Mechanism for the Nonlinear Pharmacokinetics of Erythropoietin in Rats

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ABSTRACT

The contribution of erythropoietin- (EPO) receptors in target tissues, such as bone marrow and spleen, to the nonlinear pharmacokinetics of recombinant human EPO (rh-EPO) was evaluated in rats. The total body clearance after i.v. administration of rh-EPO (0.2-5 µg/kg) decreased as the dose of rh-EPO increased, approaching a plateau at high doses. The uptake clearance of 125I-rh-EPO by the target tissues, bone marrow and spleen, exhibited clear saturation. The Km values ranged from 240 to 450 pM, which are comparable with the reported value for the dissociation constant of EPO binding to EPO-receptors (180 pM) in rat bone marrow cells. A single s.c. administration of a large dose of rh-EPO (1 µg/kg) caused a reduction in tissue uptake clearance of 125I-rh-EPO by bone marrow and spleen (down-regulation). Furthermore, repeated intravenous injection of rh-EPO caused up-regulation of the tissue uptake clearance of 125I-rh-EPO, especially by the spleen, in a dose-dependent manner. Hematopoietic parameters such as hematocrit and hemoglobin concentration were also increased by repeated rh-EPO treatment and significantly correlated with the sum of the tissue uptake clearances in bone marrow and spleen. These findings suggest that the pharmacological receptor could be an important factor in defining the nonlinear pharmacokinetics of rh-EPO.

Recent advances in recombinant DNA technology have made it possible to use biologically active polypeptides for therapeutic purposes. Some of these peptides, e.g. insulin (Whitcomb et al., 1985), tissue-plasminogen activator (Tanswell et al., 1990), epidermal growth factor (Kim et al., 1988; Sato et al., 1988) and hepatocyte growth factor (Liu et al., 1992, 1993) are, however, rapidly eliminated from the circulation by RME in the liver and other organs. This process is an important feature of the pharmacokinetics of biologically active polypeptides and contributes to not only their elimination but also distribution (Sugiyama et al., 1989). Generally, biological action is exhibited via an interaction between the peptide and its receptor. In addition, Maack et al. (1987) have reported the presence of a biologically “silent” receptor that acts only as a clearance site for peptides. Polypeptide receptors are, thus, important for both the pharmacological effects and pharmacokinetic properties of polypeptides.

The genes of hematopoietic growth factors such as EPO, G-CSF and GM-CSF have already been cloned (Jacobs et al., 1985; Lin et al., 1985; Nagata et al., 1986; Wong et al., 1985) and now their recombinant products can be used as treatments for several hematological diseases. To evaluate their pharmacological effect in vivo, it is important to understand the pharmacokinetics of these peptides. For example, hematopoietic factors such as G-CSF (Layton et al., 1989, Kuwabara et al., 1994), GM-CSF (Yoon et al., 1993) and M-CSF (Redman et al., 1992) exhibit nonlinear pharmacokinetics. Detailed studies using the G-CSF derivative, nartograstim, indicate that the RME in bone marrow contributes to the nonlinear pharmacokinetics of this polypeptide (Kuwabara et al., 1994, 1995a, 1995b). It has been shown that the nonlinear pharmacokinetics of G-CSF arises from the saturation of RME in bone marrow and spleen (Kuwabara et al., 1995a, 1995b).

In this study, we have investigated the mechanism for the nonlinear pharmacokinetics of EPO. EPO is a 34-kDa glycoprotein mainly produced by kidney and it stimulates the proliferation and differentiation of CFU-E. rh-EPO is clinically used for the treatment of anemia in patients with end-stage renal disease (Winearls et al., 1986; Eschbach et al., 1987). The pharmacokinetics of EPO exhibits nonlinearity both in humans (Flaharty et al., 1990) and rats (Kinoshita et al., 1992b) and several investigators have found that multiple administration causes a reduction in the plasma elimination half-life in the patients with renal disease (Lim et al.,

ABBREVIATIONS: EPO, erythropoietin; AUC, area under the plasma concentration-time curve; MRT, mean residence time; CLtotal, total body clearance; CLup, early-phase tissue uptake clearance; RME, receptor mediated endocytosis; CFU-E, colony-forming unit erythroid; GM-CSF, granulocyte macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; RIA, radioimmunoassay.
1989; Neumayer et al., 1989). A similar phenomenon was also observed in normal and nephrectomized rats receiving multiple doses of rh-EPO at 1 μg/kg (Kinoshita et al., 1992a). Despite the wide clinical use of rh-EPO, no quantitative analysis of its nonlinear kinetics has been reported. In this study, we examined its nonlinear pharmacokinetics and the contribution of RME to the nonlinear elimination of rh-EPO from the circulation.

Materials and Methods

Materials. rh-EPO was produced using Chinese hamster ovarian cells transfected with an expression vector containing the human erythropoietin cDNA at Production Technology Laboratories, Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). 125I-Sodium iodine (17.4 Ci/mg) and 125I-rh-EPO for RIA were obtained from Amersham plc (Amersham, UK). Iodo-GenTM (1,3,6-triethanol-3,6-diphenylglycouril) was obtained from Pierce Chemical Company (Rockford, IL). Immunobead secondary antibody was obtained from Bio-Rad Laboratories (Richmond, CA) and Amersham plc, protein A from Behring Diagnostics (La Jolla, CA). Hemoglobin B-test Wako was obtained from Wako Pure Chemical Co.,Osaka, Japan). All other reagents were obtained as the purest grade available.

Radiolabeling. 125I-rh-EPO was prepared by the Iodo-Gen method described previously (Kinoshita et al., 1991). A total of 40 μl of a 50 μCi/ml solution of Iodo-Gen in chloroform was placed in a sample tube and evaporated to dryness under nitrogen. Into another tube, 23.8 μl (30 μg polypeptide equivalent) rh-EPO, 3 μl 50 mM sodium phosphate buffer (pH 7.5) and 5 μl 125I-sodium iodide (200 μCi) were mixed and allowed to stand for 2 min in an ice bath; this was followed by the addition of 100 μl 1 mg/ml methionine solution to stop the reaction. A total of 300 μl of 50 mM sodium phosphate buffer (pH 7.5) containing potassium iodide and rat serum albumin at concentrations of 120 and 20 mg/ml, respectively, was subsequently added to the mixture. The resulting mixture was chromatographed on a Sephadex G-10 column (20 cm × 1 cm I.D.) using 50 mM sodium phosphate buffer (pH 7.4) containing 0.1% Tween 20 and 0.05% rat serum albumin as eluant, to obtain fractions of 125I-rh-EPO. The specific radioactivity was 6.87 μCi/μg as determined by gel filtration assay and radiochemical purity was 93.1% as determined by gel permeation chromatography.

Animals. Male Sprague-Dawley rats (JCL:SD, Clea Japan Inc., Tokyo, Japan) were allowed to acclimatize to the laboratory environment for one week and then the experiment was started at 7 wk of age when the animals weighed 240 to 290 g. Animal rooms were maintained at constant ambient temperature and a relative humidity of 24°C and 55%, respectively, throughout the experimental period. A standard rodent feed in pellet form (CE-2, Clea Japan Inc., Tokyo, Japan) and tap water ad libitum were available throughout the study.

Pharmacokinetic experiment. Rats were anesthetized with ether, and a polyethylene cannula (SP-31, Naruse Seisakusyo, Tokyo, Japan) was placed in the femoral artery and vein. rh-EPO was administered i.v. at doses of 0.2, 0.5, 1 and 5 μg/kg polypeptide equivalent via a femoral vein using three animals at each dose. Blood (200 μl) was withdrawn from each rat through an arterial cannula into a heparinized tube at 5, 15 and 30 min and 1, 2, 4, 6, 8 hr and centrifuged at 15,000 rpm for 3 min. In the case of continuous infusion, rh-EPO was infused i.v. into rats at rates of 16, 32, 160, and 1,600 ng/kg/hr after a loading dose of 20, 40, 200 and 2,000 ng/kg, respectively, using three animals at each infusion rate. Blood (200 μl) was obtained at 2, 4, 6 and 8 hr after the start of infusion.

RIA for rh-EPO. A total of 300 μl of a 1:150,000 dilution of anti-rh-EPO rabbit antiserum in phosphate buffer was added to 100 μl of appropriate phosphate buffer dilutions of plasma and incubated overnight at room temperature. Subsequently, 100 μl of a 100,000 cpm/ml solution of 125I-rh-EPO was added and allowed to stand for 4 hr at room temperature. Immunobead second antibody (250 μl) were then added. The mixture was further incubated for 1 hr at room temperature followed by washing the beads with 2 ml 0.9% (w/v) NaCl solution. After centrifugation at 3,000 rpm for 5 min, the supernatant was aspirated and the radioactivity of the residue measured in a gamma counter. The inter- and intra-day reproducibility for this assay was less than 10%. Endogenous EPO was not detectable (<0.32 ng/ml).

Immunoprecipitation assay. For the assay of 125I-rh-EPO, an immunoprecipitation assay was used: 200 μl phosphate buffer and 200 μl of 100-fold diluted anti-rabbit serum were added to 50 μl plasma. The mixtures were allowed to stand for 16 hr at room temperature. They were then incubated for 1 hr with 100 μl of a suspension containing 5% protein A. Then 200 μl 0.9% NaCl were added following by centrifugation at 15,000 rpm using a microcentrifuge for 3 min. The supernatant was removed and the precipitated fraction was counted in a gamma counter.

Tissue uptake of 125I-rh-EPO. 125I-rh-EPO was administered in a dose of 0.05 to 125 μg/kg polypeptide equivalent. Solutions of 125I-rh-EPO (0.025–62.5 μg/ml) were prepared with isotonic saline containing 0.05% (w/v) Tween 20 and 0.05% (w/v) rat serum albumin. Rats (n = 21) received injections with 2 ml/kg of solution into the tail vein and exsanguinated via cardiac puncture under ether anesthesia 30 min after dosing. The spleen, femoral bone marrow, liver and kidney were removed and weighed accurately. Blood was transferred to a heparinized tube and centrifuged at 15,000 rpm for 3 min. Then 1 ml plasma and all the tissues obtained were counted directly by gamma counter.

The short-term effect of rh-EPO treatment. rh-EPO was administered s.c. at a dose of 1 μg/kg to groups of three rats. 125I-rh-EPO was administered i.v. at a dose of 0.1 μg/kg before and 4, 10, 24 and 48 hr after administration of rh-EPO. Radioactivity in the bone marrow and spleen was measured 30 min after the administration of 125I-rh-EPO.

The long-term effect of rh-EPO treatment. Rh-EPO was administered i.v. at a dose of 0, 1, 5 and 25 μg/kg, twice over a 4-day period, the first dose being given on day 0 and the second on day 2. 125I-rh-EPO was administered at a dose of 0.2 μg/kg on day 4. The plasma concentration of immunoreactive radioactivity was measured. Radioactivity in the bone marrow and spleen was measured 30 min after the administration of 125I-rh-EPO in rats treated with rh-EPO 5 μg/kg. Hematocrits and hemoglobin concentrations were measured on day 7. Three or four rats per group were used.

Data analysis. The plasma concentration data were fitted to a nonlinear regression program using MULTI (Yamaoka et al., 1981); first-order kinetics was assumed. Because the plasma concentration (C) declined in a biexponential fashion, it was described by equation 1:

\[ C = A \exp(-\alpha t) + B \exp(-\beta t) \] (1)

The initial distribution volume (Vc) was calculated as dose/(A + B); steady state distribution volume (Vss) was calculated as dose/(Aβ + Bα)/(Aβ + Bα)^2. Area under the curve (AUC) and area under the moment curve (AUMC) were calculated by the trapezoidal rule with extrapolation to infinity. MRT was calculated as AUMC/AUC, and total body clearance (CLtotal) was estimated as dose/AUC. The steady-state plasma concentration in the infusion study (Css) was determined as the mean value of C at 6 and 8 hr and CLtotal was calculated as infusion rate/Css.

Nonlinear model fitting. The mean plasma concentrations of rh-EPO at each dose in rats were simultaneously fit to a two-component open model using the nonlinear least squares regression program MULTI(RUNG) (Yamaoka et al., 1983) in which each data point was weighted with a factor equal to 1/C^2. This model involved both first-order elimination and Michaelis-Menten type elimination.
of rh-EPO from the central compartment. The differential equations describing this compartment model can be expressed as:

\[
dC_1/dt = -(k_{12} + k_e + V_{\text{max}}/(V_c + C_1))C_1 + k_{12} C_2
\]

\[
dC_2/dt = k_{21} C_1 - k_{21} C_2
\]

where \(C_1\) is the concentration of rh-EPO in the central compartment, \(C_2\) is the concentration of rh-EPO in the peripheral compartment; \(K_m\) is the Michaelis-Menten constant and \(V_{\text{max}}\) is the maximum elimination rate of the saturable elimination process from the central compartment; \(k_{12}\) and \(k_{21}\) are the first-order transfer rate constants between the central and peripheral compartments, \(k_e\) is the first-order elimination rate constant from the central compartment. The nonsaturable clearance was calculated as \(k_e V_c\).

The tissue uptake clearance (CLup). When the initial tissue distribution of \(^{125}\text{I}\)-rh-EPO is measured within a short time period after its intravenous administration, back flux may be neglected and tissue uptake clearance (CLup) can be defined by equation 4.

\[
dX_t/dt = CL_{\text{up}} C
\]

where \(C\) and \(X_t\) are plasma concentration and amount in tissue.

Integration of equation 4 gives

\[
X_t(t) = CL_{\text{up}} \text{AUC}_{0-\text{t}}
\]

where \(CL_{\text{up}}\) was calculated using equation 5. CLup is the influx clearance from the central compartment into tissue. AUC_{0-\text{t}} is the AUC from 0 to time t. The decrease in the plasma concentration until 0.5 hr was approximated by a monoexponential process. Based on this approximation AUC_{0-0.5} was calculated by assuming log-linear regression from the concentration at 0.5 hr \((C_{0.5})\) after intravenous administration using the following equations:

\[
C_0 = \text{dose}/V_c, \quad k_e = (\ln(C_0) - \ln(C_{0.5}))/0.5
\]

\[
\text{AUC}_{0-0.5} = C_0/k_e(1 - \exp(-0.5 k_e))
\]

where \(C_0\) is the initial plasma concentration.

The parameters in the nonlinear process \((K_m, V_{\text{max}})\) were determined by fitting using the following equation:

\[
CL = V_{\text{max}}/(K_m + C_0) + CL_{\text{ns}}
\]

where \(CL_{\text{ns}}\) is nonsaturable clearance.

Although the blood space in the tissue was not washed before excising the tissue, the radioactivity remaining in such blood space will affect only the nonlinear portion of tissue uptake clearance, its contribution being minor to the net tissue uptake.

**Statistical methods.** Comparisons of pharmacokinetic, hematopoietic and tissue uptake parameters were performed using a one-way analysis of variance followed by the Dunnet or Scheffe test. Statistical significance was taken as \(P < .05\).

**Results**

**Pharmacokinetics.** Figure 1A and table 1 show the plasma concentration-time profiles and pharmacokinetic parameters, respectively, after i.v. administration of rh-EPO at doses of 0.2 to 5 \(\mu\)g/kg. The plasma concentration decreased biexponentially after each dose (fig. 1A). Both the terminal phase half-life \((T_{1/2}(\beta))\) and MRT were prolonged with increasing dose (table 1) but there was no significant change in \(V_c\) and \(V_{\text{ss}}\) (table 1). The CLtotal fell with increasing dose and, at high doses, approached a plateau (table 1). Figure 1B shows the plasma concentration-time profile during continuous infusion. Plasma concentration reached a steady-state at 6 hr after the start of infusion. The CLtotal calculated from Css was \(32.2 \pm 2.1, 31.1 \pm 0.8, 22.7 \pm 0.9\) and \(20.6 \pm 2.1\) ml/kg/hr (mean \(\pm\) S.E., \(n = 3\)) at infusion rates of 16, 32, 160 and 1,600 ng/kg/hr, respectively, indicating saturation of the overall elimination of rh-EPO. Additionally, CLtotal was comparable between 160 and 1,600 ng/kg/hr. These results suggest that CLtotal consists of saturable and nonsaturable components. Interestingly, the CLtotal after a bolus dose of 0.2 \(\mu\)g/kg (48.3 ml/kg/hr) was greater than the CLtotal under the linear conditions of the infusion study (31.1–33.2 ml/kg/hr). This may arise from an underestimation of AUC after bolus administration, because the plasma sampling time was not long enough due to the detection limit of the RIA.

The plasma concentration-time profiles were also kinetically analyzed using a model with linear elimination and Michaelis-Menten type elimination from the central compartment. The values of \(V_{\text{max}}\) and \(K_m\) for saturable elimi-

![Fig. 1. Plasma concentration-time profiles for rh-EPO after i.v. bolus administration to rats at doses of 0.2 (○), 0.5 (●), 1 (□) and 5 (■) \(\mu\)g/kg (A) and during continuous infusion at rates of 16 (○), 32 (●), 160 (□) and 1,600 (■) ng/kg/hr (B) to rats. Each point with vertical bar represents mean \(\pm\) S.E. for three rats. Solid lines were obtained by fitting using equations 2 and 3. Data at all doses examined were simultaneously fitted to equations 2 and 3, and the fitted lines thus obtained are also shown. The Michaelis-Menten constant and maximum elimination rate of the saturable elimination process were 4.03 \(\pm\) 3.03 ng/ml (219 pm) and 47.5 \(\pm\) 30.2 ng/kg/hr, respectively, although the nonsaturable clearance was 23.5 \(\pm\) 1.2 ml/kg/hr (calculated mean \(\pm\) S.E.). In the case of infusion, rh-EPO was infused at the rates of 16, 32, 160 and 1,600 ng/kg/hr after a loading dose of 20, 40, 200 and 2,000 ng/kg, respectively.
TABLE 1

Pharmacokinetic parameters of rh-EPO after i.v. administration of rh-EPO to rats at doses of 0.2, 0.5, 1 and 5 μg/kg

<table>
<thead>
<tr>
<th>Dose (μg/kg)</th>
<th>T1/2 (hr)</th>
<th>Vc (ml/kg)</th>
<th>Vss (ml/kg)</th>
<th>MRT (h)</th>
<th>CLtotal (ml/kg/h)</th>
<th>AUC0–8/Dose (kg · h/ml)</th>
<th>AUC0–8/Dose (kg · h/ml)</th>
<th>AUC0–8/Dose (kg · h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>1.32</td>
<td>49.6</td>
<td>85.2</td>
<td>1.10</td>
<td>48.3</td>
<td>0.0141</td>
<td>0.0208</td>
<td></td>
</tr>
<tr>
<td>(0.12)</td>
<td>(4.2)</td>
<td>(1.4)</td>
<td>(0.09)</td>
<td>(3.9)</td>
<td>(0.0006)</td>
<td>(0.0016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2.49</td>
<td>46.0</td>
<td>84.8</td>
<td>2.05*a</td>
<td>27.0*a</td>
<td>0.0193*a</td>
<td>0.0371</td>
<td>0.0436</td>
</tr>
<tr>
<td>(0.38)</td>
<td>(4.3)</td>
<td>(3.5)</td>
<td>(0.09)</td>
<td>(3.0)</td>
<td>(0.0000)</td>
<td>(0.0004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.42</td>
<td>50.0</td>
<td>82.2</td>
<td>2.35*a</td>
<td>23.7*a</td>
<td>0.0224*a</td>
<td>0.0373</td>
<td>0.0423</td>
</tr>
<tr>
<td>(0.15)</td>
<td>(1.1)</td>
<td>(2.2)</td>
<td>(0.08)</td>
<td>(0.6)</td>
<td>(0.0005)</td>
<td>(0.0006)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.36</td>
<td>47.5</td>
<td>77.8</td>
<td>2.33*a</td>
<td>24.3*a</td>
<td>0.0205*a</td>
<td>0.0377</td>
<td>0.0413</td>
</tr>
<tr>
<td>(0.16)</td>
<td>(4.5)</td>
<td>(3.8)</td>
<td>(0.03)</td>
<td>(1.8)</td>
<td>(0.0011)</td>
<td>(0.0027)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value represents mean (S.E.) of three animals.
*a Significant difference from 0.2 μg/kg (P < .05).

Short-term effect of rh-EPO treatment. Rh-EPO was administered s.c. at a dose of 1 μg/kg. 125I-rh-EPO was subsequently administered i.v. at a dose of 0.1 μg/kg at 4, 10, 24 and 48 hr after administration of rh-EPO. The CLup of 125I-rh-EPO by bone marrow and spleen was estimated from the AUC0–0.5 and tissue concentrations. Figure 3 shows the time-course of CLup by bone marrow and spleen. The CLup by bone marrow and spleen was reduced by preadministration of rh-EPO, suggesting down-regulation of the CLup (fig. 3). The CLup by both organs reached a minimum 10 hr after treatment (fig. 3) when the plasma concentration of s.c. administered rh-EPO reached a maximum (Kato et al., 1993). The CLup by bone marrow returned to the control level after down-regulation, although that by the spleen reached a significantly higher level than the control value after recovery (fig. 3).

Long-term effect of rh-EPO treatment. Figure 4 show the plasma concentration-time profiles and pharmacokinetic parameters of immunoreactive radioactivity after i.v. administration of 125I-rh-EPO (0.2 μg/kg) to rats who had received rh-EPO at 0, 1, 5, 25 μg/kg on two occasions, i.e., Plasma disappearance of 125I-rh-EPO during the first 6 hr after administration, when most of the radioactivity is due to the unchanged form, was accelerated as the pretreatment dose increased (fig. 4). Pretreatment with rh-EPO caused up-regulation of the CLup of 125I-rh-EPO that depended on the pretreatment dose (fig. 5). The CLup by bone marrow and spleen increased to 1.2 and 4 times the control value, respectively, at the pretreatment dose of 25 μg/kg (fig. 5). Thus, marked up-regulation was observed, especially in the spleen (fig. 5). Hematopoietic parameters such as the hematocrit and hemoglobin concentration also increased as the pretreatment dose increased (fig. 5). A significant correlation was also observed between hematocrit and the sum of CLup by bone marrow and spleen (r = 0.973, P < .05) (fig. 5). In rh-EPO- (5 μg/kg) treated rats, the values of Km and Vmax for tissue uptake were estimated using equation 8 (fig. 6). The values of Km, Vmax and CLns for bone marrow and spleen are shown in table 3. In the spleen, there was an increase in Vmax and a reduction in Km (table 3).

Discussion

RME contributes to the systemic clearance of bioactive polypeptides such as hepatocyte growth factor (Liu et al., 1985) and epidermal growth factor (Kim et al., 1988; Sato et al., 1988). The pharmacokinetics of these peptides exhibit nonlinearity due to saturation of RME. In this study, we examined the pharmacokinetic properties of rh-EPO in rats. After i.v. administration of 0.2 to 5 μg/kg rh-EPO, CLtotal fell as the dose increased (table 1). Additionally, CLtotal...
calculated from $C_{ss}$ in the infusion study also was reduced as the infusion rate increased (fig. 1). These results indicate that the pharmacokinetics of rh-EPO exhibits nonlinearity. At higher doses, the clearance approached a constant value (fig. 1; table 1), suggesting that $C_{\text{L, total}}$ consists of saturable and nonsaturable components. The intrinsic clearance of the saturable component ($V_{\text{max}}/K_m$) was estimated to be 11.8 ml/kg/hr, which was approximately half that of the nonsaturable component (Flaharty et al., 1990). These data suggest that $C_{\text{L, total}}$ in humans also consists of saturable and nonsaturable clearances and the saturable clearance is more than 10 ml/kg/hr, which is 2.5 times as great as the nonsaturable clearance (Flaharty et al., 1990). In humans, the saturable elimination mechanism of rh-EPO may play a more important role than the nonsaturable elimination mechanism. Such a species difference in the ratio of saturable clearance to nonsaturable clearance is attributed to the fact that the nonsaturable clearance in rats is five times greater than that in humans although the saturable intrinsic clearances in humans and rats are similar. The reported values for the nonsaturable clearance in rats (Kuwabara et al., 1995c) can be described by the allometric equation ($C_{\text{L, ns}} = 17.0 (BW)^{0.70}, r = 0.994$), where the allometric exponent, 0.70, is similar to values reported for interspecies relations involving several parameters such as glomerular filtration rate (Dedrick, 1973a). This suggests that the nonsaturable clearance of rh-EPO in humans can be predicted from animal data. The $C_{\text{L, total}}$ in 5/6 nephrectomized rats after administration of a large dose of rh-EPO was 66% that in control rats (Kinoshita et al., 1992b), and $C_{\text{L, total}}$ in anephric dogs was significantly smaller than that in intact dogs (Juan et al., 1988). These reports suggest that the kidney plays an important role in $C_{\text{L, total}}$. Furthermore, our study suggests that renal clearance is mainly due to a nonsaturable component since no dose-dependence of the $C_{\text{L, up}}$ of $^{125}$I-rh-EPO by the kidney was observed (fig. 2). These results and previous reports suggest that the kidney might, at least partially, contribute to nonsaturable clearance. It has also been reported that the kidney is the organ responsible for nonsaturable clearance of G-CSF derivatives, which are also eliminated by saturable and nonsaturable clearances such as rh-EPO (Kuwabara et al., 1995c). Such a nonsaturable clearance mechanism is thought to involve glomerular filtration. It is well known that glomerular filtration functions as a barrier discriminating between the size and electric charge of molecules in the circulating plasma (Takakura et al., 1990). Compounds with a molecular size less than 50 to 60 kDa can be filtered through the glomerulus. The molecular weight of rh-EPO is 34 kDa and, therefore, the mechanism for its nonsaturable clearance may be glomerular filtration.

Sawyer et al. (1987) reported that $^{125}$I-rh-EPO was taken

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$K_m$ (ng/ml)</th>
<th>$V_{\text{max}}$ (ng/g tissue/hr)</th>
<th>$C_{\text{L, ns}}$ (ml/g tissue/hr)</th>
<th>$V_{\text{max}}/K_m + C_{\text{L, ns}}$ (ml/kg B.W./hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>8.26 (449 pM)</td>
<td>15.9 ± 0.3</td>
<td>0.163 ± 0.002</td>
<td>41.8$^a$</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.42 (240 pM)</td>
<td>4.56 ± 0.13</td>
<td>0.143 ± 0.002</td>
<td>3.64$^b$</td>
</tr>
</tbody>
</table>

$^a$ Each value represents the calculated mean ± S.E.; 21 rats were used.
$^b$ The bone marrow weight per kilogram of body weight was measured as 3.0 g.

*$^*$ Significant difference from control ($P < .05$). Values are means ± S.E. for three or four rats.
up into Friend virus-infected erythroid cells by RME, followed by lysosomal degradation; specific binding sites on the cell surface were reduced by exposure to excess rh-EPO (down-regulation). In our study, the $K_m$ value for saturable elimination of rh-EPO from the central compartment was 220 pM (fig. 1) and this agrees with the $k_d$ value of EPO receptors in rat erythroid progenitor cells (180 pM) (Akahane et al., 1989). This suggests that the mechanism for the saturable clearance of rh-EPO is also RME by EPO receptors. In fact, saturable uptake of rh-EPO can be observed in target organs such as bone marrow and spleen but not in liver or kidney (fig. 2). These $K_m$ values of saturable uptake are also comparable with the reported $k_d$ value for EPO binding to EPO receptors (180 pM) in rat bone marrow cells (Akahane et al., 1989). Considering the weight of the bone marrow (4 g/200 g for rats; Dedrick et al., 1973b) and spleen (3.0 g/kg for rats; values observed in our study), the saturable portion of the CLup by bone marrow and spleen is 38.5 and 3.1 ml/kg/hr, respectively (table 2). Therefore, the nonlinear kinetics in plasma can be accounted for mainly by uptake into bone

![Fig. 6. The relationship between C and CLup of bone marrow (A) and spleen (B) in control (C) and EPO-treated rats (D). Solid lines were obtained by fitting using equation 8.](image)

<table>
<thead>
<tr>
<th>TABLE 3</th>
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<tbody>
<tr>
<td>Kinetic parameters for CLup by bone marrow and spleen in control and EPO-treated rats</td>
</tr>
<tr>
<td>$K_m$ (ng/ml)</td>
</tr>
<tr>
<td>Control Bone marrow</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Spleen</td>
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<td>EPO-treated Bone marrow</td>
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<td>Spleen</td>
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Each value represents the calculated mean ± S.E.; the values were estimated by equation 8; 12 rats/group were used.
marrow. However, no dose-dependence of tissue uptake was observed for the liver and kidney, known clearing organs for many peptides (fig. 2). Thus, uptake by liver and kidney does not contribute to the nonlinear elimination of rh-EPO from the body.

To confirm that RME participates in the saturable clearance, the down-regulation of the CLup was examined. The CLup for 125I-rh-EPO by bone marrow and the spleen was reduced after s.c. administration of rh-EPO (fig. 3). The CLup by both organs reached a minimum 10 hr after pretreatment (fig. 3) and the rh-EPO concentration was approximately 1 ng/ml in the plasma at that time (Kato et al., 1993). Therefore, it is possible that the uptake of 125I-rh-EPO by bone marrow and spleen was competitively inhibited by rh-EPO remaining in plasma. To exclude such a possibility, the degree of reduction in the tissue uptake of 125I-rh-EPO, which was accounted for by competitive inhibition by rh-EPO at 10 hr after pretreatment, was calculated based on the K_m and V_max obtained from the analysis of CLup by bone marrow and spleen. The calculated reductions in CLup by bone marrow and spleen were only 8% and 12%, respectively, although the actual reductions were 33% and 44%, respectively (fig. 3). These observed reductions were much larger than the calculated decreases, suggesting that down-regulation of the CLup of 125I-rh-EPO cannot be explained just by inhibition by rh-EPO and, therefore, the down-regulation of EPO receptors may contribute to the reduction in CLup.

The CLup of 125I-rh-EPO by spleen 48 hr after administration of rh-EPO significantly increased to 1.4 times the control value (fig. 3). Therefore, the CLup is down-regulated for a short period but up-regulated for a long period after pretreatment with excess rh-EPO. To investigate whether the change in the number of receptors affects the pharmacokinetics of rh-EPO, the up-regulation of the CLup was also examined. Treatment with rh-EPO accelerated the plasma disappearance of 125I-rh-EPO in a dose-dependent fashion (fig. 4). Furthermore, treatment with rh-EPO also caused a dose-dependent up-regulation of the CLup of 125I-rh-EPO (fig. 5). This result suggests that the change in plasma disappearance may be due to a change in CLup by bone marrow and spleen. This change in CLup was observed almost exclusively for the saturable component of CLup although the change in the nonsaturable component was minimal (fig. 6). In addition, significant correlations were also found among the sum of the various CLup values and hematopoietic parameters for by an increase in the number of pharmacological receptors. When multiple doses of rh-EPO were given to patients with renal failure (Lim et al., 1989; Neumayer et al., 1989) and normal subjects (McMahon et al., 1990), an increase in CLtotal was observed. This phenomenon may also be explained by an increase in the number of target cells due to the pharmacological action of rh-EPO.

In conclusion, the elimination of rh-EPO occurs through both saturable and nonsaturable clearance mechanisms and the former is mainly governed by uptake via EPO receptors in bone marrow at least under physiological condition.

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References


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