Evaluation of Phlebotomy-Induced Erythropoietin Production in the Dog

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ABSTRACT. Erythropoietin (EPO) production in dogs was studied by reducing red blood cells with phlebotomy. In this study, the hemoglobin reduction rate (Δ%Hb) was newly taken into account as the regulating factor for EPO production, and its usefulness to estimate the stimulating intensity to EPO production was examined. As the result, plasma EPO was highly correlated with Δ%Hb showing the importance related to regulation of EPO production, in the increasing plasma EPO by different degrees of phlebotomy, in the change of plasma EPO through the anemia progress and recovery period after severe phlebotomy, and in the initial variation of plasma EPO induced by chronic mild phlebotomy. On the other hand, increasing EPO production appeared at least within 6 hr after acute severe phlebotomy, which revealed significantly higher plasma level compared with the mild chronic phlebotomy, suggesting the effect of time leading to red blood cell reduction on EPO production response. Simultaneously, considering an in vivo EPO half life of 8.4 hr calculated from plasma EPO disappearance after bilateral nephrectomy, endogenous plasma EPO accumulation should be taken into consideration in rapidly increasing of Δ%Hb.—KEY WORDS: bioassay, dog, erythropoietin, hemoglobin reduction rate, phlebotomy.


It has been known that erythropoietin (EPO) plays the most important role in the mechanism of regulating red blood cell production and a reduction of red blood cell stimulates EPO production [1, 11-17, 29, 30]. From various studies on human anemic cases, it has been already clarified that plasma EPO level correlates with hemoglobin or hematocrit value [10, 30]. There are some works about EPO response to pathological conditions of the dog; hemorrhage [19, 21], polycthemia [25, 32], renal tumor [23, 26, 28], and canine cyclic hematopoiesis [8]. However, details in the relationship between the plasma EPO and red blood cells have been hardly studied, and the phenomenon of increasing EPO production under anemic state has not been sufficiently clarified at present. In addition, EPO production under anemic conditions in human beings has been little discussed in aspect of development or recovery of anemia, as well as rate of anemia progress. Further, there still remains a question whether plasma EPO value directly reflect the state of EPO production in the kidney or not, because the relationship between EPO accumulation into plasma and disappearance from plasma is equivocal.

Thus, EPO production reaction associated with the reduction of red blood cells is left unclarified because of difficulty in EPO assay in the dog. However, it has now been able to measure normal plasma EPO levels by the authors' studies [24], and may give a whole picture to evaluate EPO production.

In this study, the state of EPO production induced by phlebotomy was assessed by in vivo EPO bioassay reported previously [24]. One of major concerns was the significance of correlation of plasma EPO value with hemoglobin reduction rate (Δ%Hb), which was applicable to an indirectly suggestive regulating factor for EPO production. In addition to these evaluations, morphological changes of erythropoietic responses in bone marrow reflected on plasma EPO, the influence of reduction rate of red blood cells upon EPO production, and half life of endogenous EPO in plasma were studied.

MATERIALS AND METHODS

Animals: Forty-four adult mongrel dogs (23 males and 21 females, 1-8 years of age, weighing 7.0-18.0 kg) used for the experiments were clinically proved to be healthy.

Phlebotomies: In the phlebotomic treatment, blood was removed from jugular vein of each dog as quickly as possible. Four modes of acute phlebotomy referring to the grade of red blood cells reduction were applied. In the highest grade, Grade
1, an amount of blood equivalent to 2% of body weight was phlebotomized five times every 24 hr. The blood volume as much as 2% of body weight was phlebotomized three times every 12 hr in the mode of Grade 2, and that equivalent to 4% of body weight was done once in Grade 3. As the lowest grade, Grade 4, the blood volume equivalent to 2% of body weight was phlebotomized once. In these acute modes, a double volume of lactated Ringer's solution was transfused immediately after each phlebotomic treatment in order to keep the circulating blood volume at the initial level. On the other hand, as chronic mild phlebotomy, an amount of blood equivalent to 0.23% of body weight was phlebotomized 10 times every 24 hr.

Red blood cell parameters and plasma EPO values: The blood sample (10 ml, heparinized) was collected for measuring red blood cell parameters and plasma EPO value. Red blood cell count (RBC) was measured by using Micro Cell Counter (CC-130A, Sysmex, Kobe), hematocrit value (Ht) was measured by the microhematocrit method, and hemoglobin concentration (Hb) was measured by cyanmethemoglobin method. Plasma EPO value as in vivo bioactivity was determined by the transfused polycythemic mouse method as described in our previous study [24].

Bone marrow aspiration and examination: Bone marrow aspiration was performed by using a 18G injection needle at the site of the iliac crest in an anterior-dorsal angle, and EDTA (dipotassium ethylenediamine-tetraacetate; DOTITE 2K, Wako Pure Chemical Industries, Tokyo) was used as an anticoagulant. The prepared marrow smears were submitted to May-Grünewald-Giemsa staining, and myeloblasts and erythroblasts in 1,000 nucleated cells were counted under the optical microscope to calculate the myeloid : erythroid ratio (M/E).

Hemoglobin reduction rate (Δ%Hb): Hb value before phlebotomy was set as 100% and the Hb value after phlebotomy was converted into percentage. This differential percentage denoted Δ%Hb using as index of anemia.

Bilateral nephrectomy: After premedication with subcutaneous administration of atropine sulfate, the dogs were laparotomized under GOF inhalation anesthesia induced by intravenous administration of sodium thiopental. Both kidneys were removed by excision after ligation of the respective renal artery and vein with ureter.

RESULTS

Effect of phlebotomic grade on plasma EPO values: In four modes of acute phlebotomy (Grade 1 to 4, consisting of 5 animals in each groups), red blood cell parameters and plasma EPO values were measured before phlebotomy served to reference value and 48 hr after the accomplishment of phlebotomic procedure. Their results are shown in Table 1. In most severe anemic condition (Grade 1),

| Phlebotomic Grade | RBC (×10^6 / mm³) | Hb (g / dl) | Ht (%) | EPO (U / ml)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1</td>
<td>194.8±29.6</td>
<td>3.78±0.46</td>
<td>13.0±1.0</td>
<td>2.203±0.427</td>
</tr>
<tr>
<td></td>
<td>[71.5]</td>
<td>[72.2]</td>
<td>[70.7]</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>300.6±24.5</td>
<td>5.84±0.62</td>
<td>19.2±1.3</td>
<td>1.126±0.336</td>
</tr>
<tr>
<td></td>
<td>[56.8]</td>
<td>[57.0]</td>
<td>[56.1]</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>383.2±45.3</td>
<td>8.14±0.75</td>
<td>25.4±1.1</td>
<td>0.484±0.128</td>
</tr>
<tr>
<td></td>
<td>[41.8]</td>
<td>[41.6]</td>
<td>[41.2]</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>483.2±42.1</td>
<td>9.92±0.53</td>
<td>31.0±1.6</td>
<td>0.240±0.063</td>
</tr>
<tr>
<td></td>
<td>[25.8]</td>
<td>[25.8]</td>
<td>[25.8]</td>
<td></td>
</tr>
</tbody>
</table>

a) Grade 1. Phlebotomized 5 times 2% blood of body weight every 24 hr.
Grade 2. Phlebotomized 3 times 2% blood of body weight every 12 hr.
Grade 3. Phlebotomized once 4% blood of body weight.
Grade 4. Phlebotomized once 2% blood of body weight.
b) Red blood cell count.
c) Hemoglobin concentration.
d) Hematocrit value.
e) Plasma erythropoietin levels.
f) Values are expressed as mean±S.D. from five dogs.
g) The mean value of reduction rate; Δ% in bracket.
ERYTHROPOIETIN IN PHLEBOTOMIZED DOGS

Fig. 1. Relationships of plasma erythropoietin levels (EPO) with (a) hemoglobin concentrations (Hb) and (b) hemoglobin reduction rates (Δ%Hb) in 20 dogs phlebotomized with various degrees.

Fig. 2. Relationships of the mean plasma erythropoietin level (EPO) with (a) the mean hemoglobin concentration (Hb) and (b) the mean hemoglobin reduction rate (Δ%Hb) in 4 groups phlebotomized with different degrees.

Fig. 3. Changes of hemoglobin (Hb), myeloid : erythroid ratio (M/E), and plasma erythropoietin levels (EPO). Six phlebotomized dogs and 6 intact dogs (controls). Phlebotomy was performed three times every 12 hr as much as 2% of the body weight in each dog (△).

each red blood cell parameter extremely decreased, leading to less than 30% of reference levels. Concomitantly, plasma EPO was elevated to the mean value of 2.203 U/ml corresponding to the mean Δ%Hb of 72.2%. These changes in red blood cell parameters and plasma EPO values were well accordance with those in the more mild phlebotomic grades; the mean Δ%Hb and plasma EPO in Grade 2, 3 and 4 were 57.0% and 1.126 U/ml, 41.6% and 0.484 U/ml, and 25.8% and 0.240 U/ml, respectively. Plasma EPO was undetectable in any reference samples and below the lower limit of assay. Correlation between Hb and plasma EPO of all dogs in four phlebotomic groups was very high as shown in Fig. 1-(a); γ (correlation coefficient) = -0.968, Y = -0.15X + 0.92 (formula of regression line). Similarly, higher correlation between Δ%Hb and plasma EPO was recognized; γ = 0.991, Y = 0.02X - 1.20 (Fig. 1-(b)). When the correlation of the mean plasma EPO value with the mean Hb or the mean Δ%Hb in each phlebotomic group was studied, either correlation showed higher linearity (Fig. 2).

Changes of plasma EPO values associated with progress and recovery of anemia caused by severe phlebotomy: Providing 6 dogs phlebotomized by the acute mode of Grade 2 and 6 intact dogs as the control, blood sampling and bone marrow aspiration were performed every 6 hr for the first 3 days after phlebotomy, every 12 hr for the consecutive 4 days, and every 24 hr for the following 2 weeks. Red blood cell parameters (RBC, Ht, Hb), M/E and plasma EPO were investigated. Changes in Hb, M/E and plasma EPO following the phlebotomy were shown in Fig. 3. In Table 2-(a), plasma EPO values at 6 and 12 hr after the single phlebotomy in this follow up series were showed as the values of the initial changes in acute severe phlebotomy in order to compare with that in chronic mild phlebotomy.

The lowest level of red blood cell parameters was observed 18-24 hr after the completion of phlebotomy. Hb was 44.9% at 42 hr, which increased gradually and recovered to the original levels on the
Table 2. Effect of acute severe and chronic mild phlebotomy on red blood cell parameters and plasma erythropoietin levels

(a) an acutely phlebotomized dog\(^3\)

<table>
<thead>
<tr>
<th>Time of blood sampling (hr)</th>
<th>RBC (%)</th>
<th>Hb (%)</th>
<th>Ht (%)</th>
<th>mean Δ%Hb (%)</th>
<th>EPO (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>82.1±3.3</td>
<td>78.4±4.1</td>
<td>78.6±4.8</td>
<td>21.6</td>
<td>0.118±0.018</td>
</tr>
<tr>
<td>12</td>
<td>70.0±6.5</td>
<td>71.5±5.4</td>
<td>71.1±5.1</td>
<td>28.5</td>
<td>0.377±0.060</td>
</tr>
</tbody>
</table>

(b) a chronically phlebotomized dog\(^5\)

<table>
<thead>
<tr>
<th>Time of blood sampling (days)</th>
<th>RBC (%)</th>
<th>Hb (%)</th>
<th>Ht (%)</th>
<th>mean Δ%Hb (%)</th>
<th>EPO (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>79.4±0.9</td>
<td>77.9±2.2</td>
<td>77.4±3.0</td>
<td>22.1</td>
<td>0.065±0.006</td>
</tr>
<tr>
<td>8.5</td>
<td>75.6±2.2</td>
<td>75.7±3.4</td>
<td>75.2±2.8</td>
<td>24.3</td>
<td>0.074±0.011</td>
</tr>
<tr>
<td>9</td>
<td>73.8±1.7</td>
<td>75.0±2.7</td>
<td>72.9±2.5</td>
<td>25.0</td>
<td>0.095±0.015</td>
</tr>
<tr>
<td>9.5</td>
<td>72.2±2.2</td>
<td>73.4±1.8</td>
<td>71.5±2.1</td>
<td>26.6</td>
<td>0.174±0.115</td>
</tr>
<tr>
<td>10</td>
<td>70.4±0.4</td>
<td>71.9±2.0</td>
<td>68.4±1.8</td>
<td>28.1</td>
<td>0.224±0.115</td>
</tr>
</tbody>
</table>

\(^a\) Phlebotomized once 2% blood of body weight.
\(^b\) Phlebotomized ten times 0.23% blood of body weight every 24 hr.

14th-15th day. RBC and Ht also showed the same change as Hb (data not indicated). M/E was beginning to reduce at 6 hr after the completion of phlebotomy, and the highest erythropoietic response, 44.7%, was recognized 36 hr later. And then, it increased progressively, requiring about 15 days to recover to the original level. Plasma EPO level increased to the detectable level (≥0.05 U/ml) at 6 hr after primary phlebotomy and reached the highest level, 1.565 U/ml, within 24 hr following final phlebotomy. Thereafter, plasma EPO decreased gradually to undetectable level (<0.05 U/ml) on the 14th day. In the control group, there was no remarkable change in red blood cell parameters and M/E, and any plasma EPO was undetectable. The correlation of the mean plasma EPO values with the mean Hb or Δ%Hb values was studied in the both periods of the anemia progress (0-48 hr) and the anemia recovery (48 hr-21 day). The correlation coefficient for Hb was —0.962 in the anemia progress period, and —0.982 in the anemia recovery period. The correlation coefficients for Δ%Hb in anemia progress and recovery periods were 0.963 and 0.986, respectively (Fig. 4). In any cases, there was an intimate relationship between plasma EPO and Hb profile in which Δ%Hb is no less better than Hb.

Effect of chronic mild phlebotomic stimulation on plasma EPO: Six dogs phlebotomized chronically was provided and blood collection was performed at 12 hr after every phlebotomy for measuring red blood cell parameters and plasma EPO values. As indicated in Table 2-(b), red blood cells decreased to approximately 70% in 10 days. Plasma EPO in all
dogs reached to the measurable level only after 8 days (Fig. 5), and the mean value was 0.065 U/ml, the Hb decreased nearly to 80% at the time. After that, plasma EPO increased gradually to the mean value of 0.224 U/ml on the 10th day (Fig. 5). Table 2-(b) also shows plasma EPO values of five detectable samples (during 8–10 day) together with red blood cell parameters. Statistical correlation of the mean plasma EPO values with the mean Hb and Δ%Hb were correlation coefficient of −0.952 and 0.956 (Fig. 6). Correlation coefficient of Δ%Hb was also slightly higher than that of Hb.

The half life estimation of endogenous plasma

**Fig. 5.** Changes of hemoglobin (Hb) and plasma erythropoietin levels (EPO) in 6 dogs under chronic mild phlebotomy. Phlebotomy was performed ten times every 24 hr as much as 0.23% of the body weight in each dog (△).

**Fig. 6.** Relationships between the mean hemoglobin reduction rate (Δ%Hb) and the mean erythropoietin level (EPO) at the stage detectable EPO in 6 dogs under chronic mild phlebotomy.

**Fig. 7.** Changes of plasma erythropoietin levels in 6 anemic dogs after bilateral nephrectomy. Twenty-four hr before nephrectomy, each dog had been phlebotomized three times every 12 hr as much as 2% of the body weight.

**EPO:** Six dogs applied with the Grade 2 phlebotomy were submitted to bilateral nephrectomy 24 hr after the completion of phlebotomy to estimate the half life of EPO in plasma. The initial mean plasma EPO value of 0.976 U/ml reduced rapidly to 0.083 U/ml in 30 hr after the nephrectomy, and circulating plasma EPO was detectable only in 2 dogs at 36 hr and undetectable in all dogs at 42 hr (Fig. 7). On the basis of the disappearance of endogenous EPO from plasma, the half life was calculated to be 8.4±1.1 hr from the formula of exponential regression line in each dog.

**DISCUSSION**

It has been understood that EPO production in the kidney is stimulated by reduction of oxygen supply to the renal tissue, and there is generally a high correlation between Hb and plasma EPO [1, 12]. Such consideration has been established from the both aspects of the classical experimental facts on EPO-increasing response to red blood cell reduction in animals [4, 9, 19, 21] and the clinical data of plasma EPO obtained from numerous human patients with anemia [5, 6, 10, 12, 30, 31]. At present, plasma EPO kinetics to red blood cell reduction has not been sufficiently investigated in dogs, and details as to the correlation between Hb and plasma EPO have not yet been reported.

In this study, experimental anemia with various degrees was induced by means of phlebotomies. It is
suggested that the post-phlebotomic elevation of plasma EPO would reflect EPO production in the kidney because of its plasma half life (8.4 hr) and relatively long term retention in blood (Fig. 3). This EPO production intimately correlated with Hb or erythrocyte concentration (negative correlation) under various conditions; degree of anemia (Fig. 2), progression and recovery of acute severe anemia (Fig. 4), and chronic mild anemia (Fig. 6).

In anemic conditions induced by the phlebotomic treatment, it can be interpreted that oxygen supply to the kidney tissue is strongly restricted by Hb deficiency accompanying with little variation of tissue blood flow rate and blood oxygen partial pressure. RBC, Ht and Hb are used as routine indicators for anemia, but their levels are variable among individuals. To dissolve this problem, we introduced Δ%Hb as a more suitable index for the experimental anemia induced by phlebotomy, which has not been reported in the past experimental studies on erythropoietin production using phlebotomized animals. The correlation of plasma EPO with Δ%Hb showed higher coefficient than that with Hb through all experiments. Accordingly, Δ%Hb would be more meaningful control factor for EPO production.

It has well known that EPO production in the kidney is accelerated by decreasing of red blood cells and inhibited by increasing of them [1, 5, 6, 11, 12, 30]. Considering the variation of red blood cells the stimulation for EPO production under the anemic condition is thought to be accelerative in response to progressing anemia and moderated in the recovery period from anemia. In the comparative experiment using 4 anemic dog groups with different mode of phlebotomy, plasma EPO values were suggested to be nearly at the transitional state between progression and recovery in a series of anemia. The correlation of plasma EPO with Δ%Hb in this experiment was assessable without consideration for the both stimulative vectors of acceleration and inhibition. It seems, therefore, reasonable to presume that EPO production in the present study exclusively depended on the degree of reduction of red blood cells (anemic severity).

In the experiment following up anemia induced by acute severe phlebotomy, the relationship between plasma EPO and Δ%Hb was assessed about respective period of progressive anemia and its recovery to check their stimulatory effect upon EPO production. However, there was no difference in correlation of both periods. Plasma EPO values were maintained nearly at definite level corresponding with Δ%Hb irrespective of the stimulative vector.

Reduction rate of circulating red blood cells would play an important role as rate limiting factor in EPO synthesis. To assess this influence of the progressive rate of anemia within a limited time upon EPO production, the initial changes of plasma EPO values in acute severe phlebotomy were compared with that in chronic one. As the result, the initial plasma EPO levels in acute phlebotomy were significantly higher than that in chronic phlebotomy. Plasma EPO values in spite of the same anemic condition were different between these two anemia cases required rapid and moderate changes to reach to the same Δ%Hb level (20-30%). Accordingly, it was suggested that EPO production can be affected by the time relating to stimulative augmentation, even though EPO production is regulated by the same level of oxygen supply [1, 12].

Besides the time-relating effect of red blood cell reduction on EPO production, there still remained a problem related to accumulation and disappearance of circulating EPO. EPO productive response would be induced in a short time, and the detectable plasma level, 10-fold the normal plasma level estimated previously [24], appeared within 6 hr following the initial phlebotomy. If plasma EPO creatance is kept at a definite level, accumulation of EPO into plasma during rapidly progressing anemia was thought to be more effective than that during moderately progressing anemia. In this meaning, the half life of plasma EPO was examined by using bilaterally nephrectomized dogs. Since it has been known that plasma EPO in the dog exclusively originated from the kidney [18, 19, 22], EPO production can be eliminated by bilateral nephrectomy, and plasma EPO disappears by inactivation or degradation in the body without excretion into urine. Because urine EPO levels is high in the anemic state [20] and EPO is inactivated rapidly in urine [7], the intrinsic half life of plasma EPO in anemic condition may be shorter than that of the nephrectomized animal. There are some reports that the plasma half life of EPO was 7-10 hr and EPO excretion rate into urine was 2-5% when human EPO was administrated to normal dogs [3, 33]. Endogenous plasma half life of EPO obtained from the present experiment can at least neglect the urinary excretion and is assumed to be reasonable value in the light of the other data. On the basis of
this half life, the plasma EPO values different to the same Δ%Hb levels in anemia progress following acute and chronic phlebotomy were considered partly due to the effect of plasma EPO accumulation.

Thus, in this study, EPO kinetics was studied by using phlebotomies as the stimulation. On the other hand, the erythropoietic response to the EPO in bone marrow was also assessed additionally. In the dogs subjected to acute severe phlebotomy, the erythropoietic response was recognized as decreased M/E in 18–24 hr delaying from the increasing inclination of plasma EPO correlated with decreased Hb (Fig. 3). Such time lag between enhanced plasma EPO and decreased M/E corresponded with the time required for erythroblastic proliferation established by cultured marrow cells [2, 27]. As in vitro experiment, it has been demonstrated that red blood cell differentiation in dogs initiates morphologically at the concentration of EPO above 0.05 U/ml and requires more than 48 hr for the proliferation in cultured marrow cells [2]. M/E findings obtained from present study was well coincident with those in vitro.

REFERENCES


