Serum Erythropoietin Concentration in Chronic Renal Failure: Relationship to Degree of Anemia and Excretory Renal Function

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By use of the fetal mouse liver cell assay, serum erythropoietin (SEP) concentration was measured in 135 patients at various stages of chronic renal failure and in 59 healthy subjects. In patients with creatinine clearances (CCr) ranging from 2 to 40 ml/min/1.73 sq m, endocrine renal function was found to deteriorate in parallel to excretory renal function. The known negative correlation between SEP and hematocrit (Hct) was not apparent, probably because of the loss of renal mass accompanying progress of anemia and renal insufficiency. In contrast, in patients with minimal variation of residual excretory renal function, as in individual patients investigated repeatedly within a short period of time, changes of Hct were always accompanied by opposite changes of corresponding SEP concentrations. Thus, patients with chronic renal failure have a sustained regulatory feedback mechanism between Hct and SEP, which probably works at a lower level.

In chronic renal failure, loss of red cells from various sources and increased hemolysis of erythrocytes dwelling in an uremic environment usually cause a severe anemia. Since renal anemia, which increases with progression of renal insufficiency, is hypoproliferative in nature, indicating an insufficient compensatory increase of red cell production in response to blood loss and hemolysis, most investigators believe that it is erythropoietin (Ep) deficiency that prevents an adequate rise of erythropoiesis. This concept is in accordance with earlier studies that showed that serum erythropoietin (SEP) concentration measured in the polycythemic mouse assay was only exceptionally elevated in uremic patients.

However, recent data obtained by immunologic methods and biologic assays show that Ep levels are also elevated in uremic anemic patients. This finding questions the crucial role of Ep deficiency in the pathogenesis of renal anemia. However, only a few patients have been investigated, and little is known about the regulatory mechanism controlling erythropoiesis in patients with end-stage renal failure.

In order to find out whether excretory and endocrine renal function deteriorate in parallel in chronic renal disease, and whether the regulatory feedback mechanism between hematocrit (Hct) and SEP, as it exists in nonuremic anemic patients, is sustained in the anemia of chronic renal failure, we investigated a total of 135 patients at various stages of renal insufficiency. Using the sensitive fetal mouse liver cell assay, we followed the changes in SEP concentrations accompanying increase of anemia during the course of progressing renal insufficiency.
MATERIALS AND METHODS

Patients

The following two groups of patients were studied. The first group consisted of 117 patients (56 male and 61 female) 26-77 yr old with chronic renal disease, who were on conservative therapy and did not need hemodialysis treatment. The patients did not receive blood transfusions or iron substitution therapy. The underlying diagnoses were pyelonephritis and interstitial nephritis (n = 50), glomerulonephritis (n = 30), polycystic kidney disease (n = 16), and others (n = 21). According to the residual excretory renal function, the group was divided into 5 subgroups: (1) creatinine clearances (CCr) between 2 and 9 ml/min/1.73 sq m (18 patients), (2) CCr between 10 and 19 ml/min/1.73 sq m (34 patients), (3) CCr between 20 and 29 ml/min/1.73 sq m (18 patients), (4) CCr between 30 and 39 ml/min/1.73 sq m (18 patients), and (5) CCr between 40 and 90 ml/min/1.73 sq m (29 patients).

The second group was composed of 18 patients (10 male and 8 female), 26-57 yr old, suffering from end-stage renal failure. They were investigated at three different occasions characterized by a different degree of renal anemia: (1) 2-6 mo before onset of regular hemodialysis treatment, (2) immediately before the first dialysis in a state of severe uremic intoxication and heavy anemia, and (3) 2-6 mo after start of regular hemodialysis treatment, when anemia already had improved again. Dialysis treatment was performed 3-4 times 6-8 hr/wk either with a Kiil dialyzer or with various disposable dialyzers with 1 sq m surface area and with cuprophane membranes 11.5-13.5 μ thick. None of the patients received blood transfusions or had a major blood loss during the time of investigation.

An additional group of 59 (35 male and 24 female) healthy subjects, 17-67 yr old, served as normal controls.

Methods

SEp concentration was measured using the fetal mouse liver cell culture technique, as described by Wardle et al.28 and Dunn et al.,29 with minor modifications described in detail elsewhere.30

In principle, the livers from 13-14-day-old mouse fetuses were dissected and the fetal liver cells suspended in a culture medium (Eagle's minimal essential medium, 10 vol% fetal calf serum, 0.22% NaHCO₃, gassed with 5% CO₂ in air). After addition of test sera or Ep standards, the culture was incubated for 20 hr at 37°C. Then, radioiron (1 μCi/ml), previously bound to equivalent amounts of pure human transferrin (Behringwerke, Marburg, Germany), was added and incubated for another 4 hr for pulse labeling. After chemical extraction of heme with HC1, Drabkin's solution, and butanone, radioactivity was measured in a β-liquid-scintillation counter.

To avoid disturbances of Ep measurement due to varying dilutions of the radiolabel, which could be caused by sera containing different amounts of iron,30,31 final iron concentration in the culture was stabilized. The sources of varying amounts of iron, i.e., the test serum samples, whose iron concentrations ranged from 32 to 157 μg/100 ml, were limited to 4 vol%, i.e., 40 μl/ml culture medium, and counterbalanced by the constant iron pool of the fetal calf serum, which contained 270 μg Fe/100 ml, and by the constant amount of 0.100 μg Fe/ml culture medium added for pulse labeling as radioiron-transferrin. So, 0.013-0.063 μg Fe/ml culture medium from serum samples were opposed by the constant amount of 0.370 μg Fe/ml culture medium from the fetal calf serum (0.270 μg) and from the radiolabel (0.100 μg). Thus, the difference in the specific activity of radioiron-transferrin was 13% when sera with minimal and maximal iron content were measured, respectively, and resulting errors of the measurement were well within the limits of accuracy, with an index of precision ranging from 0.04 to 0.20 and 95% confidence limits of usually 80%-120% of the mean potency.34

As laboratory standard, we used in every assay both a crude Ep preparation (Connaught Laboratories, Toronto, Canada, Step III) and a reference serum from a healthy volunteer, which was divided in 200 portions and stored deep-frozen. These laboratory standards were calibrated against the International Standard Preparation B (courtesy of the WHO Laboratory for Biological Standards, Mill Hill, London35), so that the Ep concentration could be expressed in international units (in vitro, mU/ml).

To obtain a full log.dose–response relationship, Ep measurements of every serum were done in serial dilutions of 3-4 steps, and 5 replicates were assayed of every dilution step and compared to standard samples assayed in the same way. The validity of every measurement was checked by testing the significance of regression and the absence of a significant deviation from linearity and parallelism in the log.dose–response relationship of test and standard samples using the analysis of variance.36,37
Fig. 1. Relationship between creatinine clearance (CCr) and hematocrit (Hct) at various degrees of renal insufficiency. In the range between 2 and 40 ml/min/1.73 sq m, Hct and CCr are highly significantly correlated \( (r = +0.69; p < 0.0001) \).

RESULTS

Figure 1 shows the development of anemia during progression of renal insufficiency in patients at different stages of chronic renal failure. CCr are plotted against the corresponding Hct. As can be seen from the data, renal anemia becomes manifest in patients with a CCr below 40 ml/min/1.73 sq m. In the range between 2 and 40 ml/min/1.73 sq m, CCr and Hct are highly significantly correlated \( (r = +0.69; p < 0.0001) \), whereas no significant correlation exists in the range between 41 and 90 ml/min/1.73 sq m.

In Fig. 2, mean SEp concentration and mean Hct of every subgroup are

![Graph showing mean serum erythropoietin (SEp) concentration and mean hematocrit (Hct) in patients at various stages of renal insufficiency (A–E) and in healthy controls (F). The bars indicate 1 SD. Mean SEp concentration in patients with chronic renal failure are significantly elevated \( (p < 0.01) \).]
compared to the control values. As in the previous figure, it can be seen that Hct drops continuously with decreasing clearances. However, mean SEp concentrations are always significantly elevated ($p < 0.01$). The highest value of 299 in vitro mU/ml was found in patients with CCr between 20 and 29 ml/min/1.73 sq m and was more than twice as high as the mean of 136 in vitro mU/ml found in normal controls and was not exceeded by the mean SEp concentrations of the more anemic patients, i.e., those with CCr between 2 and 9 and 10 and 19 ml/min/1.73 sq m, respectively. Thus, despite increasing anemia, SEp concentration drops with increasing impairment of excretory renal function. In the range between 2 and 40 ml/min/1.73 sq m, CCr and SEp are significantly positively correlated ($p < 0.01$), indicating a parallel decrease of excretory and endocrine renal function.

The relationship between SEp concentration and Hct in patients with renal insufficiency of varying degree (CCr ranging from 2 to 40 ml/min/1.73 sq m) is shown in Fig. 3. In these patients, the known negative correlation between SEp and Hct has converted into a positive one of marginal significance ($p < 0.015$), most likely due to the decreasing ability of the continuously deteriorating kidney to respond adequately to the increasing hypoxic stimulus.

Evidence of a sustained feedback mechanism is derived from longitudinal studies of patients with end-stage renal failure and varying degree of anemia (Fig. 4). While Hct decreases in every patient during the predialysis interval, and increases again after institution of regular hemodialysis treatment, SEp concentration behaves in the opposite way. In every patient, it increases during the predialysis interval, together with decreasing Hct, and decreases after regular hemodialysis.
ERETHROPOIETIN IN CHRONIC RENAL FAILURE

881

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Fig. 4. Hematocrit (A) and serum erythropoietin (B) during the development of terminal renal failure and following the onset of regular hemodialysis treatment. Hct and SEp are significantly different when first and second and second and third measurements are compared (Student’s paired t test).

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Hct and SEp values are significantly different when first and second and second and third measurements are compared using the paired t test ($p < 0.001$).

DISCUSSION

SEp concentrations of healthy subjects reported in the literature vary widely. They range from 3.9–5.3 mU/ml, measured by Stockman et al. by use of a radioimmunoassay, and 7.8 mU/ml found by Erslev et al., measuring highly concentrated plasma in the transfusion-induced polycythemic mouse, to 320 mU/ml measured by Davies et al. by use of the polycythemic mouse assay. However, normal SEp values obtained by immunologic assay and in vitro bioassay methods seem now to accumulate in the range of 20–80 mU/ml, as Lange et al. found a mean of 37 mU/ml (hemagglutination-inhibition test), Lertora et al. found 52–84 mU/ml (radioimmunoassay), de Klerk et al. found a mean of 48 in male, and 29 mU/ml in female volunteers (fetal mouse liver assay), Sherwood found 15–30 mU/ml (radioimmunoassay), and Garcia found 21 mU/ml in a pooled normal serum (radioimmunoassay). Comparing the mean SEp level of 136 mU/ml found in our healthy controls with normal values obtained by other investigators who used the fetal mouse liver cell assay, they are in accordance with the data of Napier et al., who found 150

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mU/ml, but are higher than the values of de Klerk et al. of 48 and 29 mU/ml, which correspond well with the immunologic data. De Klerk et al. point out the problem of assaying a constituent of a biologic fluid against a partially purified standard. Therefore, the authors solve their standard Ep preparation already in the serum to be assayed to provide equal conditions to standard and serum samples.40 This methodological difference might be the cause of the discrepancy in regard to our normal values, e.g., we might have underestimated our crude Ep standard preparation, which might be deficient of a factor necessary for optimal cell growth that is present in the human serum. On the other hand, like Napier et al., who showed that the fetal mouse liver cell bioassay is capable of detecting erythropoiesis-stimulating activity under various physiologic and pathologic conditions in a dose-related way,27 we did not find an indication for an Ep-independent inhibitory or enhancing influence in our standard and serum samples, since the dose–response curves were always proven to be parallel, suggesting that an identical reagent has been measured.

Differences of normal control values obtained by different laboratories using similar methods might also be attributed, at least in part, to inaccuracies in calibrating the laboratory standards against the international standard, which is most critical because of the limited supply of the International Reference Preparation, e.g., when the second International Reference Preparation was calibrated against the first, results of measurement of 10 U/definition ranged from 5.4 to 19.1 U, obtained by 10 different laboratories.35

Of course, we cannot completely exclude the possibility that the exquisitely sensitive fetal liver cell assay responds to an additional factor present in the serum and/or standard, and therefore, we would like to use the term “Ep” operationally and express our SEp values in “in vitro mU/ml,” referring to the fetal mouse liver cell in vitro bioassay applied, until more work is done to exclude this possibility. Fortunately, comparative studies are relatively independent of the absolute height of normal control values as long as the data can be referred to a sufficient number of controls measured under the same conditions.

By use of a sensitive method, it could be shown that SEp concentrations are elevated in predialysis patients with chronic renal failure at various stages of renal insufficiency and anemia. However, when excretory renal function is severely impaired, the deteriorating kidney becomes more and more unable to meet the increased demand of Ep production with increasing anemia (Fig. 2). Thus, it appears that excretory and endocrine renal function do not decrease independently, but deteriorate in parallel. This view is further supported by the observation that in patients with the lowest excretory renal function, those who needed artificial kidney treatment for more than 6 mo, the lowest SEp concentrations were found. In 100 non-nephrectomized hemodialysis patients with a comparative degree of anemia investigated in another study using the same method, mean SEp concentration was 135 in vitro mU/ml, which is as low as in normal persons.41 In 13 anephric patients (8 of whom were on testosterone therapy), mean SEp concentration was only 104 in vitro mU/ml, despite an even more pronounced anemia.44

Although Ep production declines with progression of renal failure, the negative feedback mechanism between Hct and SEp (as it exists in nonuremic anemic patients) appears to be sustained, probably working at a lower level. When
individual patients with varying degrees of anemia were studied repeatedly within a short period of time that did not permit considerable changes in excretory renal function. Hct and SEp levels changed in a mirror-image way (Fig. 4). That the negative correlation between Hct and SEp concentration could not be demonstrated in our predialysis group of patients (Fig. 3) is probably due to the fact that, in addition to changing Hct and SEp concentrations, a third variable, namely residual renal function, was superimposing. Together with decreasing Hct, CCr decreased as an expression of the continuously deteriorating kidney (Fig. 1). Thus, the increasing hypoxic stimulus was progressively inadequately answered, resulting in a positive overall correlation between Hct and SEp concentration. That this explanation seems to be correct shows the presence of a significant negative correlation between Hct and SEp \( (p < 0.001) \) when only minimal variation of residual renal function occurs, as found in our aforementioned group of hemodialysis patients with normal or slightly elevated SEp concentrations.\(^\text{41}\)

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Serum erythropoietin concentration in chronic renal failure: relationship to degree of anemia and excretory renal function

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