Epoetin-Alpha: Preserving Kidney Function via Attenuation of Polymorphonuclear Leukocyte Priming

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Abstract

Background: Polymorphonuclear leukocyte priming and low grade inflammation are related to severity of kidney disease. Erythropoietin-receptor is present on PMNLs.

Objectives: To evaluate the effect of 20 weeks of epoetin-alpha treatment on PMNL characteristics in relation to the rate of kidney function deterioration in patients with chronic kidney disease.

Methods: Forty anemic chronic kidney disease patients, stage 4-5, were assigned to EPO and non-EPO treatment for 20 weeks. A group of 20 healthy controls was also studied. PMNL priming and PMNL-derived low grade inflammation were estimated, *in vivo* and *ex vivo*, before and after EPO treatment: The rate of superoxide release, white blood cells and PMNL counts, serum alkaline phosphatase and PMNL viability were measured. EPO-receptor on PMNLs was assayed by flow cytometry. The effect of 20 weeks of EPO treatment on kidney function was related to the estimated glomerular filtration rate.

Results: EPO treatment attenuated superoxide release *ex vivo* and *in vivo* and promoted PMNL survival *ex vivo*. Decreased low grade inflammation was reflected by reduced WBC and PMNL counts and ALP activity following treatment. EPO retarded the deterioration in GFR. The percent of PMNLs expressing EPO-R was higher before EPO treatment and correlated positively with the rate of superoxide release. After 20 weeks of EPO treatment the percent of PMNLs expressing EPO-R was down-regulated.

Conclusions: These non-erythropoietic properties of EPO are mediated by EPO-R on PMNLs, not related to the anemia correction. A new renal protection effect of EPO via attenuation of PMNL priming that decreases systemic low grade inflammation and oxidative stress is suggested.

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Oxidative stress and inflammation are associated with uremia already in the early stages of chronic kidney disease and are involved in the development of atherosclerotic cardiovascular events in this population [1]. Moreover, oxidative stress and inflammation, either acute or chronic, were found to aggravate tubular and interstitial damage and are recognized as mechanisms involved in the progression of chronic kidney disease [1,2]. We recently defined peripheral polymorphonuclear leukocyte priming and related PMNL counts as surrogate markers and key mediators in low grade inflammation and systemic oxidative stress associated with renal failure [3]. The PMNL, one of the main inflammatory cell types, exists in the bloodstream in one of three functional states: quiescent, primed, or activated. Under non-infectious conditions, the PMNLs are quiescent, exhibiting little or no release of reactive oxygen species. Studies have led to the concept of a two-stage activation process: PMNLs first encounter a stimulus that leaves the cells in a "primed" state. Upon encountering a second stimulus, PMNLs proceed to the second state of full activation, releasing reactive oxygen species, granule contents and inflammatory mediators [3]. We have demonstrated a positive significant correlation between PMNL priming, expressed by increased rate of superoxide release, and PMNL counts, implying that the increased PMNL counts in peripheral blood is an adaptive response to PMNL priming [3]. In patients with chronic kidney disease not yet on renal replacement therapy, both PMNL priming and PMNL counts negatively correlated with glomerular filtration rate [3]. PMNL priming and elevated peripheral counts are aggravated as kidney function deteriorates, especially with the commencement of chronic hemodialysis [3].

In previous studies we also showed that epoetin-alpha, beyond its well-known role in anemia correction, possesses non-erythropoietic properties mediated by an EPO receptor on PMNLs [4-6]. Irrespective of the correction of anemia, EPO mediated the alleviation of systemic oxidative stress and low grade inflammation contributed by primed PMNLs in chronic kidney disease patients on dialysis [4,5].

The involvement of oxidative stress and inflammation in the deterioration of kidney function, together with our data on the attenuation of oxidative stress and low grade inflammation by modulating PMNL priming with EPO, prompted us to evaluate the hypothesis that EPO treatment will also slow the rate of kidney failure progression and delay the commencement of renal replacement therapy in patients with kidney disease. We designed a prospective study to evaluate the effect of 20 weeks of EPO treatment on PMNL characteristics and their relation to kidney

PMNL = polymorphonuclear leukocytes
EPO-alpha = erythropoietin-alpha
WBC = white blood cells
ALP = alkaline phosphatase
GFR = glomerular filtration rate
EPO-R = EPO-receptor

function in these patients before initiating renal replacement therapy.

Patients and Methods

The study group comprised 40 anemic (hemoglobin 8.1–11 g/dl) chronic kidney disease patients, stage 4-5, before starting renal replacement therapy and EPO treatment. The study was conducted during the period 2000–2001. The patients were divided into two groups: one treated with EPO for 20 weeks (group 1) and the second without (group 2). Group 1 included 20 participants: 4 males and 16 females aged 70.2 ± 2.0 years, with systolic and diastolic blood pressure of 157.1 ± 5.9 and 80.8 ± 2.4 mmHg respectively. The underlying renal diseases were diabetes mellitus (n=7, 35%), hypertension (n=5, 25%), chronic glomerulonephritis (n=2, 10%), polycystic kidney (n=1, 5%), nephrolithiasis (n=1, 5%) and unknown (n=4, 20%). Subcutaneous recombinant human EPO (6000 U/week, EPREX®, Cilag Schaffhausen, Switzerland) was administered for 20 weeks. Only 17 patients completed the 20 week period as 3 were excluded due to non-compliance and commencement of dialysis.

Group 2 included 20 patients – 6 males and 14 females aged 67.2 ± 2.7 years with systolic and diastolic blood pressure of 160.8 ± 5.9 and 85.5 ± 6.1 mmHg respectively. The underlying renal diseases were diabetes mellitus (n=11, 55%), hypertension (n=3, 15%), polycystic kidney (n=1, 5%), chronic glomerulonephritis (n=1, 5%), and unknown (n=4, 20%). Only 11 patients completed their 20 weeks of follow-up without EPO as 9 were excluded mainly due to aggravation of anemia and commencement of dialysis.

All patients received oral maintenance iron supplementation (320 mg/day; ferrous sulfate, slow Fe, Novartis), calcium bicarbonate, statins, beta-blockers, and calcium channel blockers; no patient received angiotensin-converting enzyme inhibitors. Data of serum creatinine levels and PMNL counts 10 weeks prior to starting EPO treatment were taken from the medical files of group 1 patients.

The control group comprised 20 healthy subjects matched for age and gender. The inclusion of these participants was based on a clinical examination with laboratory confirmation. Excluded were patients and healthy controls with evidence of infection, heavy smoking, malignancy, severe hyperparathyroidism or blood transfusions within 3 months before the study. Patients and subjects gave informed consent for blood sampling, which was approved by the institutional committee in accordance with the Helsinki Declaration.

Blood withdrawal and PMNL separation

Blood was withdrawn after an overnight fast for biochemical and hematological parameters and for PMNL separation. Ten milliliters of heparinized blood (50 U/ml) was used for PMNL isolation as previously reported [3]. The separated PMNLs (> 98% pure, approximately 10^7 cells per isolation) were resuspended in a minimal volume (0.1–0.3 ml) of phosphate-buffered saline containing 0.1% glucose, immediately counted, and diluted for the different experimental needs. We defined *in vivo* conditions where PMNLs were exposed to EPO in the circulation of patients treated with EPO, while in the *ex vivo* settings separated PMNLs from all subjects and patients were treated with EPO *in vitro*. Sera were frozen at -20ºC and saved for determination of serum EPO measurements as described below. Blood creatinine levels were measured using Hitachi 917 Automatic analyzer (Boehringer Mannheim, Germany). Kidney function was estimated by the calculated GFR according to the MDRD formula (modification of diet in renal disease) [7].

Evaluation of PMNL priming

- R*ate of superoxide release from separated* PMNL*s*. The rate of superoxide release was assayed after stimulation with $0.32x10^{-7}$ M phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO, USA) as a measure of PMNL priming [3]. The assay is based on superoxide dismutase inhibitable reduction of 80 µM cytochrome C (Sigma) to its ferrous form [8].

- W*hite blood cells and* PMNL *counts*. WBC and PMNL counts in blood withdrawn in EDTA were performed by a Coulter STKS counter (Miami, FL, USA).

- A*lkaline phosphatase*. Previous reports have shown that increased plasma ALP activity can serve as a measure of PMNL degranulation derived from PMNL priming [8-10]. Plasma ALP was measured using the Hitachi 917 Automatic analyzer (Boehringer Mannheim).

Effect of EPO on PMNL functions – *ex vivo*

PMNLs from 10 randomly chosen chronic kidney disease patients before starting EPO treatment – with a mean creatinine of 3.7 ± 1.04 mg/dl (range 2.1–5.4), similar to the creatinine range in groups 1 and 2 – were isolated, incubated with EPO and used for the following *ex vivo* studies:

- R*ate of superoxide release from* PMNL*s*. Isolated PMNLs were incubated for 15 minutes with increasing concentrations of EPO (0–40 U/ml) (Eprex®). The rate of superoxide release was determined as described above.

- *PMNL survival*. Separated PMNLs were suspended at 10^6cells/ml and incubated in 25% autologous serum at 37ºC for 90 min, with increasing concentrations of EPO (0–40 U/ml). PMNLs were counted by Coulter STKS before and after 90 min of incubation with EPO, with confirmation of cell viability by trypan blue (0.1% w/v) exclusion.

Levels of endogenous serum erythropoietin

In both healthy controls and chronic kidney disease patients s-EPO was always measured before EPO treatment was started, using the Human EPO Immunoassay ELISA kit (Quantikine™ IVD™, R & D systems, MN, USA).

s-EPO = serum erythropoietin

Table 1. Biochemical and hematological parameters of chronic kidney disease patients determined before, and at 10 and 20 weeks of EPO treatment (group 1), compared with patients (group 2) not treated with EPO and followed for 20 weeks

	Group 1 EPO treatment			Group 2 Without EPO	
	Before	10 weeks	20 weeks	Before	20 weeks
Cholesterol (mg/dl)	218.4 ± 9.7	205.7 ± 11.2	211.4 ± 11.8	196.7 ± 18.2	184.4 ± 12.2
Triglycerides (mg/dl)	201.3 ± 25.5	200.5 ± 31.9	227.9 ± 37.5	193.1 ± 32	158.4 ± 21.5
Creatinine (mg/dl)	4.2 ± 0.3	3.9 ± 0.4	2.9 ± 0.1*	3 ± 0.3	3.8 ± 0.4*
Range	2.3–8.6	2.5–7.4	2.1–6.9	2.4–7.3	2.6–6.8
Calculated GFR (ml/min/1.73 m^2)	14.7 ± 1.2	14.8 ± 1.2	14.1 ± 1.1	14.4 ± 1.3	11.1 ± 1.0*
Range	8.3–23.9	7.9–24.8	5–23.2	8.4–22.1	4.9–18.2
Albumin (g/dl)	3.8 ± 0.05	3.9 ± 0.1	3.9 ± 0.1	3.7 ± 0.1	3.6 ± 0.1
Hemoglobin (g/dl)	10.6 ± 0.1	10.9 ± 0.3	11.5 ± 0.4	10.7 ± 0.2	9.4 ± 0.2*
Hematocrit (%)	31.1 ± 0.6	32.7 ± 1	34.3 ± 1.2*	31.2 ± 0.9	28.5 ± 0.5*
Transferrin saturation (%)	18.1 ± 1.6	16.0 ± 0.9	19.9 ± 1.7	16.6 ± 3	12.6 ± 3.2
Ferritin (ng/ml)	98 ± 14.3	66 ± 9.2	63 ± 7.9	65.8 ± 17	48.5 ± 5.3
WBC (x 10^9/L)	9.2 ± 0.4	7.9 ± 0.5	7.6 ± 0.5*	8.4 ± 0.2	8.8 ± 0.4
PMNLs (x 10^9/L)	6.4 ± 0.3	5.3 ± 0.3*	5.2 ± 0.4*	5.7 ± 0.1	6.1 ± 0.4
ALP (U/ml)	239.4 ± 17	192.9 ± 18.4	131.1 ± 12.7*	229.1 ± 45.6	268.7 ± 60.2*
EPO-R (%) **	5.27 ± 1.3	4.00 ± 0.3	2.16 ± 0.5*	4.75 ± 1.07	4.55 ± 1.1

Values are means ± SEM.
* $P < 0.05$ in group 1 vs. values before EPO treatment; in group 2 vs. time 0.
** The percentage of PMNLs expressing EPO receptors.

Detection of PMNLs expressing EPO-R

EPO-R expression on PMNLs from all kidney disease patients before and after EPO treatment and from healthy control PMNLs was detected by flow cytometry using fluorescent monoclonal anti-human EPO-R antibodies (R&D systems). The non-specific mouse anti-immunoglobulin G fluorescence was subtracted from EPO-R fluorescence, as previously described [6]. The results are presented as percent of PMNLs expressing EPO-R (%).

Statistical analysis

Data were expressed as means ± SEM. Differences between the study parameters were compared by paired or unpaired student *t*-test, or by one-way analysis of variance, according to the experimental requirements. The correlation between different study parameters was performed by linear and non-linear regression analysis using Spearman and Pearson correlation coefficients. $P < 0.05$ was considered significant.

Results

Effect of EPO on biochemical parameters and GFR

The EPO-treated kidney patients and the non-EPO treated patients showed non-significant differences in the levels of blood glucose, cholesterol, triglycerides and albumin during the study [Table 1]. Hemoglobin and hematocrit levels increased in the EPO-treated group, reaching significance for hematocrit [Table 1], while in group 2 both hemoglobin and hematocrit demonstrated a significant decrease. A stable GFR was observed in the EPO-treated group 1, compared to a significant increase in serum creatinine and a decrease in GFR in the absence of EPO in group 2 [Table 1].

The individual GFR profiles of group 1 are depicted in Figure 1 (left panel). The calculated GFR in these EPO-treated patients is shown 10 weeks before (as recovered from the patients' files) and after 10 and 20 weeks of treatment with the hormone. A significant ($P = 0.001$) deterioration in the average GFR from 17.4 ± 1.3 to 14.7 ± 1.2 ml/min/1.73 m^2 was demonstrated during 10 weeks prior to EPO treatment, similar to the rate of deterioration observed in group 2. However, during the two periods of 10 weeks of EPO treatment in group 1, the GFR decline was retarded. It showed similar calculated GFR values of 14.7 ± 1.2, 14.8 ± 1.2 and 14.1 ± 1.1 ml/min/1.73 m^2 at 0, 10 and 20 weeks of EPO treatment, respectively.

PMNL priming – in vivo

- *Rate of superoxide release from separated* PMNL*s of kidney patients*. The rate of superoxide release from PMA-stimulated PMNLs was monitored in group 1 after 10 and 20 weeks of EPO treatment and in group 2 after 20 weeks without EPO. Both 10 and 20 weeks of EPO treatment in kidney patients induced a significant reduction in the rate of superoxide release from PMA-stimulated PMNLs, with a significant 40% further reduction between 10 and 20 weeks [Figure 2]. The rate of superoxide release after 20 weeks of EPO treatment was similar to that of PMNLs separated from healthy control subjects (11 ± 1.2 nmoles/10^6 cells/10 min). In PMNLs separated from patients not treated with EPO, the rate of superoxide release during 20 weeks was not attenuated and

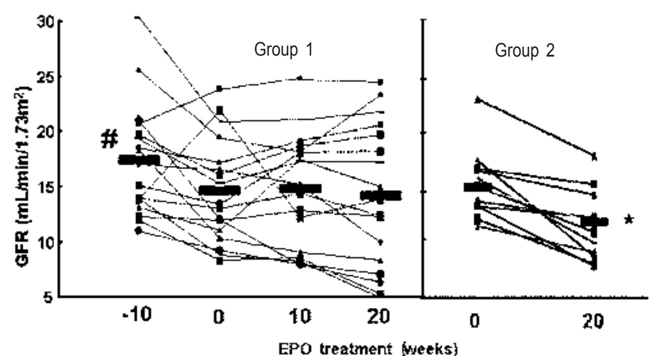

Figure 1. Individual GFR curves and longitudinal evaluation of GFR by MDRD at 10 weeks before, at EPO administration (time 0), and 10 and 20 weeks following EPO treatment, for each patient (group 1, left panel). Individual GFR curves during 20 weeks without EPO treatment (group 2, right panel). # $P < 0.05$, group 1: calculated GFR values, 10 weeks before vs. EPO administration (time 0). *$P < 0.05$, group 2: calculated GFR values, time 0 vs. 20 weeks without EPO administration.

PMA = phorbol 12-myristate 13-acetate

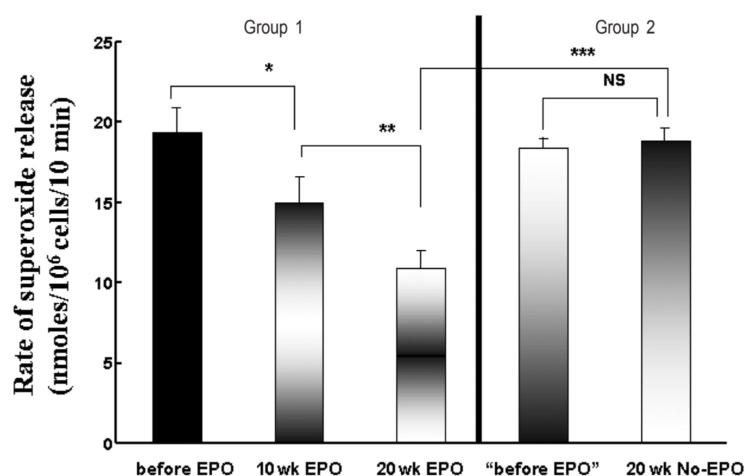

Figure 2. Rate of superoxide anion release from PMA-stimulated PMNLs of chronic kidney disease patients, with (group 1) and without (group 2) 20 weeks of EPO treatment. Superoxide was measured by SOD-inhibitable reduction of cytochrome C after PMA stimulation of 10^6 isolated PMNLs at 22ºC. Data are means ± SEM.
* $P = 0.04$, PMNLs after 10 weeks of EPO treatment vs. before.
** $P = 0.03$, PMNLs from kidney disease patients after 20 weeks vs. 10 weeks of EPO treatment.
*** $P = 0.0002$, PMNLs from kidney disease patients after 20 weeks of EPO treatment vs. patients undergoing 20 weeks without EPO treatment.
NS = not significant, group 2 before EPO vs. group 2 after 20 weeks.

stayed significantly elevated compared to patients after 20 weeks of EPO treatment [Figure 2]. No correlation could be found between the rates of superoxide release from separated PMNLs and the hemoglobin levels following 10 weeks ($r = 0.5$, $P = 0.1$) or 20 weeks ($r = 0.17$, $P = 0.6$) of EPO treatment. Nevertheless, the rate of superoxide release from separated PMNLs from all participants, without or before EPO treatment, negatively correlated with GFR ($r = -0.315$, $P = 0.002$, n=58). This correlation is similar to the one previously reported [3].

- WBC *and* PMNL *counts*. EPO treatment caused attenuation of the increase in WBC and PMNL counts, while without EPO treatment the counts tended to increase [Table 1]. EPO treatment caused a significant decrease in WBC and PMNL counts in kidney patients, compared to the pretreatment values, reaching the cell counts of healthy controls (WBC 6.7 ± 0.2 10^9/L; PMNLs 3.9 ± 0.2) [Table 1].

- *Alkaline phosphatase*. ALP activity measured after 20 weeks of EPO treatment decreased significantly compared to its level before EPO treatment, reaching healthy control levels (141 ± 11 U/ml). Without EPO treatment ALP activity increased [Table 1]. A linear regression analysis revealed strong positive correlations between ALP activity and the rate of superoxide release from separated PMNLs ($r = 0.37$, $P = 0.02$), and between ALP activity and PMNL counts ($r = 0.4$, $P = 0.0049$).

The ex vivo effects of EPO on PMNL functions

- PMNL *priming*. When PMNLs from untreated kidney patients were incubated *ex vivo* with increasing concentrations of EPO, a dose-dependent reduction in the rate of superoxide release was detected already at 5 U/ml, a concentration within the therapeutic range, reaching significance at 10 U/ml ($P < 0.05$) [Figure 3A].

- PMNL *survival*. When EPO was added to PMNLs isolated from non-EPO-treated patients, already 5 U/ml of EPO significantly promoted PMNL survival

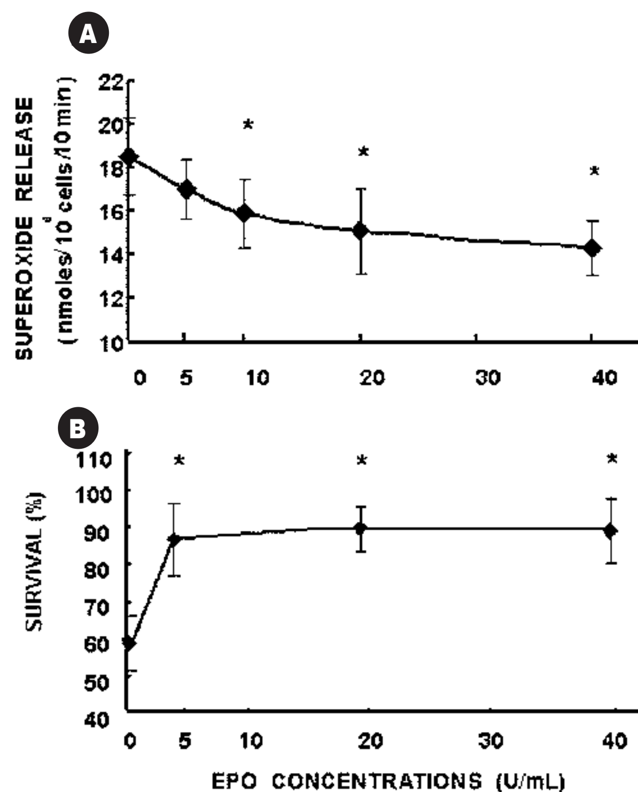

Figure 3. *Ex vivo* effects of EPO on PMNLs from chronic kidney disease patients before EPO treatment: **[A]** Rate of superoxide release by PMA-stimulated PMNLs incubated with 0–40 U/ml EPO. Superoxide was measured by SOD-inhibitable reduction of cytochrome C, after PMA stimulation of 10^6 PMNLs in 22°C. Data are means ± SEM.
* $P < 0.05$, kidney disease PMNLs incubated with various concentrations of EPO vs. no EPO.
[B] Survival of PMNLs incubated with increasing concentrations of EPO. Kidney disease PMNLs were incubated in 25% autologous serum with increasing concentrations of EPO (0–40 U/ml) and counted Coulter STKS before and after 90 min of incubation at 37ºC. Cell viability was confirmed by trypan blue (0.1% w/v) exclusion. Results are means ± SEM.
* $P < 0.05$, kidney disease PMNLs incubated with various concentrations of EPO vs. no EPO.

($P < 0.05$) [Figure 3B]. Higher EPO concentrations had no additional effect on PMNL survival.

Measurement of EPO-R and s-EPO

To elucidate the EPO/EPO-R interactions in PMNLs and the mechanism that underlies the expression of the EPO-R *in vivo*, the percentage of cells expressing EPO-R from all kidney patients not treated or before EPO treatment and from healthy controls was correlated to endogenous s-EPO levels. These parameters were negatively exponentially correlated ($r = -0.3$, $P < 0.05$, n=58), reflecting receptor-ligand interactions, suggesting a down-regulation of EPO-R by increasing endogenous EPO (s-EPO) levels. The percentage of kidney disease PMNLs expressing EPO-R before EPO treatment was relatively high compared to healthy control subjects (5.5 ± 1.3 vs. 2.6 ± 0.65%, respectively). After 20 weeks of hormone administration, a twofold decrease in the percentage of EPO-R-expressing cells was observed [Table 1], similar to its level in healthy controls.

EPO regulation of PMNLs is distinct from its role in erythropoiesis, as there was no significant correlation between PMNL EPO-R and blood hemoglobin levels ($r = 0.3$, $P = 0.13$, n=58). Nevertheless, PMNLs expressing EPO-R positively correlated with the rate of superoxide release from these cells ($r = 0.4$, $P = 0.0004$); the higher the priming state the more PMNLs carrying EPO-R are present. In addition, the percentage of PMNLs expressing EPO-R negatively correlated with GFR ($r = -0.32$, $P < 0.05$, n=58); the lower the GFR the higher the percentage of PMNLs expressing EPO-R.

Discussion

This study adds a new renal protection effect of EPO in patients with chronic kidney disease, via attenuation of PMNL priming and related inflammation: EPO attenuated PMNL priming and PMNL mediated inflammation concomitantly with retarding the decline of estimated GFR; EPO modulated PMNL priming both *ex vivo* and *in vivo*. In a longitudinal follow-up, PMNLs from kidney disease patients treated with EPO showed a reduced rate of superoxide release, similar to the *ex vivo* results, where EPO inhibited the rate of superoxide release from separated PMNLs in a dose-dependent manner. EPO caused a decrease in WBC and PMNL counts, reflecting a decrease in systemic low grade inflammation. Promotion of PMNL survival *ex vivo* by EPO suggests a new property of the hormone as a viable anti-inflammatory factor. These non-erythropoietic effects of EPO in kidney disease patients are not related to the correction in hemoglobin levels and are probably mediated by the EPO-R on PMNLs. It should be noted that the increased percentage of cells expressing EPO-R is down-regulated by increased s-EPO levels, namely EPO treatment.

We have recently shown that chronic kidney disease is accompanied by PMNL priming and increased low grade systemic inflammation directly related to the severity of kidney function [3]. The present study supports these findings in another group of kidney disease patients. When these patients were treated with EPO for 20 weeks, a complete correction of the PMNL priming state to healthy control levels occurred. EPO treatment attenuated the rate of superoxide release to levels observed in healthy control subjects, while during 20 weeks in a similar group of patients but not receiving EPO, the priming state of PMNLs was unchanged or even worsened. It must be mentioned that the correction of PMNL priming to healthy control levels by EPO was not achieved in patients on maintenance hemodialysis treated with EPO: In these chronic hemodialysis patients the amelioration in PMNL priming, although pronounced, did not reach normal levels [4]. The lowering of superoxide anion release to normal levels by EPO treatment renders antioxidative properties to the hormone. Hence, it is easy to envisage that in the face of lower antioxidant levels in kidney disease patients [11] systemic oxidative stress will increase.

Low grade PMNL-related inflammation, reflected by an increase in WBC and PMNL counts, was shown by us in many clinical states: in early pregnancy [12], in cigarette smokers [13], in type 2 diabetic patients [14], in hyperlipidemic patients [15], in essential hypertensive patients [8], at all stages of uremia [3], and in patients on hemodialysis [4] or on continuous ambulatory peritoneal dialysis [5], and is also supported here by another group of kidney disease patients before renal replacement therapy. In addition, EPO treatment ameliorated the PMNL-related low grade systemic inflammation as reflected by the reduction in WBC and PMNL counts; whereas during 20 weeks without EPO treatment the PMNL counts did not change and even increased slightly. The *ex vivo* improvement in PMNL survival by EPO lowering cell disintegration provides an explanation for the *in vivo* reduction in peripheral PMNL counts: less cell death and a reduced release of inflammatory and chemotactic mediators from PMNLs, decelerating the inflammatory vicious cycle and ending in less PMNL recruitment. This anti-inflammatory characteristic of EPO was also shown for hemodialysis and continuous ambulatory peritoneal dialysis patients in our previous studies [4,5].

PMNL-mediated inflammation resulting from PMNL activation and degranulation is also suggested by elevated serum ALP activity. Increased ALP activity in kidney disease patients in the absence of liver disease usually reflects increased secondary hyperparathyroidism and high bone turnover. Although PMNLs are not usually identified as the source of increased serum ALP activity, they can still serve as a source of neutrophil alkaline phosphatase [10]. We suggest that the increased serum ALP may reflect PMNL degranulation and priming, as already reported for hypertensive patients, for Dahl hypertensive rats and in early pregnancy [8,9,12]. This increased serum ALP approaches normal levels after 20 weeks of EPO treatment in kidney disease patients. The correction of ALP levels, together with the significant correlation shown here between serum ALP and PMNL priming and counts, strengthen the notion that the primed PMNLs contribute to the increased serum ALP levels. In the absence of liver disease, the decrease in ALP to normal levels together with the preservation of GFR by EPO treatment plausibly rules out the bone origin of this enzyme and emphasizes the beneficial non-erythropoietic effects of EPO.

The percentage of kidney disease PMNLs expressing EPO-R

does not correlate with hemoglobin levels. Similar results were previously reported for such patients, but on renal replacement therapy [4,6], suggesting a regulatory mechanism different from the traditional erythropoiesis. After 20 weeks of EPO administration the percentage of PMNLs expressing EPO-R decreased twofold, probably due to down-regulation of the receptor expressed on circulating PMNLs. This phenomenon is common to various receptor-ligand interactions, where internalization of this complex results in a decreased expression of the receptor [16]. It should be mentioned that these results are inconsistent with our previous data showing that the expression of EPO-R on PMNL positively correlates with s-EPO levels [6]. This inconsistency derives mainly from using PMNLs from healthy subjects who have normal s-EPO levels very similar to one another. These similar values caused biased results, as all points were concentrated in a narrow range. We can strongly suggest from this *in vivo* study that treatment with EPO causes a reduction in the percentage of EPO-R expressing PMNLs.

We report a negative correlation between PMNLs expressing EPO-R and GFR; the lower the GFR the higher the percentage of PMNLs expressing EPO-R. Since a positive correlation also exists between PMNLs expressing EPO-R and the priming state of PMNLs – namely, the higher the priming state the more cells expressing EPO-R – we suggest that primed PMNLs are more sensitive to EPO treatment, resulting in the amelioration of oxidative stress and inflammation contributed by PMNLs in these patients.

Oxidative stress and inflammation are involved in the deterioration of kidney function through various mechanisms [2,17,18]. According to the results of the Second National Health and Nutrition Examination Survey (NHANES II) [19], elevated WBC counts are associated with future risk for developing chronic kidney disease, supporting our new mechanism: priming of PMNLs mediates systemic low grade inflammation and oxidative stress in kidney disease patients and is directly related to the severity of uremia [3]. Hence, the next step is to link the attenuation of PMNL priming and the related reduction in low grade inflammation and oxidative stress by EPO, to the retardation in GFR deterioration. Nevertheless, the contribution of a direct effect of EPO on renal cells cannot be ruled out, since renotropic mitogenic properties of EPO were found in the renal cortex, medulla, mesangial and tubular cells via renal EPO-R [20]. Several studies confirmed the ability of EPO to retard the progression of kidney disease, reporting a significant slowing of renal failure progression and delay in the commencement of renal replacement therapy [21-23]. Although it looks like a low percentage of PMNLs (5–20%) expressing EPO-R and correlating with GFR, these cells may not be the only ones carrying EPO-R, as the non-erytropoietic effect of EPO can also benefit from other cells.

In a recently published research, Siamopoulos et al. [24] reported a decrease in renal function after 12 months follow-up, which was greater in untreated patients than in those treated with EPO, a tendency similar to the one reported here. In a median duration 6 months follow-up of EPO treatment, Rossert and co-workers [25] also noted a decrease in the rate of decline in GFR compared to patients without EPO treatment.

Our study has several limitations: The main limitation is the relatively small sample size: only 40 patients were enrolled and 30% of them did not complete the study. The second limitation was the small group of control kidney disease patients not treated with EPO. This was due to the anemia that developed in this group, which ethically compelled us to initiate EPO treatment. Yet, the statistically significant effect of EPO on GFR, despite the small sample size, emphasizes the importance of our findings. This study focuses on PMNL-related inflammation, hence only WBC and PMNL counts and serum ALP levels were used as markers of low grade inflammation.

Despite these limitations, we believe that our findings are important and further support the previously reported beneficial non-erythropoietic characteristics of epoetin-alpha. Moreover, since we show a possible retardation in the rate of progression of chronic kidney disease, even in its advanced stages, it is tempting to recommend early EPO replacement therapy, as recommended by the Dialysis Outcomes Quality Initiative, when GFR is above 60 ml/min. The combined non-erythropoietic effects of EPO, the local renoprotection exhibited through EPO-R on renal cells, and the interaction with EPO-R on PMNLs, decreasing systemic oxidative stress and inflammation, may retard the rate of deterioration of renal functions and postpone the initiation of renal replacement therapy. Obviously, additional large-scale studies with control groups are warranted.

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