PHARMACOKINETICS OF ERYTHROPOIETIN IN GENETICALLY ANEMIC MICE

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ABSTRACT:

We examined the pharmacokinetics of recombinant human erythropoietin (rh-EPO) in genetically anemic mice (W/W\textsuperscript{v} genotype) to clarify its disposition mechanism in hematopoietic injury such as occurs in aplastic anemia. After rh-EPO was administered to W/W\textsuperscript{v} and control (+/+ genotype) mice once a day for 1 week at different doses, both the hematocrit (Hct) and tissue uptake clearance (CLup) of \textsuperscript{125}I-rh-EPO by spleen and bone marrow in the femur were estimated on the eighth day. The hematocrit increased on eighth day, depending on the dose administered. Compared with +/+ mice 10 times more rh-EPO was needed in W/W\textsuperscript{v} mice to produce an almost equivalent pharmacological effect. In +/+ mice, the CLup of \textsuperscript{125}I-rh-EPO by spleen increased to 4-fold that of controls after treatment with rh-EPO, 4.8 \mu g/kg, whereas that by bone marrow remained unchanged, irrespective of the dose administered. On the other hand, the increase in both the Hct and CLup in spleen was minimal in W/W\textsuperscript{v} mice. The CLup by bone marrow and spleen in both types of mice showed saturation with similar \(K_m\) values (389–619 pm), comparable with the dissociation constant of the EPO receptor. In addition, the Hct correlated with the sum of the CLup by bone marrow and spleen in both types of mice, and the correlation lines were superimposable. These results suggest that the pharmacological receptors govern the saturable tissue uptake not only in normal mice but also in those that are anemic.

Erythropoietin (EPO) is a 34-kD glycoprotein that is mainly produced by the kidney and stimulates the proliferation and differentiation of colony-forming unit erythroid (CFU-E) (Krantz, 1991). EPO was purified from the urine of patients with aplastic anemia by Miyake et al. (1977). The cDNA of the EPO gene has been cloned (Jacobs et al., 1985). At present, recombinant human EPO (rh-EPO) is used as a treatment for the anemia seen in patients with end-stage renal disease. The treatment of rh-EPO improved the anemia in those patients (Winearls et al., 1986; Eschbach et al., 1987). On the other hand, despite high concentration of endogenous EPO in plasma, the patients with a more “aggressive” type of anemia, such as aplastic anemia and myelodysplastic anemia, exhibited no improvement (Hirashima et al., 1990). Although aplastic anemia may be a result of injury to hematopoietic stem cells or their microenvironment, the mechanism leading to such anemia has not been fully clarified yet.

Recently it has been reported that treatment with large amounts of rh-EPO improved the condition of some patients with aplastic anemia (Hirashima et al., 1990; Musolino et al., 1994; Urabe et al., 1993). Both W/W\textsuperscript{v} mice and Sl/Sl\textsuperscript{d} mice are known to be suitable models of aplastic anemia (Lewis et al., 1967; Bernstein, 1970). W/W\textsuperscript{v} mice have a genetic defect in their stem cells and Sl/Sl\textsuperscript{d} mice have a genetic defect in their stromal microenvironment. The point mutation in the W gene encoding stem cell factor receptor (c-kit) in W/W\textsuperscript{v} mice causes the reduction in tyrosine kinase activity (Nocka et al., 1990). Treatment with large doses of rh-EPO also improved the anemia W/W\textsuperscript{v} mice (Cynshi et al., 1990). In the present study we used the W/W\textsuperscript{v} mouse as a model of aplastic anemia.

We have reported that the pharmacokinetics of rh-EPO exhibits nonlinearity because of saturation of the tissue uptake by target tissues such as bone marrow and spleen (Kato et al., 1997). In that study we clarified the contribution of receptor-mediated endocytosis (RME) to the nonlinear elimination of rh-EPO from the circulation in rats (Kato et al., 1997). Our study also indicated that the administration of rh-EPO caused up- and down-regulation of receptor-mediated uptake by target tissues. Repeated administration of rh-EPO caused both an increase in hematocrit (Hct) and tissue uptake clearance, especially in the spleen. The Hct correlated well with the sum of the tissue uptake clearance, suggesting that repeated administration of rh-EPO increases the receptor density and/or target cells (CFU-E) in spleen, resulting in an increase in receptor-mediated tissue uptake. Thus, the pharmacokinetics of rh-EPO is affected by saturation of receptor binding and/or receptor-mediated uptake as well as up- and down-regulation. To evaluate the effect of rh-EPO on aplastic anemia, it is important to understand the pharmacological properties and pharmacokinetics of rh-EPO in W/W\textsuperscript{v} mice. In the present study, we examined the effect of rh-EPO administration on the hematocrit and the specific tissue uptake of rh-EPO by target organs.

Materials and Methods

Materials. rh-EPO was produced using Chinese hamster ovarian cells transfected with expression vector harboring the human erythropoietin cDNA at Production Technology Laboratories, Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). \(\text{^{125}}\text{I}-\text{sodium iodide (17.4 Ci/mg)}\) and \(\text{^{125}}\text{I-rh-EPO for radioimmunoassay (RIA)}\) were obtained from Amersham (Amersham, UK). Iodo-Gen\textsuperscript{TM} (1,3,6-tetrachloro-3,6- diphenylglycouril) was obtained from Pierce.
Chemical Company (Rockford, IL). All other reagents were the purest grade available.

**Radiolabeling.** $^{125}$I-rh-EPO was prepared by the Iodo-Gen method described previously (Kinoshita et al., 1991). The specific radioactivity was 2.6 $\mu$Ci/µg as determined by gel filtration assay. The radiochemical purity was 96.3% as determined by gel filtration.

**Animals.** Male WBB6F1-W/W$^v$ and WBB6F1-+/+ strain mice (Japan SLC Inc., Shizuoka, Japan) were allowed to acclimate to the laboratory environment for 1 week and then the experiment was started when the animals were 8–9 weeks of age. 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.** $^{125}$I-rh-EPO was administered at a dose of 0.48 µg/kg polypeptide equivalent to rh-EPO via the tail vein. Solutions of $^{125}$I-rh-EPO (0.24 µg/ml) were prepared with isotonic saline containing 0.05% (w/v) Tween 20 and 0.05% (w/v) mouse serum albumin. The injected volume was 2 ml/kg. The mice were bled to death via cardiac puncture under ether anesthesia at 5 and 30 min and 1, 2, 4, 8 hr after injection with rh-EPO. Blood was transferred into a heparinized tube and centrifuged at 15,000 rpm for 3 min.

**TCA-precipitation Assay.** Two hundred milliliters of 25% TCA solution and 150 µl 1 M NaF were added to 50 µl plasma. The reactive mixture was allowed to stand for 10 min at room temperature, centrifuged for 5 min at 3,000 rpm, and then the radioactivity in the TCA-precipitated fractions was measured.

**Tissue Uptake of $^{125}$I-rh-EPO.** $^{125}$I-rh-EPO was administered in a dose of 0.1–125 µg/kg into the tail vein. The mice were bled to death via cardiac puncture under ether anesthesia 30 min after dosing. The spleen and femur were removed and weighed. Blood was transferred into a heparinized tube and a glass tube and centrifuged at 15,000 rpm for 3 min to obtain plasma and measure the hematocrit, respectively. Then 0.1 ml plasma and all the tissue obtained were counted directly by gamma counter. In the present study, blood space in the tissue was not washed before excising the tissue. Therefore, the radioactivity remaining in this space may affect the nonlinear portion of tissue uptake clearance.

**The effect of rh-EPO treatment.** Unlabeled rh-EPO was administered intravenously at a dose of 0, 0.48, 4.8, and 48 µg/kg once a day for 7 days. $^{125}$I-rh-EPO was administered at a dose of 0.48 µg/kg on the eighth day after the rh-EPO. Radioactivity in the bone marrow and spleen was measured 30 min after the administration of $^{125}$I-rh-EPO. Hct was measured on the seventh day after the dose of rh-EPO.

**Data Analysis.** A biexponential equation was fitted to the plasma concentration data using a nonlinear regression program MULTI (Yamaoka et al., 1995). The specific radioactivity was 2.6 $\mu$Ci/µg as determined by gel filtration assay. The radiochemical purity was 96.3% as determined by gel filtration.

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**Statistical Method.** Comparisons of pharmacokinetic, hematopoietic, and tissue uptake parameters were performed using a one-way analysis of variance followed by the Scheffe test. Statistical significance was taken as $p<0.05$.

**Results**

**Pharmacokinetics.** Fig. 1 shows the plasma concentration of TCA-precipitable radioactivity after intravenous administration of $^{125}$I-rh-EPO to +/+ and W/W$^v$ mice. Injected dose was 0.48 µg/kg. Solid lines were obtained by fitting using eq. 1.

Where $C_0$ and CLns are the initial plasma concentration and nonsaturable clearance, respectively. After repeated administration of nonlabeled rh-EPO, the rh-EPO remains in the body and can compete with $^{125}$I-rh-EPO. Therefore, the CLup calculated in this way is the apparent value (CLup, app). CLup was corrected using eq. 6 after the nonlabeled rh-EPO concentration (Cmin) was estimated using eq. 5.

The CLup by bone marrow was calculated by multiplying the CLup by the femur by 17, assuming that the femur contained approximately 6% of the total bone marrow (Loeffler and Wichmann, 1985).

**Fig. 1.** Plasma concentration of TCA-precipitable radioactivity after iv administration of $^{125}$I-rh-EPO to +/+ (■) and W/W$^v$ (●) mice. Injected dose was 0.48 µg/kg. Solid lines were obtained by fitting using eq. 1.

**Effect of Repeated rh-EPO Treatment.** Rh-EPO was administered to W/W$^v$ and +/+ mice once a day for 1 week. The Hct and CLup of $^{125}$I-rh-EPO by femur and spleen were estimated on the 8th day. In both W/W$^v$ and +/+ mice, rh-EPO treatment caused an
increase in Hct depending on the dose administered (fig. 3). At 0.48 μg/kg, Hct increased significantly in +/+ mice (1.2-fold that before treatment) (fig. 3). In W/Wv mice, a much larger dose of rh-EPO (4.8 μg/kg) was needed to produce a significant increase in Hct (1.16-fold that before treatment) (fig. 3). Thus, 10 times more rh-EPO was needed in W/Wv mice to obtain a similar pharmacological effect to that in +/+ mice (fig. 3). In +/+ mice, the CLup by the spleen increased significantly to 4-fold that of the control after treatment with rh-EPO 4.8 μg/kg while that by the femur remain unchanged irrespective of the dose (fig. 4). In W/Wv mice, the CLup by the femur increased significantly, but only to 1.5-fold of the control after treatment with rh-EPO 4.8 μg/kg and that by the spleen remained unchanged, irrespective of the dose (fig. 4). Thus, the degree of the increase in Hct and up-regulation of CLup in spleen was minimal in W/Wv mice compared with that in +/+ mice (fig. 4). Fig. 5 shows the relationship between Hct and the sum of CLup by bone marrow and spleen using the data from both types of mouse. There exists a statistically significant correlation (fig. 5).

Discussion

The most common treatment of aplastic anemia involves immunosuppressive therapy as well as bone marrow transplantation. However, the immunosuppressive therapy is not always effective for all patients with aplastic anemia. Recently, recombinant DNA technology has made it possible to use hematopoietic growth factors such as EPO and granulocyte colony stimulating factor. It has been reported that such treatment improves aplastic anemia (Hirashima et al., 1990; Musolino et al., 1994; Urabe et al., 1993). Also as an experimental model of aplastic anemia, the present study shows that treatment with rh-EPO produces a pharmacological effect (increase in Hct) in W/Wv mice (fig. 3). However, the pharmacokinetics of rh-EPO in patients with aplastic anemia and also in model animals was unknown. Here, we compared the pharmacokinetics of rh-EPO in W/Wv mice with that in normal mice.

There is no difference in CLtotal between the two strains (fig. 1 and table 1). This result suggests that the elimination of rh-EPO by both strains of mouse is similar and, in addition, gives an insight into the cause of the different endogenous plasma levels exhibited by the two strains: we measured endogenous EPO levels before rh-EPO treatment in both types of mouse. The level in W/Wv mice was approximately 1 ng/ml (data not shown), which was 10 times higher than that in +/+ mice. The endogenous EPO level (Cepo) can be described by the following equation:

\[
Cepo = \frac{\text{production rate}}{\text{CLtotal}}
\]

Therefore, the increased endogenous EPO level in W/Wv mice is not caused by a difference in its elimination but might be caused by an increased EPO production rate.

The Vc in W/Wv mice was greater than that in +/+ mice (67.5 and 52.3 ml/kg in W/Wv and +/+ mice, respectively) (table 1). We also calculated the central compartment distribution volume (Vb) based on the blood concentrations in both types of mouse and found it to be similar (97.8 ml/kg and 98.8 ml/kg in W/Wv and +/+ mice, respectively). The Hct in W/Wv and +/+ mice was 0.366 and 0.519, respectively. Thus, the Vc was nearly equal to Vb (1-Hct) in each type of mouse, indicating minimal distribution of rh-EPO into red blood cells. Therefore, the difference in Vc might be a result of the difference in plasma volume per body weight, which comes from the difference in Hct.

Our previous pharmacokinetic analysis demonstrated that RME by target organs such as bone marrow and spleen contributes to the saturable tissue uptake in rats (Kato et al., 1997). In this study, the CLup by target organs in both types of mouse exhibited clear saturation (fig. 2). The \(K_m\) value for both strains was similar and close to the Kd of the EPO-receptor (Kabaya et al., 1995) (table 2). Repeated administration of rh-EPO caused up-regulation of CLup by spleen in +/+ mice and by bone marrow in W/Wv mice (fig. 4). Our previous study (Kato et al., 1997) also demonstrated that the up-regulation of CLup by target organs (especially spleen) occurs after repeated administration of rh-EPO in rats, suggesting that RME contributes to the tissue uptake. Good correlations between the Hct and sum of the CLup by bone marrow and spleen were observed in both types of mouse (fig. 5). Moreover, the correlations in W/Wv and +/+ mice could be described by a single regression line (fig. 5). These results suggest that the pharmacological receptors govern the saturable tissue uptake not
the spleen results in up-regulation of CLup in the spleen (fig. 4). The
1993). Therefore, we can speculate that such an increase in CFU-E in
than that in bone marrow after rh-EPO administration (Nijhof
et al.
V
for such a reduction in
Hct in W/Wv mice was lower than that in
W/W
v mice was smaller than that in
V
comparable in both strains (table 2). On the other hand, the
by target organs between W/Wv and
W/W
v mice since the
1
max might be a smaller number of receptors
might reflect the low number of target cells in W/Wv mice.
TABLE 2
Kinetic parameters for tissue uptake clearance in +/+ and W/Wv mice

|        | K
\text{app} | V
\text{max} | CLns |
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>W/Wv</td>
<td>femur</td>
<td>10.0 (544 pM) ± 4.1 ng/ml</td>
<td>3.35 ± 1.17 ng/g tissue/hr</td>
</tr>
<tr>
<td></td>
<td>spleen</td>
<td>7.93 (431 pM) ± 8.14 ng/ml</td>
<td>2.01 ± 1.70 ng/g tissue/hr</td>
</tr>
<tr>
<td>+/+</td>
<td>femur</td>
<td>11.4 (619 pM) ± 4.2 ng/ml</td>
<td>7.80 ± 2.36 ng/g tissue/hr</td>
</tr>
<tr>
<td></td>
<td>spleen</td>
<td>7.15 (389 pM) ± 2.97 ng/ml</td>
<td>5.94 ± 1.88 ng/g tissue/hr</td>
</tr>
</tbody>
</table>

*mean ± calculated SD.

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only in normal mice but also in their anemic counterpart. There may
not be any difference in the affinity of receptor-mediated tissue uptake
by target organs between W/Wv and +/+ mice since the K
\text{m}
was
comparable in both strains (table 2). On the other hand, the V
\text{max}
max in W/Wv mice was smaller than that in +/+ mice (table 2). The reason
for such a reduction in V
\text{max}
max might be a smaller number of receptors
on the cell surface or a smaller number of target cells (CFU-E). The
Hct in W/Wv mice was lower than that in +/+ mice (fig. 3). This
might reflect the low number of target cells in W/Wv mice.

The number of CFU-E in spleen increased much more markedly
than that in bone marrow after rh-EPO administration (Nijhof et al.,
1993). Therefore, we can speculate that such an increase in CFU-E in
the spleen results in up-regulation of CLup in the spleen (fig. 4). The
differentiation of CFU-E to erythrocytes was stimulated by rh-EPO,
and the Hct subsequently increased. The slight increase in Hct in
W/Wv mice (fig. 3) might be a result of the slight increase in CFU-E
numbers in the spleen. Nijhof et al. (1993) reported that a redistribu-
tion of BFU-E from bone marrow to spleen occurs with rh-EPO and
conclude from the data that there is a change in CFU-E and BFU-E
numbers in bone marrow and spleen after treatment with rh-EPO.
There are two possible mechanisms for this discrepancy in the up-
regulation between each type of mouse according to Nijhof’s hypoth-
thesis: in one case, the cells cannot be released from bone marrow in
W/Wv mice; in the other, the released cells cannot remain in the
spleen. No reports support the former possibility, while some reports
support the latter: the stem cell factor is the ligand for c-kit. The c-kit
is a membrane-binding protein in stromal cells that is involved in
maintaining the microenvironment in hematopoietic tissues (Flanagan
et al., 1991). The binding of c-kit on normal bone marrow cells of
mast cells to stem cell factor activates cell-surface integrin causing
those cells to bind strongly to fibronectin (Kodama et al., 1994;
Dastych and Metcalf, 1994). However, the integrin of those cells in
W/Wv mice cannot be activated because of the mutation of c-kit in
W/Wv mice (Kinashi and Springer, 1994.). Therefore, the cells of
W/Wv mice are unable to remain in spleen. The present result is also
consistent with the hypothesis that c-kit may contribute to the up-
regulation of CLup in the spleen.

The pharmacokinetics of rh-EPO in rats and humans exhibited
nonlinearity. We have already reported that the nonlinear pharma-
kinetics of rh-EPO in rats might be a result of the saturation of tissue
uptake by bone marrow and spleen (Kato et al., 1997). The CLtotal of
rh-EPO in healthy human volunteers fell from 15 ml/kg/hr to 4
ml/kg/hr as the dose increased, suggesting that a saturable clearance
mechanism may predominantly contribute to the elimination of rh-
EPO in humans (Flaharty et al., 1990). We performed the infusion
study in rats at different infusion rates. The CLtotal at lowest (linear)
and highest (excess) infusion rates was 32.2 and 20.6 ml/hr/kg,
respectively (Kato et al., 1997). This result suggests that the contribu-
tion of saturable clearance to the CLtotal under the linear conditions
was, at most, 30% in rats. There is a species difference in the
contribution of saturable clearance to total body clearance between
rats and humans. In the present study using mice, the tissue uptake by
target organs also exhibited nonlinearity (fig. 2). Although there is a
difference in the V
\text{max} between the two mouse strains (table 2), there
is only a very small difference in CLtotal (fig. 1 and table 1). This
result can be explained if the contribution of saturable tissue uptake to
CLtotal is low in mice. In fact, the plasma concentration of 125I-rh-
EPO 30 min after iv administration of 125I-rh-EPO (0.1–125 μg/kg)
increased in proportion to the dose. Additionally, since the sum of
CLups by bone marrow and spleen in mice (18.5 ml/hr/kg) was 50%
in rats (38.5 ml/hr/kg), the contribution of saturable clearance to
CLtotal in mice might be smaller than that in rats. Thus, it is possible

Fig. 3. The effect of repeated administration of rh-EPO on Hct in +/+ (A) and
W/Wv (B) mice. Rh-EPO was administered to +/+ and W/Wv mice at doses of
0, 0.48, 4.8, and 48 μg/kg once a day for 7 days. Hct was measured on the 8th
day. Each bar represent mean and SE of three to five animals. The open bar
represents the Hct before rh-EPO treatment. The closed bar represent the Hct
after rh-EPO treatment.
that the pharmacokinetics of rh-EPO in mice may differ from that in humans.

A saturable clearance mechanism may predominantly contribute to the elimination of rh-EPO in humans (Flaharty et al., 1990). Although the pharmacokinetics of rh-EPO in patients with aplastic anemia have not been investigated, the saturable clearance in such patients might be smaller because of a reduction in the number of bone marrow cells. This might be one reason why plasma EPO concentrations in patients with aplastic anemia or myelodysplastic syndrome are higher than those in patients with other types of anemia (Hirashima et al., 1990). It has been reported that the plasma half-life of rh-EPO was greater as the endogenous EPO level was higher in patients with myelodysplastic syndrome (Browen et al., 1991), implying that endogenous EPO levels might be affected by the saturable clearance governed by a patient’s bone marrow cells.

We conclude that the pharmacological receptors for rh-EPO govern the saturable uptake by bone marrow and spleen in both +/- and W/Wv mice. The affinity of tissue uptake by target organs in W/Wv mice was similar to that in +/- mice, whereas the capacity of tissue uptake in W/Wv mice was lower than that in +/- mice.

References


