Effects of Recombinant Human Erythropoietin on Porphyrin Metabolism in Uremic Patients on Hemodialysis

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ABSTRACT

Recombinant human erythropoietin (r-HuEPO) is being successfully used for the treatment of uremic anemia. Several abnormalities of heme biosynthetic pathway have been described in patients with end-stage renal failure. In this condition, the activity of erythrocyte porphobilinogen deaminase has been found to be slightly increased. If this enzyme were to be the key enzyme in erythroid heme regulation, its activity would be increased to an even greater degree during the correction of uremic anemia. To assess this hypothesis, this study followed the variations of this and other parameters of porphyrin metabolism over 12 months of erythropoietin therapy in eight patients with nephrogenic anemia who underwent hemodialysis. By the first month of therapy, an increase of the previously depressed erythrocyte activity of aminolevulinate dehydratase was already evident, in coincidence with a nonsignificant increase of the reticulocyte count. The activity of this enzyme reached its maximal level by Month 3, and did not change up to Month 10. The porphobilinogen deaminase hyperactivity normalized at Month 4. By Month 12, in coincidence with the reduction of erythropoietin doses, the maximal levels of erythrocyte protoporphyrin, and the decrease in aminolevulinate dehydratase activity, the porphobilinogen deaminase values started to increase once again. In conclusion, the administration of r-HuEPO to hemodialyzed patients induced transient normalization of the previously observed porphyrin metabolism abnormalities. However, erythrocyte porphobilinogen deaminase activity did not rise concomitantly with the increase in hematocrit or hemoglobin values, but it did diminish during treatment. Therefore, porphobilinogen deaminase did not behave as a controlling enzyme in heme synthesis during the r-HuEPO-induced correction of uremic anemia.

Key Words: End-stage renal failure, uremic anemia (therapy), heme biosynthesis pathway, porphobilinogen deaminase, aminolevulinate dehydratase

Anemia markedly contributes to morbidity in patients with chronic renal failure (CRF) who undergo hemodialysis therapy. Although the pathogenesis of this anemia is multifactorial, inadequate erythropoietin (EPO) secretion secondary to damage of the renal secretory site(s) is probably its most important cause (1–3).

The availability of recombinant human erythropoietin (r-HuEPO) has opened new perspectives in the management of CRF-associated anemia (1,4), yet little is known regarding the effects of this glycoprotein on heme biosynthesis.

In patients with CRF, and particularly in those on hemodialysis, a number of derangements in the heme biosynthesis pathway have been reported (5–8), which might in theory be related to the nephrogenic form of anemia. However, studies in which the actions of r-HuEPO on porphyrin metabolism and on the hematological parameters are assessed simultaneously are very scarce, and such studies have never exceeded an observation period of 3 months.

Porphobilinogen deaminase (PBG-D) may be considered to be the primary enzyme controlling heme synthesis during EPO-induced normal erythroid differentiation (9). However, the erythrocyte activity of this enzyme is generally increased in patients with CRF (7–8), although this increase is insufficient to prevent or avoid the hemoglobin deficit. If PBG-D were to be the key enzymatic activity in the regulation of erythroid porphyrin synthesis, then its activity—in erythrocytes—should increase in parallel with the EPO-induced increment in hemoglobin (Hb) concentration. To examine this hypothesis, the work presented here was designed to assess, over an extensive period of time, the evolution of the porphyrin synthesis derangements during partial correction of anemia with r-HuEPO in a group of CRF patients on hemodialysis.

PATIENTS AND METHODS

We studied the evolution of some hematologic and porphyrin parameters over 13 months of r-HuEPO therapy in eight patients (five women; age range, 27 to 80 yr; median, 57 yr) with CRF who were enrolled in a periodic hemodialysis
program (median, 71 months; range, 8 to 154 months). The residual diuresis was less than 50 mL/day in all cases, and the hematocrit level did not exceed 28% in any patient.

R-HuEPO was administered IV at each of the three weekly dialysis sessions. The dosage was 40 U/kg per session during the first 6 wk, and was thereafter titrated to the needs of each patient to maintain hematocrit values at between 30 and 35%.

This therapy was interrupted on Month 6 and definitively suspended on Month 7 in one case because of a cerebrovascular accident. In addition, one patient successfully underwent renal transplantation on Month 11, and was withdrawn from the study at that time.

Fifty-two healthy subjects (16 women) aged between 17 and 76 yr (median, 48 yr) formed the control group.

Blood was collected at monthly intervals for assessment of the porphyrin and hematologic parameters, and the iron and ferritin concentrations were measured every 3 months in each patient.

We assessed the red blood cell (RBC) activities of aminolevulinic acid dehydratase (ALA-D, EC 4.2.1.24; μmol ALA-D/L RBC min⁻¹) and porphobilinogen deaminase (PBG-D, EC 4.3.1.8; μmol URO/MM RBC h⁻¹), as well as the porphyrin concentrations in plasma and red blood cells (μg/dL) (12). The zinc (Zn) protoporphyrin/free protoporphyrin ratios were estimated by fluorometric scanning after ethanol extraction (13). Also, the ALA-D activity was restored by addition of Zn and dithiothreitol (DTT) to the incubation medium (restored ALA-D) (14); the levels of restored ALA-D activity may be held to represent the full expression of the enzyme activity in RBC.

To assess the capability of uremic plasma to inhibit the ALA-D activity, RBC from the heparinized blood of control subjects were first washed twice with normal saline; equal amounts of washed erythrocytes and uremic plasma were then mixed and preincubated for 20 min at 37°C, and the ALA-D activity was then quantitated by the standardized European method (10). The inhibition of ALA-D was expressed as the percentage of the activity measured in washed control RBC preincubated with their own plasma.

The measurements of hemoglobin, hematocrit, and cell count and volume were carried out in a Technicon H-11 System autoanalyzer (Technicon Instruments Co., Tarrytown, NY), and those of creatinine and BUN in a Ciba-Corning 550 Express analyzer (Ciba-Corning Diagnostics Corporation, Medfield, MA). The ferritin concentrations were quantified by immunoenzymatic methods (Tanden®-E-Ferritin; Hybritech, Liège, Belgium).

At the beginning and at the end of the study, the plasma Zn concentrations were measured by atomic absorption spectrophotometry (15).

The results are expressed as medians, this being the most appropriate measure of the central trend, considering the variability in the r-HuEPO requirements and the number of patients, and compared with the baseline values. A repeated-measurement analysis of variance with the data previously normalized through Log(1+X) was used for sample comparison except in the case of the plasma Zn concentrations, for which the nonparametric Wilcoxon’s test was used. Differences were considered statistically significant if the P value was 0.05 or lower. Tests of the Spearman (nonparametric) correlation were used to assess the association between pair of variables.

RESULTS

All of the patients responded to r-HuEPO therapy with a progressive increase of their hematocrit and hemoglobin levels (Table 1). The transfusion requirements dropped from 30 in the year preceding the r-HuEPO treatment to just five in the course of the study.

The increase in the reticulocyte count was prompt (30 days); the values were highest by the third month, and decreased toward the baseline values from the following month onward. The correlation between this parameter and the r-HuEPO dose was very weak (r = 0.38, P < 0.05).

The ferritin concentrations, which showed a great dispersion at baseline (mean, 297 ± 344 ng/dL), showed no significant changes with treatment, even though some patients also received iron supplementation.

Collectively, the patients exhibited baseline ALA-D hypoactivity with compensatory PBG-D hyperactivity and increased plasma and erythrocyte porphyrins, as compared with the control subjects (Table 2). The time course of the studied parameters of porphyrin metabolism is shown in Table 2.

**TABLE 1. Evolution of hematological data in eight patients on hemodialysis during r-HuEPO treatment**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basal</th>
<th>Month 1</th>
<th>Month 2</th>
<th>Month 3</th>
<th>Month 6</th>
<th>Month 9</th>
<th>Month 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose r-HuEPO (U/kg per session)</td>
<td>0 (0 to 0)</td>
<td>(40 to 40)</td>
<td>(80 to 80)</td>
<td>(80 to 80)</td>
<td>(45 to 120)</td>
<td>(45 to 120)</td>
<td>(20 to 100)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>23.5 (21.6 to 28)</td>
<td>24.6 (20 to 28)</td>
<td>28.2b (21.6 to 31)</td>
<td>29.6c (25 to 36)</td>
<td>31.4 (21 to 39)</td>
<td>34.0c (32 to 39.5)</td>
<td>30.6b (25.6 to 40)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>8.0 (6.9 to 9.2)</td>
<td>7.7 (6.3 to 9.4)</td>
<td>8.9 (6.3 to 9.8)</td>
<td>9.3b (8.4 to 11)</td>
<td>10.6b (7 to 13.5)</td>
<td>11.7c (10.5 to 14)</td>
<td>10.3d (8.4 to 13.3)</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>11 (8 to 23)</td>
<td>19 (11 to 26)</td>
<td>21 (13 to 25)</td>
<td>26b (11 to 36)</td>
<td>8 (2 to 25)</td>
<td>9 (5 to 30)</td>
<td>10 (9 to 39)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>91.6 (84 to 109)</td>
<td>89.2 (83 to 107)</td>
<td>95.6 (83 to 108)</td>
<td>94.5 (89 to 105)</td>
<td>91.6 (86 to 111)</td>
<td>94.0 (90 to 112)</td>
<td>92.0 (82 to 109)</td>
</tr>
</tbody>
</table>

a Data are median (range).

b P < 0.05.

c P < 0.01 versus basal (Month 0).
By the first month of r-HuEPO therapy, a significant increase of the ALA-D activity was already evident, in coincidence with a (nonsignificant) increase in the reticulocyte count. The activity of this enzyme reached its maximum levels by Month 3, and remained stable up to Month 10. The erythrocyte ALA-D values did not correlate with any hematological parameter.

The variations observed in the activity of this erythrocyte enzyme were no longer evident upon in vitro restoration by the addition of Zn and DTT. The assessment of ALA-D activity by this latter method showed a significant increase by Month 1, followed by a return to baseline values by Month 6, which persisted to the end of the study period. The dose of r-HuEPO correlated weakly with both the ALA-D and restored ALA-D activities (r = 0.48 and 0.39; P < 0.05, respectively). The ability of the unemic plasma to inhibit ALA-D activity in vitro was not modified throughout the study.

Therapy with r-HuEPO normalized the PBG-D hyperactivity as from Month 4 (Figure 1). By Month 12, and in coincidence with the reduction in the dose of r-HuEPO and the decrease in ALA-D activity, the PBG-D activity again started to increase (Figure 1, Table 2). There was no correlation between the activities of both enzymes.

The PBG-D activity correlated weakly and inversely with the hemoglobin (r = -0.44, P < 0.05) and hematocrit (r = -0.41, P < 0.05) values and also with the Zn protoporphyrin/free protoporphyrin ratio (r = -0.53, P < 0.05), which evidenced a trend toward normality during r-HuEPO therapy.

After an initial increase, which reached its maximum by Month 2, the erythrocyte free protoporphyrin progressively decreased in the following months even to below the baseline levels at Month 9 and then increased again, in parallel with the reduction of ALA-D activity and PBG-D hyperactivity (Figure 1). Differences only have statistical significance at the times of the maximal increments (Months 2 and 12). The protoporphyrin levels correlated weakly with the plasma porphyrins (r = 0.44, P < 0.05), but without statistical significance in the latter.

The serum Zn concentrations, measured at the beginning (median 55; range, 47 to 72 µg/dL) and at the end (median, 60; range, 44 to 78 µg/dL) of the study, did not change significantly with r-HuEPO treatment.

**DISCUSSION**

The evolution of the hematological parameters in our patients is similar to that described in the already extensive literature about the correction of nephroanemic anemia with r-HuEPO (16). On the other hand, r-HuEPO treatment induced modifications of some parameters of porphyrin metabolism.

The ALA-D activity was the first one of the studied parameters to change toward normal levels. It has been reported that patients on hemodialysis develop

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**TABLE 2. Evolution of heme pathway parameters in eight patients on hemodialysis during r-HuEPO treatment**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Basal</th>
<th>Month 1</th>
<th>Month 2</th>
<th>Month 3</th>
<th>Month 6</th>
<th>Month 9</th>
<th>Month 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA-D (µmol ALA/L RBC/min)</td>
<td>12.6</td>
<td>16.5b</td>
<td>19.2b</td>
<td>20.3b</td>
<td>19.7b</td>
<td>18.4b</td>
<td>12.8</td>
</tr>
<tr>
<td>Restored ALA-D (µmol ALA/L RBC/min)</td>
<td>(6.4 to 18.8)</td>
<td>(14 to 29.6)</td>
<td>(8.2 to 31)</td>
<td>(13 to 26.5)</td>
<td>(16 to 28.6)</td>
<td>(8.4 to 27.2)</td>
<td>(7.3 to 24)</td>
</tr>
<tr>
<td>PGA-D (nmol URO/mL RBC/h)</td>
<td>36.0</td>
<td>33.0</td>
<td>32.0</td>
<td>31.0</td>
<td>30.0</td>
<td>29.0</td>
<td>28.0</td>
</tr>
<tr>
<td>Zn/Proto/Free Proto (µg/dL)</td>
<td>36.6</td>
<td>43.6</td>
<td>50.6</td>
<td>55.6</td>
<td>55.6</td>
<td>50.6</td>
<td>32.6</td>
</tr>
<tr>
<td>Plasma Porphyrins (µg/dL)</td>
<td>1.7</td>
<td>2.1</td>
<td>2.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>In Vitro ALA-D Inhibition (%)</td>
<td>-14.9</td>
<td>-12.2</td>
<td>-10.1</td>
<td>-12.9</td>
<td>-11.9</td>
<td>-11.8</td>
<td>-10.1</td>
</tr>
</tbody>
</table>

Data in square brackets are those of the control group. Data are expressed median (range). ALA-D, aminolevulinate dehydratase; PBG-D, porphobilinogen deaminase; Proto, protoporphyrin.
Figure 1. ALA-D (µmol ALA/L Ht/min) and PBG-D (µmol URO/ml RBC/h) and erythrocyte protoporphyrin concentration (µg/dL) in eight CRF patients on dialysis during r-HuEPO therapy. The r-HuEPO dose is expressed in U/kg per session.

low blood Zn concentrations, which could be related to their ALA-D hypoactivity (5). Hosokawa and Yoshida (17) observed a significant increase of the serum Zn concentration in patients treated with r-HuEPO. However, in our study, the serum Zn concentrations measured at the beginning and at the end of the study showed no changes; it can thus be speculated that the increase in ALA-D activity is not related to variations in serum Zn, but rather to a greater youth of the patients’ erythrocyte population and/or to the direct action of the glycoprotein. The lack of a significant correlation between the reticulocyte count and the ALA-D activity is not surprising, as it is well known that r-HuEPO administration causes an early but transient increase of reticulocytes, which later stabilizes at intermediate levels (18), the early response not being strictly proportional to the dose of r-HuEPO (19). Beguin et al. (18) showed that this glycoprotein induces a slow and gradual expansion of erythropoietic activity (as measured by serum transferrin receptor levels) that then reaches a plateau phase, returning to baseline values when r-HuEPO is discontinued.

It has been observed in experimental situations that juvenile erythroid cells are endowed with greater enzymatic activity than are more mature ones, which gradually lose it as they age (20). A striking finding, however, is that of Leber et al. (21), who demonstrated that the ALA-D activity in the reticulocytes of patients on hemodialysis was only 20% of that observed in healthy subjects.

As previously observed (8), the restored (with Zn and DTT), ALA-D erythrocyte activity was low in our CRF patients. Because this parameter may be considered to represent the full expression of the enzyme activity present in RBC, the low values of restored ALA-D suggest that, besides an inhibition of enzyme catalytic activity, the enzyme synthesis is also partially defective in CRF. R-HuEPO therapy only induced a moderate and transient increase of restored ALA-D by Month 3. These results suggest that the increase in the ALA-D activity might have been the result of an activating effect of r-HuEPO, the enzyme synthesis having been stimulated only slightly.

This activating effect has been observed by other investigators. Kraupp et al. (22) reported a marked increase (approximately threefold) in the activity of this enzyme in just 27 days of therapy; unfortunately, the lack of data on the dosages used in their work preclude comparison with our own results. Djordjevic et al. (23) reported that the administration to four patients of 50 U/kg per session for 8 wk increased the ALA-D activity to within the normality limits of their laboratory.

The ALA-D activity did not correlate with any hemato logical parameter. Because ALA-D is not a rate-limiting enzyme for heme biosynthesis, the effectiveness of r-HuEPO in correcting the anemia of CRF should not be attributed or related to the observed modifications in ALA-D activities. In fact, throughout the study, the uremic plasma maintained the same capability to inhibit the ALA-D activity of control RBC. Thus, the presence of such an ALA-D inhibitor in the plasma of CRF patients (8) helps to explain the decreased activity of erythrocyte ALA-D, but it should not be related to the pathogenesis of renal anemia. It might be interesting to assess the presumptive effects of r-HuEPO on the plasma concentrations of substances such as polyamines, furan carboxylic acid, or “middle molecules.”

The control of heme biosynthesis in erythroid cells is regulated differently from that in hepatocytes, in which aminolevulinic acid synthase is the rate-limiting enzyme (9). PBG-D appears to be the key enzyme during in vitro EPO-induced erythroid differentiation of both normal and suppressed marrow cells (24,25). Therefore, an increase in PBG-D activity should be expected during EPO-treatment of patients with CRF. In fact, Rosenlöf et al. (26) observed an increase in erythrocyte PBG-D activity 2 wk after the institution of r-HuEPO therapy (310 U/kg per wk). In comparison with the control subjects, our patients exhibited a twofold increase of PBG-D over the baseline values. This finding may reflect a compensatory mechanism for ALA-D hypoactivity (8).

During r-HuEPO administration, PBG-D activity showed a tendency to rise further by Month 1 (no significant difference versus basal values), but dropped to normal levels as from the third month. At the end of the study (when the median r-HuEPO dose was only 20 U/Kg per session), the decrease in the ALA-D activity was also associated with a return of the PBG-D hyperactivity. Thus, the correction—perhaps normalization—of the PBG-D hyperactivity during r-HuEPO treatment seems to be merely a secondary event to the induction of ALA-D, but does not represent the behavior of a key enzyme regulating
EPO-induced heme synthesis. In this latter case, the evolution of the PBG-D levels should have followed a course parallel to those of the hemoglobin and hematocrit levels. On the contrary, and surprisingly, there is a weak but significant inverse correlation between enzymatic activity and hematocrit levels.

The erythrocyte protoporphyrin levels increased even further after 2 months of therapy, possibly as a consequence of the stimulus to erythropoiesis and to porphyrin synthesis. Later on, and in parallel with the increase in the hemoglobin concentration and to the normalization of the PBG-D activity, a decrease was seen in the protoporphyrin concentration, followed by a new rise toward the end of the study with the return of the ALA-D and PBG-D activities to their baseline levels. Piazza et al. (27) reported a decrease of the high concentrations of erythrocyte free protoporphyrin after 3 months on r-HuEPO therapy. This reduction in free protoporphyrin may be the result of an increase in the activity of the enzyme ferrochelatase (26), which would allow increased incorporation of iron to the protoporphyrin ring and thus reduce the amounts of this porphyrin. However, in vitro, ferrochelatase was not modified by EPO in suppressed marrow cells (24).

On the other hand, it is known that periodic administration of r-HuEPO increases the rate of iron incorporation to erythroid cells (1); once it is within the cells, iron is reduced and incorporated to the protoporphyrin IX molecule to form heme. Free protoporphyrin is thus consumed, leading to a rise in the Zn-protoporphyrin/free protoporphyrin ratio.

R-HuEPO did not change the concentrations of plasma porphyrins, the accumulation of which is the result of the impossibility of their urinary elimination and to the scarce effectiveness of conventional hemodialysis in removing these substances (28). Fortunately, the porphyrin concentration does not become excessively high in chronic renal patients (8), though there have been some reports of a bullous dermatosis quite similar to that seen in porphyria cutanea tarda (29); the pathogenesis of this skin lesion, however, is still obscure.

To summarize, the administration of r-HuEPO to CRF patients on periodic hemodialysis induced transient normalization of the previously observed porphyrin metabolism abnormalities. Erythrocyte PBG-D activity did not rise in a parallel fashion with the increase in hematocrit or hemoglobin values; the PBG-D values even decreased in the course of therapy. Therefore, PBG-D did not behave as a primary enzyme that controls heme synthesis during the r-HuEPO-induced correction of uremic anemia.

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