The impact of early normalization of haematocrit by erythropoietin on renal damage in the remnant kidney model

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Abstract

**Background.** Correction of anaemia in moderate to advanced renal failure is still a matter of debate because of postulated detrimental effects of erythropoietin on the progression of renal damage.

**Methods.** The renal effects of early normalization of haematocrit (Htc) by erythropoietin (rHuEpo) were investigated from the time of 5/6 nephrectomy up to 8 weeks post-intervention in three groups of remnant kidney model rats: untreated controls (CON), rats receiving 100 UI/kg body-wt of rHuEpo i.p. twice a week (EPO), and rats receiving rHuEpo in which periodic phlebotomies maintained Htc similar to the value of the control group (PHL). The latter group was included to evaluate the direct effects of rHuEpo on renal damage, i.e. independent from Htc correction.

**Results.** Two weeks after renal ablation (basal), Htc decreased in CON and PHL rats (from 49.3±1.4% to 43.2±1.1, P<0.05 and from 49.6±1.1 to 43.3±1.5%, P<0.05 respectively), while it remained consistently normal in EPO rats (48.9±1.2% to 48.9±1.5%, P<0.05 vs other groups). Thereafter Htc did not change throughout the remaining period in all groups. At the end of the study, with respect to basal, resting blood pressure increased significantly by the same extent in CON (+13±2%) and EPO rats (+15±5%), while it remained constant in PHL rats. Notably, creatinine clearance significantly decreased in CON (−53±8% vs basal) and EPO (−38±8% vs basal), while it did not change in PHL rats. Likewise the degree of proteinuria as well as renal morphologic alterations and glomerular hypertrophy/sclerosis was similar in CON and EPO rats, and was significantly more severe than in the phlebotomized group. The only difference detected between CON and EPO group was the greater mesangial hypercellularity in rHuEpo-treated rats.

**Conclusion.** In uraemic rats, chronic treatment with rHuEpo aimed at normalization of Htc beginning the early stage of renal failure does not inevitably account for a rise in systemic blood pressure. In addition, neither erythropoietin per se nor the correction of haematocrit accelerates the progression of renal damage when blood pressure remains constant.

**Key words:** erythropoietin; haematocrit; morphometry; progression of renal failure; proteinuria; remnant kidney model rats

Introduction

In uraemic predialysis patients, treatment with erythropoietin (rHuEpo) is efficacious in correcting anaemia and its related symptoms, and therefore in improving the quality of life [1–4]. Importantly, preliminary evidence suggests that correction of uraemic anaemia diminishes the risk of death for cardiovascular disease; anaemia, in fact, is a main determinant of cardiac hypertrophy in chronic renal failure (CRF) [5,6]. Therefore it has been proposed to extend the use of erythropoietin to CRF of moderate degree and to administer this drug at doses which correct haematocrit completely [7–9].

Such an intriguing new frontier of rHuEpo therapy is matter of debate because of the controversial data suggesting that this approach leads to the progression of renal damage. Indeed, clinical studies have indicated that rHuEpo treatment does not worsen, but eventually slows, the decline of renal function in predialysis patients [2,3,10–13]. Most of these studies, however, are limited not only by a short observation period but also by the advanced stage of renal failure in which the effect of rHuEpo was evaluated. Indeed, the glomerular and interstitial sclerosis, extensively present in advanced renal failure [14], does not allow the correct assessment of the potential influence of erythropoietin on renal damage. On the contrary, this critical issue should be addressed in renal failure of moderate degree, that is, a condition preceding extensive glomerular sclerosis and obsolescence.

The issue becomes even more puzzling because experimental studies indicate that erythropoietin accel-
erates the progression of CRF. Specifically, it has been hypothesized that anaemia has a protective role on residual renal function, while correction of haematocrit enhances renal injury [15, 16]. In these studies, however, a major hypertensive effect of erythropoietin was observed which possibly influenced the outcome of renal function. In addition, further detrimental effects of erythropoietin may occur as a consequence of its recently identified broad cellular proliferative activity; including stimulation of the red cell-lineage, other marrow progenitors and non-haematopoietic cells [17–19], and induction of the release of fetal proteins [20]. Under this view, erythropoietin may also accelerate glomerulosclerosis by directly stimulating the proliferation of renal cells. To date, this hypothesis remains unexplored.

The aims of the study include: (i) assessment of both the structural and functional effects of chronic rHuEpo administration at doses able to maintain the haematocrit in the normal range since the initial stage of disease, and (ii) discernment between possible direct and indirect effects of rHuEpo on the progression of renal insufficiency. Our studies were performed in remnant kidney model rats, untreated and treated with rHuEpo from the time of 5/6 nephrectomy, and followed for a prolonged period of 8 weeks. In order to investigate the renal effects of erythropoietin independently from the correction of haematocrit, a third group of remnant rats underwent rHuEpo treatment while receiving periodic phlebotomies to maintain a persistently reduced haematocrit.

Subjects and methods

Experimental model

Twenty-seven male Sprague–Dawley rats at an average initial weight of 301 ± 7 g were anaesthetized (Nembutal 40 mg/kg BW, i.p.), placed on a heated table and the right kidney removed after flank incision (3/6 nephrectomy). Nine of these kidneys were randomly chosen and used for morphological and morphometric analysis as normal kidneys. After 2 weeks, 5/6 nephrectomy was completed by ligating two or three branches of the left renal artery, thus infarcting 2/3 of the residual kidney. Although the infarction model is characterized by hypertensive renal injury [21], it was preferable to the other model of CRF, that is, the surgical excision of renal mass, because of the earlier development of the typical changes of human CRF such as proteinuria and glomerulosclerosis [22].

Groups

After subtotal nephrectomy, rats were randomly assigned to one of the three experimental groups and studied for 8 weeks. Rats of CON group (n = 9) were uraemic untreated controls, receiving sham-injection. The EPO rats (n = 9) received 100 UI/kg BW of rHuEpo i.p. twice a week (Dompé Biotec, Milan, Italy) from the first day after renal artery ligation. This was the minimal dosage of erythropoietin required to correct the haematocrit to normal values according to preliminary studies (n = 8), in which lower doses of 50 and 75 UI/kg BW did not normalize haematocrit. The rats in the PHL group (n = 9) received the drug as in the EPO group; Htc was measured weekly, starting from the time of renal artery ligation, and periodic phlebotomies of 0.5–1.5 ml/week were performed from the tail-vein to maintain haematocrit levels equal to those of the untreated control rats. All rats received iron sulphate supplementation (1 mg/100 g BW/day in tap water).

Measurements

Systolic blood pressure was measured by the tail-cuff method prior to blood sampling in all rats and/or bleeding in the PHL group (BP recorder W-4 W Electronic 8005 Base, Como, Italy), as previously described [23]. Briefly, the animals were pre-warmed in a temperature and time-controlled system and then each rat was confined in a heated cage and the tail-cuff was connected to the recorder. Each blood pressure measurement represents the average of five consecutive values (variance coefficient, range 1–3%).

The assessment of creatinine clearance and proteinuria was performed after blood pressure measurement. Rats were housed in individual metabolic cages at temperature-controlled conditions (25–26 °C), with a standard pellet diet (protein content: 14 g/100 g as casein; NaCl 782 mg/100 g) and tap water ad libitum. After 48 h of acclimation, three consecutive 24-h complete urine collections were obtained and both creatinine and protein urinary concentrations were measured. At the end of the third 24-h period, a tail vein blood sample was withdrawn to determine plasma creatinine level and the haematocrit value. Each value of creatinine clearance and proteinuria is the mean of three consecutive daily measurements (variance coefficient, range: 24-h urinary creatinine 4–19%; 24-h urinary protein 5–18%). Creatinine clearance values are per 100 g of body-weight. Each haematocrit measurement is the average of five consecutive determinations (variance coefficient, range 0.5–2%).

Systolic blood pressure, creatinine clearance, proteinuria and haematocrit were measured in the normal state (initial value), 2 weeks after 5/6 nephrectomy (baseline value of chronic renal failure) and, then, every 2 weeks for 6 more weeks.

Analytic procedures

Blood and urine creatinine levels were assessed by the Jaffé method, using an autoanalyser (Creatinine Analyzer 2, Beckman, Fullerton, California), and creatinine clearance was calculated by standard formula. Urine protein concentration was measured by phosphoric acid (55%) and methanol (15%) protein assay (Bio-Rad, Munchen, Germany), detecting the absorbance with a spectronic 301 spectrophotometer (Milton Roy, New York) at a wavelength of 595 nm. For haematocrit determinations, NH₄ heparinized microtubes (Brand, Germany) and micro-centrifuge (Bicasa, Sesto S.Giovanni, Italy) at 15 000 r.p.m. were used.

Microscopic analysis

After the 8-week-study period, rats were anaesthetized with Nembutal (40 mg/kg BW i.p.) and the remnant kidney was excised and processed for histological analysis. Two coronal sections of the kidneys were cut, plastic-embedded and processed by using conventional techniques [24]. Ten serial sections were cut (2 μm thick) with a microtome Reichert–Jung Autocut 2040 (Heidelberg, Germany) with
glass knives, and the sections stained with periodic–Schiff stain and haematoxylin–eosin. The glomerular lesions were defined as: (i) glomerulosclerosis: increased extracellular matrix with or without segmental collapse of the glomerular capillaries evolving to solidification of the tuft and disorganization of normal architecture (advanced lesions), with possible formation of synechiae between capillary loops and Bowman’s capsule; (ii) mesangial expansion: mild increase of extracellular matrix, without any other glomerular abnormalities and (iii) mesangial hypercellularity: when mesangial areas in one-half or two-thirds of the tuft away from the vascular pole [25] contained more mesangial cells than the mean + 2 standard deviations of those counted in the kidneys from normal rats (our normal value was 5 cells). Thickening of the mesangial basement membrane was evaluated qualitatively by comparison with glomeruli from normal control kidneys. The tubulointerstitial lesions were examined and graded as previously described [26]. Microscopic analysis was performed in a blinded fashion by one observer, evaluating at least 75 different glomeruli in each animal. If a single histological preparation did not contain enough glomeruli, a second microscopic section, at least 350 µm away, was examined to avoid the evaluation of the same glomerulus. The interval of 350 µm was considered adequate since the glomerular diameter did not exceed 300 µm in any glomerulus.

Morphometry

Three-dimensional quantitative morphometric analysis using Videoplan, Kontron Bildanalyse GMBH-Image Analysis System (Zeiss, Germany) was performed on the same sections used for morphology.

The cross-sectional capillary tuft areas and the sclerotic areas were measured with the above-mentioned system by outlining the perimeter of each glomerulus and sclerotic lesion respectively. The mean values of these parameters were then calculated for each kidney. Since more than 70 glomeruli per kidney were evaluated, it was possible to calculate the glomerular volume, as previously described [27,28]. Specifically, the following formula was applied: $GV = B/k(GA)^{3/2}$, where $GV$ is glomerular volume, $B (= 1.38)$ is the shape coefficient for the sphere, $k (= 1.1)$ is the size distribution coefficient and $GA$ is glomerular area. The fractional glomerular sclerotic area was calculated dividing the mean cross-sectional sclerosis area by the mean cross-sectional capillary tuft area.

Statistics and calculations

Glomerular morphologic data were analysed by chi-square test. The scores of the tubular and interstitial lesions were analysed by the Kruskal–Wallis (ANOVA multiple comparison) test. For all the other data, the one-way analysis of variance for comparison among different groups was used, and ANOVA for repeated measurements with Bonferroni’s correction for multiple comparison analysis in the same group. Results are reported as mean ± 1 standard error (SE). A $P < 0.05$ was considered statistically significant.

Results

Water and food intake was equal in the three groups of rats throughout the study. Body weight was similar among the groups, both after subtotal kidney ablation (321 ± 14 g in CON, 305 ± 10 g in EPO and 305 ± 10 g in PHL) and at the end of the experimental period (CON = 360 ± 18 g, EPO = 338 ± 17 g and PHL = 325 ± 13 g). All animals survived until the end of the study.

Systemic and renal functional data

As depicted in Figure 1, haematocrit (Htc) was similar in the three groups prior to surgery, and it similarly decreased in all rats after nephrectomy. At 2 weeks, a mild degree of anaemia was observed in CON and PHL ($P < 0.05$ vs initial Htc) groups, while Htc was restored to normal values in rHuEpo-treated rats ($P < 0.05$ vs CON and PHL) (Figure 1). Thereafter, these levels remained steady in all rats.

Two weeks after nephrectomy creatinine clearance was significantly reduced to the same degree in the three groups (Figure 2). Thereafter creatinine clearance further decreased in CON and EPO groups, with a final variation of $−53±8\%$ and $−38±8\%$ vs the respective 2-week value. At the end of the experimental period, it was significantly higher in the PHL group than the CON and EPO groups ($P < 0.05$). No difference in creatinine clearance was detected between EPO and CON during the whole study period.

Systolic blood pressure (SBP), was significantly increased in all groups at 2-weeks (Figure 3). Blood pressure further increased in both CON and EPO rats ($+13±2\%$ and $+15±5\%$ respectively), while it remained unchanged in PHL rats. Consequently, at the end of the study, SBP was significantly greater in both CON and EPO groups compared to PHL ($P < 0.05$).

As depicted in Figure 4, urinary protein excretion was increased in all groups at 2 weeks ($P < 0.05$ vs pre-surgery value); thereafter it increased only in CON and EPO.
Effects of rHuEpo on renal injury in rats

Fig. 2. Creatinine clearance in untreated remnant rats (CON, ——), remnant rats treated with rHuEpo (EPO, ······) and remnant rats treated with rHuEpo and phlebotomy (PHL, ——). *P<0.05 vs 2-week value; †P<0.05 vs PHL.

Fig. 4. Urinary protein excretion in untreated remnant rats (CON, ——), remnant rats treated with r-Hu-Epo (EPO, ······) and remnant rats treated with r-Hu-Epo and phlebotomy (PHL, ——). *P<0.05 vs 2-week value; †P<0.05 vs PHL.

tubular dilatation, interstitial infiltrates were present to the same extent in the three experimental groups.

Discussion

This study provides evidence that in remnant rats the normalization of haematocrit by erythropoietin administration since the very early phase of renal disease does not worsen either the decline of GFR or the degree of proteinuria and glomerulosclerosis.

Previous work in similar experimental models demonstrated that chronic erythropoietin treatment accelerates the progression of glomerular sclerosis and renal failure [15,16]. However, in these studies, blood pressure was further increased by rHuEpo treatment compared to untreated controls. The influence of erythropoietin-induced hypertension on renal damage may have been critical, since in the remnant rat, systemic arterial pressure greatly accounts for the development of glomerulosclerosis [29]. Therefore, these experimental studies could not adequately explore the direct effects of erythropoietin and correction of anaemia on the progression of renal damage.
Table 1. Prevalence of glomerular lesions in untreated remnant rats (CON), remnant rats treated with rHuEpo (EPO), and remnant rats treated with rHuEpo and phlebotomy (PHL).

<table>
<thead>
<tr>
<th>Group</th>
<th>CON</th>
<th>EPO</th>
<th>PHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesangial enlargement</td>
<td>150/573 (21)</td>
<td>138/574 (19)</td>
<td>63/672 (9)</td>
</tr>
<tr>
<td>Mesangial sclerosis</td>
<td>526/197 (73)</td>
<td>518/194 (73)</td>
<td>440/295 (60)</td>
</tr>
<tr>
<td>Mesangial hypercellularity</td>
<td>212/511 (29)</td>
<td>293/419 (41)</td>
<td>200/526 (28)</td>
</tr>
<tr>
<td>Thickness of Bowman's capsule</td>
<td>38/685 (5)</td>
<td>43/669 (6)</td>
<td>0/725</td>
</tr>
<tr>
<td>Adhesion of Bowman's capsule</td>
<td>110/613 (15)</td>
<td>99/613 (14)</td>
<td>0/735</td>
</tr>
<tr>
<td>Obsolete glomeruli</td>
<td>24/699 (3.3)</td>
<td>19/693 (2.5)</td>
<td>2/733 (0.3)</td>
</tr>
</tbody>
</table>

Abnormal/normal glomeruli ratio (n/n) and percentage of damaged glomeruli (n) are shown. *P < 0.001 vs PHL; **P < 0.001 vs CON.

In the current study, in the presence of complete correction of haematocrit as in the above-mentioned work [15,16], the arterial pressure was similar in EPO and CON rats. The reason for the different blood pressure response to erythropoietin observed in the two studies is not readily apparent. Indeed, the discrepancy cannot be related to the slight differences in either the dose of rHuEpo used or the Htc level reached, but it may be ascribed to the different species of rats employed (Sprague–Dawley in our study and Munich–Wistar in the previous) and/or to a diverse experimental environment. Nevertheless, the absence of a further rise in blood pressure in our rHuEpo-treated rats allowed us to investigate the renal effects of the correction of haematocrit by erythropoietin independent from arterial pressure. Interestingly, both the functional and histological studies demonstrated that the progression of renal disease was similar in rats treated with rHuEpo and untreated controls. In fact, no difference was observed between the two groups in the rate of GFR decline, the increment of proteinuria and the extent of glomerulosclerosis. This major finding indicates that the correction of anaemia by rHuEpo within a normal range of haematocrit does not affect the outcome of renal failure when CRF-related hypertension is not further worsened.

More insights into the effects of rHuEpo on the progression of experimental CRF were attained by comparing erythropoietin-treated rats kept anaemic by periodic phlebotomies (PHL) with untreated remnants. This comparison allows one to assess the effect of erythropoietin independent from any changes in haematocrit. PHL rats were characterized by less diffuse and severe glomerular sclerotic lesions and a further decline in renal function did not occur. These findings reasonably rule out the hypothesis of a direct ‘nephrotoxic’ effect of erythropoietin, previously drawn on the basis of its proliferative activity [17–20]. In contrast to anaemic untreated control rats and rHuEpo-treated rats, hypertension in the PHL group did not get worse throughout the follow up, possibly explaining the better renal outcome in these rats. Phlebotomy, reducing circulatory volume, may have prevented the further increase in hypertension [30].

Of note, the blood pressure levels in the rats of this study were consistently higher, regardless the type of treatment, than those attained in other studies [16,17]. Again, the variance may be ascribed to the rat species and/or experimental conditions. One could argue that the major hypertensive state may have masked an additional effect of rHuEpo on glomerulosclerosis. This is unlikely since, despite of the presence of high blood pressure in all the three groups of animals, it was possible to observe remarkable differences in the outcome of renal damage. Indeed, PHL rats were
markedly hypertensive throughout the study, and exhibited only minor renal injury. If any additive detrimental effect of erythropoietin were present this should have been apparent in PHL rats. Interestingly, in EPO rats the glomeruli exhibited a 10% greater mesangial hypercellularity compared to other groups. This is the sole morphological difference detected between untreated CON and EPO groups. We do not have an explanation for this finding; however, such a slight difference in mesangial hypercellularity did not affect the severity of renal damage in EPO rats even after the prolonged period of observation of this study. Furthermore, it was not related to erythropoietin administration itself, since mesangial proliferation was similar in the PHL group and untreated controls.

In conclusion, both the functional and histological findings of this study suggest that in moderate to advanced experimental CRF, chronic treatment with rHuEpo aimed at normalization of Htc from the early stage of renal failure, does not inevitably account for a rise in systemic blood pressure levels. In addition, neither erythropoietin per se nor the correction of haematocrit accelerates the progression of renal damage when blood pressure remains constant. As there is great interest in modifying both doses and indications for rHuEpo in CRF patients, clinical studies should be performed to confirm this favourable experimental evidence in humans.

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References


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