Evidence of Receptor-Mediated Elimination of Erythropoietin by Analysis of Erythropoietin Receptor mRNA Expression in Bone Marrow and Erythropoietin Clearance During Anemia

Demet Nalbant, Mohammad Saleh, Frederic D. Goldman, John A. Widness, and Peter Veng-Pedersen

Division of Pharmaceutics, College of Pharmacy (D.N., M.S., P.V.-P.) and Department of Pediatrics, College of Medicine (J.A.W.), University of Iowa, Iowa City, Iowa; and Division of Pediatric Hematology Oncology, School of Medicine, University of Alabama, Birmingham, Alabama (F.D.G.)

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ABSTRACT

Erythropoietin (Epo) is the primary hormone that stimulates erythroid proliferation and differentiation through its cell surface receptor (EpoR) on erythroid progenitor cells. Previous studies have suggested that the bone marrow plays an important role in Epo's elimination. The changes in the EpoR mRNA levels and Epo's clearance in the bone marrow of 11 newborn lambs were studied to elucidate the role of EpoR in Epo's clearance under anemic conditions. Epo mRNA levels were measured by real-time polymerase chain reaction, and relative expression of EpoR was calculated by using the comparative CT method. The glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was chosen as a control gene for the calculations. All lambs showed significant increase in bone marrow EpoR mRNA levels after phlebotomy-induced anemia. Epo's clear-

ance determined from simultaneous pharmacokinetic studies with ^{125}I -recombinant human Epo showed a significant increase after phlebotomy-induced anemia that was similar to the increase in EpoR. By day 28 after phlebotomy, EpoR mRNA levels and Epo clearance had returned toward baseline. These results indicate that the changes in Epo's clearance are not caused by body growth but result from significant changes in the pool of EpoR. A linear mixed-effect model was used to evaluate the quantitative relationship between EpoR and Epo's clearance. This analysis demonstrated a highly significant positive linear correlation between EpoR and Epo clearance. Together, these findings provide strong evidence that receptor-mediated Epo clearance is an important route for Epo's elimination.

Erythropoietin (Epo) is a glycoprotein hormone that stimulates erythroid proliferation and differentiation (Jelkmann, 2007). Epo exerts its erythropoietic effects through a cell surface receptor (EpoR) on the erythroid progenitor cells [burst-forming unit-erythroids and colony-forming unit-erythroids (CFU-Es)] (Jelkmann, 2004; Richmond et al., 2005). Although EpoR expression has been reported in other cell types and tissues, the highest density of EpoRs is detected on erythroid progenitor cells in bone marrow (Rossert and Eckardt, 2005).

Binding of Epo to the cell surface induces dimerization of two EpoR molecules, which in turn initiates intracellular signal transduction required for the production of mature red blood cells (RBC)

(Remy et al., 1999; Elliott et al., 2008). Human *EpoR* gene has been cloned from a placenta genomic library and characterized intensively (Maouche et al., 1991; Noguchi et al., 1991). Different EpoR isoforms derived by alternative splicing have been reported for the human, mouse, rat, and ovine EpoR genes (Kuramochi et al., 1990; Nakamura et al., 1992; Yamaji et al., 1996; David et al., 2002).

Although the mechanisms involved in Epo elimination and site of degradation are still not completely understood, several studies suggest that receptor-mediated elimination of Epo plays an important central role in its clearance (Chapel et al., 2001a,b; Freise et al., 2007; Widness et al., 2007).

The hypothesis of an Epo elimination mechanism via nonrecycled EpoRs is supported by several observations. First, Epo's clearance shows transient perturbations in conjunction with large transient perturbations in endogenous Epo levels (Chapel et al., 2001b; Freise et al., 2007; Widness et al., 2007). Second, the Epo

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ABBREVIATIONS: Epo, erythropoietin; EpoR, Epo receptor; rHuEpo, recombinant human Epo; $\Delta\Delta\text{CT}$, comparative CT method; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RBC, red blood cells; PK, pharmacokinetic; PCR, polymerase chain reaction; CFU-E, colony-forming unit-erythroid; ANOVA, analysis of variance; pO_2 , tissue oxygen.

clearance is significantly reduced after busulfan-induced bone marrow ablation (Chapel et al., 2001a). Although these observations are compelling, they provide only indirect evidence for Epo elimination being mediated primarily by EpoR. Thus, the current study was undertaken to provide additional direct evidence of the proposed EpoR-based elimination mechanisms by analysis investigating the relationship between the EpoR mRNA levels and Epo clearance.

This was done experimentally by investigating the changes in Epo clearance in association with serial, simultaneously measured EpoR mRNA levels in the bone marrow after phlebotomy-induced anemia in lambs.

Materials and Methods

Animals and Study Outline. All animal experimental procedures were approved by the University of Iowa Institutional Animal Care and Use Committee before the study. Eleven lambs (3–4 weeks old) were studied. The lambs were housed in an indoor, light- and temperature-controlled environment alongside their mothers. Jugular venous catheters used for blood sampling were inserted under pentobarbital anesthesia. Ampicillin (1g/day) was administered for the first 3 days after the catheter insertion. Phlebotomy-induced anemia was achieved by removing 60% of the estimated total body RBC volume from the lambs starting from initial basal Hb levels of 9.7 ± 1.1 g/dl (mean $\pm$ S.D.). This was accomplished by exchange phlebotomy where equal volumes of autologous plasma (or saline when the plasma volume was insufficient) were transfused for each volume of blood removed. Doing so decreased the lamb's Hb level to 3.7 to 4.2 g/dl. For all 11 lambs a baseline Epo pharmacokinetic (PK) study and bone marrow aspiration procedure were performed the day before the major phlebotomy and repeated at approximately 9 days after phlebotomy. In 6 of 11 lambs an additional Epo PK study and bone marrow aspiration were performed approximately 28 days after the major phlebotomy (Fig. 1) when the Hb levels reached were not statistically different from the basal levels ($p < 0.05$) (9.5 ± 1.5 g/dl). Throughout the study, whole blood samples were analyzed by using a XT 2000 automated hematology system analyzer (Sysmex America Inc., Mundelien, IL).

PK Studies. After an intravenous bolus dose of a tracer amount of ^{125}I -rHuEpo (less than 0.1 U rHuEpo/kg), 14 blood samples were taken over the subsequent 7-h period. Plasma concentrations of ^{125}I -rHuEpo were analyzed by a double-antibody immunoprecipitation assay as described previously (Widness et al., 1992) with a lower level of detection of 0.004 mU/ml and a coefficient of variation of 7% in accuracy.

Fig. 1. Graphical representation of the study outline. BM, bone marrow aspirate collection; PK, PK assay; n, sample numbers.

Bone Marrow Aspirate and Peripheral Blood Collection and RNA Isolation. Bone marrow aspirates (2 ml) were collected from the iliac crest into a 5-ml syringe containing heparin (1000 units/ml) by using a sterile 18-gauge bone marrow aspiration needle. Bone marrow aspirates were transferred into 2-liter centrifuge tubes and spun at 1000g for 5 min after which the plasma was removed and the cell pellet was mixed with RNeasy Lysis Solution (RiboPure-Blood Kit; Ambion, Austin, TX) and stored at -20°C . Total RNA was isolated from stored bone marrow samples by using the RiboPure-Blood Kit from Ambion. Samples were treated with amplification-grade DNaseI (Invitrogen, Carlsbad, CA) to ensure the DNA-free RNA. Peripheral blood samples (2 ml) were obtained simultaneously as marrow samples, and RNA was isolated in a similar manner.

Reverse Transcription and Real-Time PCR. cDNA was generated from 0.5 μg of total RNA by using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time polymerase chain reactions (PCRs) were performed in triplicate in 384-well plates with an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Minus reverse transcription reactions were also run each time to test for genomic DNA contamination. Real-time PCR primers for sheep EpoR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (house-keeping gene) genes were designed by using partial *Ovis aries* cDNA sequences (GenBank accession nos. AY029231 and U94889, respectively). All primers were designed either to span exon–exon junctions or anneal to different exons, thereby preventing genomic DNA amplification. The comparative CT method ($\Delta\Delta\text{CT}$) was used to calculate relative expression of EpoR. The amplification efficiencies of EpoR primer sets (EpoRI, EpoRII, and EpoRIII) and GAPDH primer sets were confirmed to be approximately equal before the study samples were analyzed.

The primer sequences used were: ovGAPDH F, 5'-ACAGCGA-CACCTCACTCTTCTACCT-3'; ovGAPDH R, 5'-CCC TGT TGC TGT AGC CGA ATT CAT-3'; ovEpoR I F, 5'-TCG TGC TCA TTC TAC TGC TGC T-3'; ovEpoR R, 5'-ATG CCA GGC CAG ATC TTC TGC TT-3'; ovEpoR II F, 5'-ACA AGG GTA ACT TCC AGC TGT GG-3'; ovEpoR II R, 5'-AGC ACT CAG AGA GGA CTT CCA AGG-3'; ovEpoR III F, 5'-CTC CTG TGG CCG AAC TCC AAG TCT-3'; and ovEpoR III R, 5'-AGA CAG CCG TCA GTC TGG TAC A-3'.

Pharmacokinetic Analysis. The Epo plasma concentration profile from a single intravenous bolus of ^{125}I -rHuEpo was best described by a biexponential distribution function. Curve fitting was performed by using WINFUNFIT, a Windows (Microsoft, Redmond, WA) version evolved from the general nonlinear regression program FUNFIT (Pedersen, 1977). To account for weight change, Epo clearance values throughout this article are clearance values normalized to body weight.

Statistical Analysis. The statistical analysis was conducted by using R software (version 2.6.1; <http://cran.r-project.org>). Repeated-measure analysis of variance (ANOVA) was conducted to assess the effect of phlebotomy on mRNA levels and clearance values. Because our data include both fixed and random effects, repeated-measure ANOVA was applied by using the lme (linear mixed effect) function in R (nlme library). Tukey's test for multiple comparisons was applied by using the glht (general linear hypothesis testing) function in R (multcomp library). The following linear mixed model was used to examine the relationship between Epo clearance values and EpoR mRNA levels:

$$\text{Cl}_{i,j} = \alpha_i + \beta_i \cdot \text{mRNA}_{i,j} + \varepsilon_{i,j} \quad (1)$$

$$\varepsilon_{i,j} \sim N(0, \sigma_e^2) \quad (2)$$

$$\begin{pmatrix} \alpha_i \\ \beta_i \end{pmatrix} \sim \text{BVN} \left(\begin{pmatrix} \alpha \\ \beta \end{pmatrix}, \begin{pmatrix} \sigma_\alpha^2 & 0 \\ 0 & \sigma_\beta^2 \end{pmatrix} \right) \quad (3)$$

where $\text{Cl}_{i,j}$ is the j th Epo clearance for the i th subject. The $\text{mRNA}_{i,j}$ term denotes the j th EpoR mRNA relative value for the i th subject; α_i and β_i are the slope and the intercept for the i th subject; $\varepsilon_{i,j}$

is residual error, and σ^2 is the variance of the bivariate normal distribution (BVN).

Normal distribution was used to express interindividual variability with diagonal variance covariance matrix (eqs. 1–3). An additive model was used to describe the residual error. Because the absolute number of EpoR mRNA molecules cannot be ascertained by real-time PCR, the relative number of EpoR mRNA molecules was derived from the cycle number of the gene of interest, *EpoR*, and housekeeping gene, *GAPDH*, as shown below. The relative number of EpoR mRNA molecules does not indicate the exact number of genes; instead it gives a value proportional to the actual number (Pfaffl, 2001). Although this proportionality constant cannot be determined with the current analysis, it was the same for all samples analyzed. This assumes that the efficiency of the target gene is two.

$$\text{mRNA}_{i,j} = 2^{-\Delta \text{CT}(\text{Sample})} \quad (4)$$

and

$$\Delta \text{CT} = \text{CT}_{\text{EpoR}} - \text{CT}_{\text{GAPDH}} \quad (5)$$

where CT_{EpoR} is the cycle number of EpoR mRNA and CT_{GAPDH} is the cycle number of GAPDH.

Results

The partial ovine EpoR cDNA sequences, spanning from exons 2 to 8, has been published (David et al., 2002). The PCR primers designed to amplify ovine EpoR amplified the two distinct expected bands (Fig. 2A). A 93-bp insert between exons 7 and 8 potentially results in expression of a longer EpoR protein as described earlier (David et al., 2002). We designed three different primer sets (EpoRI, EpoRII, and EpoRIII) (Fig. 2B) to amplify the different isoforms independently. The EpoRI primer set amplifies both isoforms, whereas the EpoRII and EpoRIII primer sets specifically amplify longer and shorter isoforms of the EpoR cDNA, respectively. Total RNA isolated from bone marrow and peripheral blood samples was used to generate cDNA. Cell surface EpoR protein expression is found on burst forming unit-erythroids and CFU-Es located within the bone marrow space and is not found on mature RBC or other cellular components of peripheral blood (Broudy et al., 1991). Thus, peripheral blood samples served as negative controls for EpoR mRNA expression profiles. As expected, EpoRI, EpoRII, and EpoRIII amplified EpoR cDNA only in bone marrow samples (Fig. 2C). The internal housekeeping gene, GAPDH, was equally expressed in bone marrow and peripheral blood (Fig. 2C). Experiments were undertaken to determine

whether different isoforms had different expression levels and isoform expression levels are affected by phlebotomy-induced anemia. PCR analysis revealed that under steady-state baseline (nonanemic) conditions the shorter isoform tends to be more abundant than the longer EpoR mRNA isoform (Fig. 2A). In addition, the levels of these two isoforms were affected in a very similar manner under conditions of phlebotomy-induced anemia. Therefore, we have used real-time PCR results obtained from EpoRI primers for the statistical analyses.

Real-time PCR analyses revealed that 9 days after the major phlebotomy EpoR mRNA levels were higher than prephlebotomy baseline levels in all study lambs. The increases for the EpoR mRNA levels ranged from 1.2- to 12.6-fold (Table 1). The six lambs that had an additional bone marrow aspirate collected at day 28 after phlebotomy had Epo mRNA levels that were significantly lower than those measured at day 9.

As shown in Table 2, repeated-measure ANOVA analysis of EpoR mRNA levels revealed that, compared with baseline day -1, there was a significant ($p < 0.05$) increase in EpoR mRNA levels by 9 days after phlebotomy, which subsequently decreased in all six lambs by 28 days after phlebotomy. There was no significant difference between EpoR mRNA levels before phlebotomy and 28 days after phlebotomy.

These sequential changes observed in EpoR mRNA over the 4-week study period paralleled those observed for Epo clearance. Ten of 11 subjects showed an increase in Epo clearance on day 9 after phlebotomy, decreasing toward baseline levels by day 28. The repeated-measure ANOVA analysis conducted to examine the effect of phlebotomy on Epo's clearance demonstrated that there is a significant increase in Epo's clearance 9 days after phlebotomy compared with both the Epo clearance before phlebotomy and 28 days after phlebotomy ($p < 0.05$). Similar to what was observed for EpoR mRNA levels, Epo's clearance before the phlebotomy was not significantly different from that at 28 days after phlebotomy (Table 2).

The linear mixed-effect modeling of Epo's clearance using mRNA as a covariate showed that the clearance is positively and linearly related to mRNA by a population slope of 76.8 and a population intercept of 63.1. The 95% confidence interval of the population estimate for the slope is 13.7 to 139 and for the intercept it is 51.1 to 75.1. Regarding interindividual variability, the coefficient of variation as percentage for the individual estimates for the slope is 125.8 and for the inter-

Fig. 2. Two isoforms of EpoR and EpoR real-time PCR primer locations. A, ethidium bromide-stained agarose gel. The two isoforms of EpoR are indicated by arrows. B, exons are shown as gray solid boxes, and the 93-bp insertion is the crossed-line pattern box. Arrows indicate real-time PCR locations. C, ethidium bromide-stained agarose gels for each EpoR real-time primer pair and GAPDH. M, DNA base pair marker; BM, bone marrow; PB, peripheral blood.

TABLE 1

Fold changes in EpoR mRNA levels and Epo clearance before and after phlebotomy

Lamb ID No.	Fold Change in EpoR mRNA Levels Relative to Before Phlebotomy			Fold Change in Epo Clearance Relative to Before Phlebotomy		
	Before Phlebotomy	9 Days After Phlebotomy	28 Days After Phlebotomy	Before Phlebotomy	9 Days After Phlebotomy	28 Days After Phlebotomy
1	1.0	12.6	N.D.	1.0	2.3	N.D.
2	1.0	5.0	N.D.	1.0	1.3	N.D.
3	1.0	9.5	N.D.	1.0	1.2	N.D.
4	1.0	2.8	N.D.	1.0	1.4	N.D.
5	1.0	3.7	N.D.	1.0	1.8	N.D.
6	1.0	3.5	2.3	1.0	2.2	1.4
7	1.0	1.2	0.5	1.0	1.5	0.8
8	1.0	2.4	1.1	1.0	1.4	0.8
9	1.0	2.2	1.6	1.0	1.3	0.6
10	1.0	10.2	3.0	1.0	0.9	0.6
11	1.0	1.6	0.8	1.0	1.8	1.0

N.D., not done.

TABLE 2

Repeated measure ANOVA results for EpoR mRNA levels and Epo linear clearance

	Estimated Difference	Standard Error	P Value
$Cl_L^2 - Cl_L^1$	32.7	6.16	<0.0001*
$Cl_L^3 - Cl_L^1$	-9.3	7.41	0.42
$Cl_L^3 - Cl_L^2$	-42.0	7.41	0.0001*
$mRNA^2 - mRNA^1$	0.16	0.03	0.0001*
$mRNA^3 - mRNA^1$	0.04	0.04	0.58
$mRNA^3 - mRNA^2$	-0.12	0.04	0.01*

Cl_L^1 , Cl_L^2 , and Cl_L^3 are clearance (ml/h/kg) before phlebotomy, 9 days after phlebotomy, and 28 days after phlebotomy, respectively. $mRNA^1$, $mRNA^2$, and $mRNA^3$ are mRNA values before phlebotomy, 9 days after phlebotomy, and 28 days after phlebotomy.

* Indicates significant difference from zero at level of significance <0.05.

Fig. 3. Estimated clearance values determined by using linear mixed-effect model versus actual clearance values (ml/h/kg).

cept it is 51.3. The clearance values estimated by the linear mixed-effect model plotted against the actual measured clearance are shown in Fig. 3.

Discussion

The present study provides compelling new additional evidence that EpoR mRNA is up-regulated as a result of phlebotomy-induced anemia. In further support of this hypothe-

sis is the report that the incubation of murine cell lines with Epo for 6 days increases the number of EpoR per cell without changing EpoR binding affinity (Broudy et al., 1990). EpoR up-regulation was also reported in a rat brain endothelial cell line and cortical astrocyte cell cultures as a result of hypoxic conditions (Bernaudin et al., 1999). Our observation that EpoR mRNA levels decreased toward baseline 28 days after phlebotomy relative to the 9-day sample supports our hypothesis that EpoR up-regulation is caused by increased endogenous Epo levels after phlebotomy and is not a developmental growth phenomenon.

Because anti-ovine EpoR antibody is not commercially available, it was not possible to show the changes in EpoR protein levels. Spandou et al. (2004) reported that after hypoxia-ischemia up-regulation of EpoR mRNA and protein levels show a very similar temporal pattern in rat brain. Therefore, we expect the EpoR protein levels to show a similar pattern to EpoR mRNA levels.

Our previous studies provided significant evidence that bone marrow plays a major role in Epo elimination (Chapel et al., 2001a; Widness et al., 2007) and EpoR is up-regulated as a result of phlebotomy-induced anemia in sheep (Freise et al., 2007). Similarly, the present study showed an increase in Epo clearance 9 days after phlebotomy. As was observed with EpoR mRNA, the increase observed in Epo clearance is not likely a natural developmental event resulting from growth. This speculation is supported by the observation that Epo clearance decreased toward baseline by 28 days after phlebotomy. Taken together, these findings strongly support the hypothesis that Epo elimination is largely the result of a receptor-mediated mechanism.

There was a close similarity observed in the behavior of changes in EpoR mRNA and Epo clearance throughout the study during the transition from baseline conditions followed by anemic stress conditions as a result of phlebotomy with a subsequent return toward baseline. This observation supports our previously published receptor-mediated model of Epo elimination (Chapel et al., 2001b). Furthermore, results from our linear mixed-effect model show a linear relationship between EpoR mRNA and Epo clearance. The positive slope observed is significantly different from zero. This indicates that Epo's clearance increases linearly in parallel with the amount of EpoR mRNA in erythroid progenitor cells. The positive intercept can be explained by a simultaneous Epo elimination through a nonhematopoietic elimination pathway. Studies by other research groups also provide a sub-

stantial indirect evidence of receptor-mediated elimination of Epo. Sawyer et al. (1987) demonstrated receptor-mediated elimination of Epo as a result of lysosomal degradation in erythroid cells. In rats, higher organ-specific uptake of ^{125}I -rHuEpo in the bone marrow was also observed (Spivak and Hogans, 1989). Gross and Lodish (2006) also reported that 40% of Epo internalized in cells carrying EpoR is intracellularly degraded.

EpoR mRNA splicing variants have been reported previously for human, mouse, rat, and sheep (Kuramochi et al., 1990; Nakamura et al., 1992; Yamaji et al., 1996; David et al., 2002). However, the role of different EpoR transcripts has not been well understood. In humans the splice variant with the 95-bp insert between exons 6 and 7 introduces an early stop codon generating a truncated receptor (Nakamura et al., 1992). However, the sheep EpoR transcript variant potentially encodes a longer protein in addition to wild-type EpoR, because the 93-bp insert does not interrupt the open reading frame (David et al., 2002).

We investigated whether phlebotomy-induced anemia has separate effects on expression of EpoR mRNA isoforms. We observed that the two EpoR mRNA isoforms were expressed significantly higher after phlebotomy, with no significant change in the ratio of the two isoform transcripts. Further studies are required to investigate whether the longer EpoR isoform is translated into a functional protein and has different characteristics than wild-type EpoR.

Although it is difficult to predict whether the current findings extrapolate to EpoR-expressing nonhematopoietic cells, we propose that Epo's actions are mainly on the hematopoietic system. Epo has been shown to exert cardioprotective and neuroprotective effects (Calvillo et al., 2003; Jelkmann, 2007). As mentioned above, EpoR up-regulation was also reported in a rat brain endothelial cell line and cortical astrocyte cell cultures as a result of hypoxic conditions (Bernaudin et al., 1999). Thus, one would expect a similar regulatory effect on Epo's neuroprotective properties.

A linear mixed model was used because it is suitable for dealing with sparse sampling and unbalanced design as in the current study. The data analyzed by the mixed model seemed challenging, and several programs were tried, including NONMEM (ICON, Dublin, Ireland) and SAS (SAS Institute, Cary, NC), both of which did not converge successfully. Fortunately, successful convergence was obtained by using the lme function available in R, but the relative standard error for interindividual estimates could not be determined with R.

In summary, based on the analysis of data from this study and previous findings in the literature, we suggest that under conditions of severe tissue hypoxemia, such as occurs with anemia severe enough to result in a high degree of Epo stimulation, hematopoietic EpoRs are up-regulated as a part of a feedback regulation. The feedback regulation is triggered by a decrease in tissue oxygen (pO_2). The pO_2 in turn depends on different physiological variables, including hemoglobin concentration, arterial pO_2 , hemoglobin affinity to oxygen, and rate of blood flow. The decrease in pO_2 induces an increase in the endogenous Epo production. Increases in Epo stimulate erythropoiesis by several mechanisms that include preventing the apoptosis of CFU-E (Koury and Bondurant, 1990). Each CFU-E will produce 8 to 60 erythroblasts (Jelkmann, 2007). We also propose that Epo stimulates erythropoiesis by up-regulating EpoR number per cell. Increasing

EpoR number will result in an increase in the efficacy of Epo in stimulating erythropoiesis.

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Address correspondence to: Dr. Peter Veng-Pedersen, University of Iowa College of Pharmacy, 115 S. Grand Ave., Iowa City, IA 52242. E-mail: veng@uiowa.edu