced by the weekly C.E.R.A. administration,\(^{12}\) indicating that the hypoxic milieu of the ISAM kidneys was ameliorated. Analyses of tissue sections from
the ISAM-REC kidneys also demonstrated that the increased expression of EpoGFP in the REP cells of ISAM disappeared after the weekly C.E.R.A. administration, without a loss of tdTomato-positive REP cells (Figure 3F). These results demonstrate that Epo transcription is activated in the REP cells, which sense hypoxia/anemia, and that the total number of REP cells is stable in the kidneys regardless of the oxygen conditions.

This study proposes that ISAM provides a remarkable experimental system to assess ESA efficacy and to elucidate the in vivo mechanisms of erythropoiesis that are linked to iron metabolism. The increased serum and tissue iron levels, which are considered the source of cytotoxic hydroxyl radicals, were decreased immediately and sustainably after C.E.R.A. administration. Both the hypoxic milieu and cardiomegaly in ISAM were ameliorated by ESA administration. Renal anemia in chronic kidney diseases is often linked to chronic heart failure (cardio-renal-anemia syndrome, CRAS). We propose that ESA may interfere with the CRAS linkage by inducing erythropoiesis, and ISAM may help elucidate the molecular basis of CRAS.
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References


Figure legends

Figure 1. ESA administration stimulates erythropoiesis and induces erythroferrone expression in hematopoietic organs, followed by the suppression of hepcidin production in ISAM. (A) rHuEPO, DA or C.E.R.A. was subcutaneously injected into 12- to 14-week-old ISAM at a 3.0 µg/kg BW dose on day-0, and the hemoglobin concentration, red blood cell (RBC) count and mean corpuscular volume (MCV) in the peripheral blood were measured 3 and 7 days after injection. The spleen weights were also measured. n=4–6. *P<0.01, #P<0.05 compared with vehicle-treated mice (white circles) using Dunnett’s test at each time point. (B) The serum hepcidin concentrations were measured 2, 3 and 7 days after administration of each ESA. Data from the untreated control mice are also shown. n=3 for each point. *P<0.01 compared with ISAM on day 0 using nonparametric Steel test. (C) At 6 and 48 hours after the injection of ESAs into ISAM, the expression levels of Hamp (hepcidin) and Fam132b (erythroferrone) mRNA were measured in the livers and hematopoietic organs (bone marrow [BM] and spleen), respectively. Data from untreated control mice are also shown. n=3 for each group. *P<0.01 compared with the vehicle-treated samples using Student’s t test at each time point.

Figure 2. A single dose of C.E.R.A. continuously induces erythropoiesis and iron utilization in ISAM. (A) C.E.R.A. was subcutaneously injected at a 3.0 µg/kg BW dose into ISAM on day-0. Changes in the levels of the indicated parameters were
Suzuki et al measured. \( n=3 \) for each point. Data from untreated control mice are also shown.

*\( P<0.01 \) compared with vehicle-treated ISAM at each time point using Student’s \( t \) test.

(B) Fixed-point observation of the inside of the back skin of a living ISAM 3 and 7 days after C.E.R.A. administration. Red blood cells filled the vascular networks of ISAM 7 days after injection. Scale bar, 500 \( \mu m \). (C) Berlin blue staining of the spleen sections from ISAM 7 days after the injection of vehicle or C.E.R.A. shows a decrease in hemosiderin deposition (blue) following C.E.R.A. administration. Scale bar, 30 \( \mu m \).

**Figure 3.** C.E.R.A. administration improves the cardiomegaly and hypoxic milieu of ISAM. (A) Hematoxylin-eosin staining of ISAM heart sections 7 days after C.E.R.A. or vehicle administration. An image from an untreated control mouse is also shown. LV, left ventricle; RV, right ventricle. (B) The weight (\% body weight, BW) and maximum width of the ISAM hearts were measured 7 days after C.E.R.A. or vehicle administration. The data from the untreated control mice are also shown. \( n=3 \) for each group. (C) The weights of ISAM hearts were measured at 28 days after C.E.R.A. or vehicle administration in ISAM with recurring anemia. The data from the untreated control mice are also shown. \( n=3 \) for each group. (D) mRNA expression levels of the *Egln3* (Phd3) gene in the heart and the *Slc2a3* (Glut3) gene in the kidney were measured 10 days after C.E.R.A. or vehicle injection in ISAM. Data from untreated control mice are also shown. Male mice at 12 to 16 weeks of age were analyzed. \( n=3 \) for each group. (E) Changes in the mRNA expression of the indicated genes were examined in the kidneys of ISAM-REC mice injected with C.E.R.A. or vehicle every
week for 28 days. n=3 for each group. The arrow indicates an undetectable level.

*P < 0.01, #P < 0.05 compared with vehicle-treated ISAM-REC mice using Student’s t test.

(F) EpoGFP (green) and tdTomato (red) fluorescence was detected in kidney sections from ISAM-REC mice injected with C.E.R.A. or vehicle every week for 28 days. The right panels are the merged images of EpoGFP and tdTomato expression, with DAPI counterstaining. c, cortex; m, medulla.
Figure 2

(A) Graphs showing the changes in hemoglobin, iron, MCV, UBC, spleen mass, Hepcidin, and Hamp mRNA in liver over 14 days after injection with Vehicle, C.E.R.A., and Control conditions.

(B) Images of tissue sections on Day 3 and Day 7.

(C) Hematoxylin and Eosin stained sections of liver tissue for Vehicle and C.E.R.A. conditions, with scale bars of 500 µm and 30 µm respectively.
Supplementary Appendix


Methods

Mice

Inherited super anemia mouse/mice (ISAM, \(Epo^{GFP/GFP} \cdot Tg^{3,3K-EpoE}\) genotype) and ISAM-REC mice (\(Epo^{GFP/GFP} \cdot Tg^{3,3K-EpoE} \cdot Rosa26^{SL-tdTomato} \cdot Tg^{EpoCre}\) genotype)\(^1,2\) were backcrossed with the C57Black/6 strain more than 6 times, and male mice were used for experiments. In the ISAM-REC mice, a \(GFP\) cDNA is homozygously knocked into the \(Epo\) gene (\(EpoGFP\)), and \(EpoGFP\) expression in REP cells is strongly activated by severe anemia. Additionally, anemia-activated \(EpoCre\) transgene expression efficiently causes recombination of the \(Rosa26^{SL-tdTomato}\) locus and induces the expression of the \(tdTomato\) fluorescent protein from the recombined \(Rosa26^{SL-tdTomato}\) locus in REP cells permanently.\(^2\) All mice were maintained under the Regulations for Animal Experiments and Related Activities of Tohoku University.

Blood analysis

Peripheral blood (0.2–0.3 mL) was collected from the mouse heart or submandibular vein into a 1.5-mL tube containing 5.0 \(\mu\)L of 0.5 M EDTA. To measure long-term changes of the hematocrit values in living mice, approximately 60 \(\mu\)L of peripheral blood was taken weekly from the tail using a heparinized microtube (Drummond) followed by centrifugation. The effect of this weekly small-volume phlebotomy was negligible.

Measurement of iron indices

Serum iron levels were measured using an automatic biochemistry analyzer (TBA-2000FR, Toshiba). Serum hepcidin levels were measured by a sensitive liquid chromatography/electrospray ionization tandem mass spectrometry method using a Triple Quad 5500 system (AB Sciex) equipped with a Prominence UFLCXR system (Shimadzu) as previously reported (the lower limit of quantitation is 10 ng/mL).\(^3,4\) The hepatic and splenic iron contents were measured via inductively coupled plasma atomic emission spectroscopy using an Optima 8000 spectrometer (PerkinElmer).
Erythropoiesis stimulating agents (ESAs)
rHuEPO (epoetin beta, Epogen, Chugai Pharmaceutical), Darbepoetin alpha (DA, NESP, Kyowa Hakko-Kirin) and C.E.R.A. (epoetin beta pegol, Mircera, Chugai Pharmaceutical) were reconstituted with PBS containing 0.02% Tween 80. Each ESA was subcutaneously injected at a dose of 3.0 µg/kg body weight (BW). The dose corresponds to the peptide weight of each ESA, excluding their carbohydrate chains, to compare the efficacies of the ESAs at the same molar concentrations.

DA contains 2 additional glycans by genetic modification compared with rHuEPO, and C.E.R.A. is a methoxy polyethylene glycol (PEG)-conjugated rHuEPO. These carbohydrate modifications reduce the affinities between ESAs and EPOR, which determine the plasma half-lives of ESAs because EPO-EPOR complexes are degraded following their endocytosis into erythroid cells after signal transduction. PEG may also affect the stability of glycans in ESAs, and asialoglycans of ESAs may be associated with ESA half-life through degradation in hepatocytes after the capture of asialo-ESAs by asialoglycoprotein receptors.

Reverse transcription quantitative PCR (RT-qPCR)
Total RNA was extracted using ISOGEN (Nippon Gene). cDNAs was synthesized using a SuperScript III system (Invitrogen). Quantitative PCR (qPCR) was performed with the primers listed in Table 1 using the FastStart reagent (Roche). Hprt mRNA expression levels were used as an internal control for the qPCR experiments.

Dorsal chamber window and surface PO2 measurement
A dorsal chamber window was prepared in the mouse back skin as previously described. To monitor the oxygen concentration inside the back skin, oxygen sensor foil (PreSens, Germany) was placed between the hole and the cover glass, and the surface oxygen tension was measured using a VisiSens A1 detector camera and imaging software (PreSens).

Histological analyses
Heart size was measured as the maximum width using a slide caliper. Sections (4 µm thickness) were prepared from paraffin-embedded formalin-fixed organs. Hemosiderin
deposition was assessed using Berlin blue staining. The heart sections were stained with hematoxylin-eosin (Muto). To detect fluorescent protein expression, frozen kidney sections (10 µm thickness), which were fixed in 4% paraformaldehyde for 4 hours at 4 °C, were observed after DAPI counterstaining using a BZ9000 microscope (Keyence).

Statistics
The data are presented as the means ± standard deviation (SD). The $P$ values were calculated using two-tailed, unpaired Student’s $t$ tests. Dunnett’s test or the nonparametric Steel test were also used for multiple comparison.
Online Supplementary Table S1.
Sequences of the oligonucleotide primers used in the RT-qPCR analyses.

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<th>Target gene</th>
<th>Primer 1 (5’-3’)</th>
<th>Primer 2 (5’-3’)</th>
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Online Supplementary Figure S1. The anemic phenotype appears in ISAM approximately 4 weeks after birth. (A) The levels of hematocrit, hemoglobin (Hb), iron, unsaturated iron binding capacity (UIBC), and hepcidin were measured in the peripheral blood of 4-week-old ISAM and control mice. The iron concentrations in the livers were also determined. n=9 and n=5 for the control and ISAM, respectively. *P<0.01, #P<0.05 using Student’s t test. (B) The surface oxygen concentrations on the inside of the back skin of 12-week-old control and ISAM were measured using a VisiSens A1 oxygen imaging system. (C) A gross view of the hearts demonstrates that the ISAM hearts are larger than the control hearts at 12 weeks of age.
Online Supplementary Figure S2. Long-term observation of hematocrit values after C.E.R.A. administration into ISAM.  (A) C.E.R.A. (3.0 µg/kg BW) or vehicle was subcutaneously injected into ISAM on day-0, and the change in hematocrit values was measured on the indicated days. On day-28, the increased hematocrit values returned to the levels of untreated ISAM. Data from untreated control mice are also shown. n=3 for each time point.  (B) Weekly C.E.R.A. administration sustains a normal level of erythropoiesis in ISAM. C.E.R.A. (3.0 µg/kg BW) or vehicle was injected into ISAM-REC mice every 7 days beginning at day-0, and the hematocrit value was measured on day-14, 21 and 28. The weekly injection of C.E.R.A. is optimal to maintain the normal hematocrit value in ISAM, not inducing polycythemia. n=4 for each time point. Male mice at 12 to 16 weeks of age were analyzed. *P<0.01 compared with vehicle-treated mice at each time point using Student’s t test.
Online Supplementary Figure S3. Cardiomegaly and hypoxic milieu of ISAM are corrected by 1-week administration of rHuEPO. rHuEPO (3.0 µg/kg BW) or vehicle was injected into ISAM every 2 days (day-0, 2, 4 and 6), and the mice were analyzed for the indicated parameters at 7 days after the first injection (day-7). n=3 for each group. *P<0.01, #P<0.05 compared with vehicle-treated ISAM using Student’s t test.
References for Supplementary Appendix


- S 8 -