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Improving anemia by hemodialysis: Effect on serum erythropoietin

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Improving anemia by hemodialysis: Effect on serum erythropoietin. Serum erythropoietin (S_{EP}) concentration was measured on two occasions in 42 patients with terminal renal failure *(1)* immediately before the first hemodialysis, and *(2)* 3 to 27 months following the onset of regular hemodialysis treatment. Although the hematocrit (Hct) showed an increase in every patient, the S_{EP} concentration decreased in every patient. The mean Hct rose from 21.7 to 28.6% (volume per volume) ($P < 0.001$), and the S_{EP} dropped from 509 to 182 mU/ml ($P < 0.001$). This shows that anemia improvement is not a consequence of increased erythropoietin production but that it is most likely due to elimination of an inhibitor of the bone marrow by hemodialysis treatment. The decrease of S_{EP} concentration has to be interpreted as a response to the improved tissue oxygenation that correlates with the higher hematocrit or as a consequence of further reduction of renal mass with progress of the renal disease.

L'amélioration de l'anémie de l'insuffisance rénale: Effet sur l'érythropoiëtine sérique. La concentration d'érythropoiëtine sérique (S_{EP}) a été mesurée chez 42 malades atteints d'insuffisance rénale terminale, à deux reprises: *(1)* immédiatement avant la première hémodialyse et *(2)* 3 à 27 mois après le début de l'hémodialyse intérative. Alors que l'hématocrite (Hct) augmentait chez tous les malades, S_{EP} diminuait. L'hématocrite moyen est passé de 21,7 à 28,6% ($P < 0,001$), S_{EP} moyen a baissé de 509 à 182 mU/ml ($P < 0,001$). Cela montre que l'amélioration de l'anémie n'est pas la conséquence d'une augmentation de la production d'érythropoiëtine, mais plus probablement liée à l'élimination par l'hémodialyse d'un inhibiteur de la moelle osseuse. La diminution de S_{EP} doit être interprétée comme la réponse à une meilleure oxygénation tissulaire du fait de l'augmentation de l'hématocrite ou la conséquence d'une diminution de la masse rénale liée à l'évolution de l'affection.

An elevated serum erythropoietin (S_{EP}) concentration is only occassionally found in anemic patients with end-stage renal failure [1–3], and it is generally accepted that erythropoietin deficiency is a cause of renal anemia.

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Because peripheral hemoglobin concentrations increase significantly in almost every patient following the onset of regular hemodialysis treatment [1, 4, 5], and because there are sporadic reports of increase of S_{EP} that coincides with a shortening of radioactive iron-marrow transit time [1, 6], some investigators concluded that improvement of erythropoiesis is at least partly due to a gradual increase of renal or extrarenal erythropoietin production that was originally depressed by inhibitors accumulating in uremia [1, 6–8]. Others did not find the concentration of S_{EP} to be elevated after regular hemodialysis treatment was begun [9–11], and Erslev reported the production of erythropoietin to be unaffected by uremic serum added to the perfusion fluid of the isolated hypoxic kidneys of rabbits [12].

In the attempt to solve the controversy, we measured, by using the sensitive fetal mouse liver cell assay, the S_{EP} concentrations in a large group of patients with terminal renal failure before and after the onset of regular dialysis treatment. Our purpose was to find out if the improvement of anemia following regular hemodialysis treatment is accompanied by a consistent change in S_{EP} concentration.

Methods

On two occasions, we determined the S_{EP} concentrations and the hematocrits of 42 (27 male and 15 female) nonbilateral nephrectomized patients with end-stage renal failure. This was done *(1)* immediately before the first dialysis treatment when the patients were in a state of severe uremic intoxication, and *(2)* 3 to 27 months after the start of regular hemodialysis treatment (three 6- to 8-hr treatments per week). For controls, we used 59 healthy subjects. Dialysis was performed either with a Kiil dialyzer or with various disposable dialyzers with

an effective surface area of 1 m^2 and cuprophane membranes of 11.5 to 13.5 μ. All patients were on oral or parenteral iron substitution therapy. No blood transfusions or androgens were given, and no major accidental blood loss occurred during the study.

S_{EP} was measured with the fetal mouse liver cell bioassay described elsewhere [13, 14] with minor modifications [15]. Briefly, livers from 14-day-old mouse fetuses were dissected and cells suspended in a culture medium (Eagles Minimal Essential Medium) containing 10% (volume/volume) fetal calf serum and 5% (volume/volume) bicarbonate and carbon dioxide buffer with test sera or erythropoietin standard added. After 20 hours of incubation at 37° C, 1 μCi of radioactive iron previously bound to an equivalent amount of pure human transferrin (Behring Marburg) was added for pulse labeling for another 4 hours of incubation at 37° C. Heme was chemically extracted by using hydrochloric acid, Drabkin's solution, and butanone, and its radioactivity was measured in a β-liquid scintillation counter.

In another series of experiments, the specifity of the in vitro assay was tested. Human urinary erythropoietin (50 and 200 mU/ml) was preincubated for 3 hours with rabbit antiserum having an estimated potency capable of neutralizing 50 mU/ml of human erythropoietin. The effect in the fetal mouse liver cell culture was compared to a saline control and to the effect of an erythropoietin control culture (50 mU/ml) without antiserum.

To minimize the differences of the specific activity of radioactive iron transferrin due to different iron concentrations in the various test serum samples, we limited the test serum portion in the culture to 4% (volume/volume), we counterbalanced the variation of iron concentration in the serum samples by the constant iron pool of the fetal calf serum, which contained 270 μg of iron per deciliter, and we added the transferrin-bound iron in a constant amount (5 to 10 μg/dl of culture medium) for pulse labeling after 20 hours of incubation [15]. Because the iron concentrations of the test sera ranged from 38 to 142 μg/dl, causing a 10% (maximum) difference of the specific activity of radioactive iron transferrin in the culture medium, the resulting error of the measurement was well within the limits of accuracy of the method, with an index of precision ranging from 0.04 to 0.20 and 95% confidence limits of usually 80 to 120% of the mean potency [16]. So, no individual correction for iron concentration of the test serum sample was made. For laboratory standard, we used both a crude erythropoietin preparation (Connaught Laboratories, Toronto, Canada; step 3) and a healthy volunteer's serum sample, which was divided in 200 portions and kept deep-frozen until assayed. The laboratory standards were calibrated against the International Standard Preparation B (courtesy of the WHO Laboratory for Biological Standards, Mill Hill, London [17]). S_{EP} concentrations were expressed in international units (milliunits per milliliter). To have an exact estimate of the validity of every erythropoietin measurement, we determined a complete log dose-response relationship by assaying every serum sample in serial dilutions of three to four steps of five replicates, and we compared it to the standard samples assayed in the same way. Accordingly, the estimation of each serum sample was made from 15 to 20 cultures.

The two-paired serum samples of each individual patient were always measured in the same assay, to allow a direct comparison of the corresponding samples. By doing this, we increased the accuracy of the estimate. We proved the validity of every measurement by checking the significance of regression and by checking the absence of a significant deviation from linearity and parallelism in the log dose-response relationship of test and standard samples simultaneously, using the analysis of variance [8, 19]. The analysis of variance and the calculation of the mean erythropoietin potency of the serum samples and their 95% confidence limits were performed in the computer center of the Johann Wolfgang Goethe University at Frankfurt am Main. We used Student's paired t test to evaluate the clinical data.

Results

The S_{EP} concentrations and hematocrits are shown in Fig. 1. The solid lines represent the mean values. S_{EP} concentrations decreased in all patients, but the hematocrits increased. The S_{EP} concentrations before hemodialysis varied widely and ranged from 76 to 3070 mU/ml, whereas the hematocrits before dialysis ranged from 13 to 30% (volume/volume). Three to twenty-seven months after onset of regular hemodialysis treatment, S_{EP} concentrations ranged from 57 to 667 mU/ml; the corresponding hematocrits ranged from 19 to 41%. The differences between values before and after treatment are highly significant, when tested with the Student's combined t test ($P < 0.001$).

Table 1 shows the mean S_{EP} concentrations and the mean hematocrits before and after dialysis treatment in comparison with the mean values of the

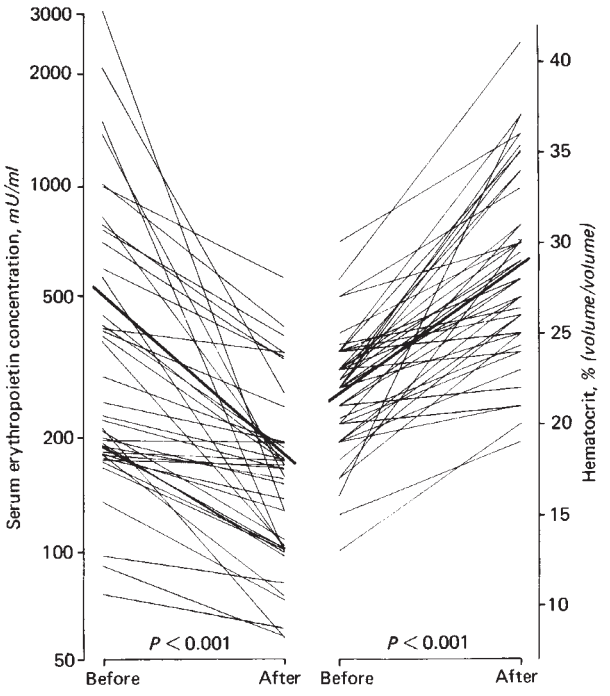

Fig. 1. *Serum erythropoietin concentrations* (left panel, logarithmic scale) *and hematocrits* (right panel, linear scale) *before and 3 to 27 months after the start of regular hemodialysis treatment.* The *thick line* denotes the mean values.

control group. Mean S_{EP} concentrations on both occasions were significantly higher than the mean S_{EP} concentrations of normal controls (509 ± [SD] 440 mU/ml, and 182 ± [SD] 110 mU/l, vs. 136 ± [SD] 66 mU/ml; $P < 0.01$). At the same time, mean hematocrits were significantly lower than the mean control values (21.6 ± [SD] 3.6%, and 28.7 ± [SD] 4.9% [volume/volume], vs. 42.7 ± [SD] 3.9%; $P < 0.001$).

Figure 2 shows the effect of rabbit antiserum against human urinary erythropoietin. Fifty neutralizing milliunits completely blocked the iron incorporation generated by 50 mU of human erythropoietin, as compared to the saline control culture.

Table 1. Serum erythropoietin (S_{EP}) and hematocrit before and after 3 to 27 months of regular dialysis treatment[a]

S_{EP} *mU/ml*			Hematocrit *% (vol/vol)*		
Before	After	Normal controls	Before	After	Normal controls (N = 59)
509 ± 440	182 ± 110	136 ± 66	21.6 ± 3.6	28.7 ± 4.9	42.7 ± 3.9
← $P < 0.001$ →	← $P < 0.01$ →		← $P < 0.01$ →	← $P < 0.001$ →	
← $P < 0.001$ →			← $P < 0.001$ →		

[a] Values are the means ± SD.

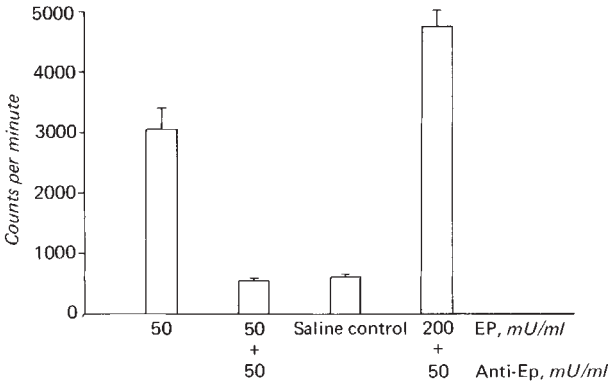

Fig. 2. *Neutralizing effect of rabbit antiserum against human urinary erythropoietin (EP).* Complete blocking (column 2) by equivalent amount of antiserum can be overcome by human erythropoietin added in excess (column 4). Values are the means ± SD of three cultures.

The blocking effect of the antiserum was overcome when erythropoietin was added in excess to the culture (200 mU/ml), proving the neutralizing capability of the antiserum to be specific and not due to a cytotoxic influence on the cell culture.

Discussion

The serum erythropoietin (S_{EP}) concentrations of normal subjects reported in the literature vary widely. The range from 3.7 to 11 mU/ml when measured with a radioimmunoassay [20] to 320 mU/ml when measured with the polycythemic mouse assay [11]. Large differences of S_{EP} concentrations of normal sera are also found when identical or similar methods are used. With the most frequently applied bioassay (the polycythemic mouse assay), most investigators are unable to detect S_{EP} concentrations in normal subjects [1–3, 9, 10]. When S_{EP} concentrations can be measured, however, reported mean concentrations range from 32 mU/ml [21] to 320 mU/ml [11]. Using a plasma concentration technique and the polycythemic mouse assay, Caro et al recently found the normal plasma erythropoietin concentration to be 7.8 mU/ml [22]. Even in the more sensitive radioimmunoassay, the reported normal values range from 3.7 to 11 mU/ml [20], to 52 to 84 mU/ml [23].

Using the fetal mouse liver cell assay, Napier et al found a normal mean value of 150 mU/ml [24], and De Klerk et al found a mean value of 48 mU/ml for males and 29 mU/ml for females [25]. Therefore, the mean S_{EP} concentration of 136 mU/ml found in our normal controls is in accordance with the results of Napier et al [24], but it is higher than the values reported by De Klerk et al [25], who used the

same method as Napier et al used. The value we found is also higher than that found by Lange and Ichiki (mean of 37 mU/ml) who used the hemagglutination-inhibition test [26], and higher than that found by Lertora et al (52 to 84 mU/ml) who used a radioimmunoassay [23].

From this discrepancy in the absolute values for normal S_{EP} concentrations, two questions arise. The first is related to the specifity of the method. Hemopoietic tissue cultures have been shown to be responsive to erythropoietin by Krantz, Gallien-Lartigue, and Goldwasser [27], Cole and Paul [28], Stephenson and Axelrad [29], Wardle et al [13], and Dunn, Jarvis, and Greenman [14]. More recently, Napier et al showed that the fetal mouse liver cell bioassay provides a quantitative measurement of erythropoiesis stimulating activity in a variety of hematologic disturbances, where the erythropoietic activity ranged from 6000 to a few milliunits per milliliter, and was significantly inversely correlated to the corresponding hemoglobin concentrations. They also reported that the erythropoietin in the human serum could be completely blocked by anti-erythropoietin antibodies [24]. This finding, which was also reported by De Klerk et al [25], is in accordance with our data in Fig. 2.

Dukes et al [30] and Goldwasser, Eliason, and Sikkema [31] showed that the polycythemic mouse assay is not sensitive to desialated erythropoietin, in contrast to cultures of hemopoietic cells, resulting in discrepancies of the measured erythropoietin content when different samples with varying portions of desialated erythropoietin were assayed. In vitro assays, where the target organ alone (that is, the hemopoietic cells) is directly exposed to the erythropoietin, seem to be more specific than are in vivo bioassays, where the sample has to be applicated to specially prepared animals. More data, however, will be necessary to confirm the specificity of the immunologic and biologic assays. Like most investigators, we wish to use the name "erythropoietin" operationally as a convenient and well-known term, for erythropoietin preparations are not yet proven to be chemically homogenous [32].

The second question concerns the accuracy of the measurement. The large differences of normal control values might be attributed, at least in part, to difficulties in calibrating the laboratory standard against the international reference preparation. Because of the limited supply of the international reference material, calibration can be performed only once or twice, and every accidental disturbance of the measurement leads to an erroneous result, which cannot be corrected by a larger number of measurements. Even in the most experienced laboratories, when the second International Reference Preparation was calibrated against the first, results of measurements of 10 U of erythropoietin by definition ranged from 5.4 to 19.1 U [17].

Fortunately, comparative studies are relatively independent of the absolute levels of normal control values, as long as the data can be referred to a sufficient number of normal control sera samples measured under the same conditions.

Concentrations of S_{EP} in patients with uremia are usually not detectable or equal to those of normal subjects when the polycythemic mouse assay is applied [9–11]. With immunologic methods, however, S_{EP} concentrations in anemic patients with uremia turn out to be higher than they are in normal persons. Lertora et al, using the radioimmunoassay, found S_{EP} concentrations in the range of 200 mU/ml in patients with uremia, compared with 52 to 84 mU/ml in normal controls [23]. With a plasma concentration technique, Caro et al found erythropoietin concentrations of 3.4 to 53.1 mU/ml in nephric and 2.8 to 5.5 mU/ml in anephric patients [22]. Interestingly, Lange and Ichiki found S_{EP} concentrations in 10 patients with uremia that ranged from 50 to 700 mU/ml when the hemagglutination-inhibition-assay was used. Of these 10 sera samples, the S_{EP} concentrations in 9 were significantly lower when the polycythemic mouse assay was applied. The authors claim that serum samples from patients with uremia are obviously underestimated in the polycythemic mouse assay [26]. Because the volume of the test serum sample applicated to the assay animal is usually 1 ml, which is in the range of 50% of the total blood volume and approximately 20% of the extracellular volume of the test animal, a bone-marrow depressing effect of the uremic serum cannot be excluded.

The very high S_{EP} concentrations found in our severely uremic patients before the start of regular hemodialysis treatment must be interpreted as a regulatory response to the extremely severe tissue hypoxia, resulting from the pronounced anemia as well as from fluid overload with respiratory and cardiac insufficiency of these decompensating patients. Erythopoietin concentrations of patients in the terminal phase of renal failure immediately before the onset of dialysis treatment have not been studied systematically by other investigators. There are, however, sporadic data demonstrating that an extreme hypoxic stimulus may cause a significant increase of erythropoietin production even in end-stage renal failure [8, 21, 33]. Fisher et al reported

two patients, one anephric and the other without detectable excretory renal function, who exhibited S_{EP} concentrations of approximately 420 mU/ml following a hemolytic episode resulting from a copper intoxication [33]. The extraordinary high S_{EP} concentrations have to be related to the pronounced hypoxic state of the patients because in every patient S_{EP} concentration dropped significantly to values slightly higher than those of normal subjects when regular hemodialysis treatment had been carried out for at least 3 months, and anemia, as well as the clinical situation, including respiratory and cardiac function, had improved. The mean S_{EP} concentration of 182 mU/ml at that time compares very well with the mean of 130 mU/ml measured in 88 nonnephrectomized patients who had been on regular hemodialysis treatment for more than 6 months [34], and with that of 104 mU/ml found in 13 anephric hemodialysis patients on testosterone therapy [16]. In both groups of patients, we also used the fetal mouse liver cell assay. In all three groups of dialysis patients, mean hematocrit did not exceed 30% (volume/volume). Thus, under a less severe hypoxic stimulus, the measured S_{EP} concentrations, which were in the same range as those of normal controls, have to be considered as being relatively decreased in regard to the degree of anemia. In patients without renal disease and with a comparable degree of anemia, S_{EP} concentrations were found to be considerably higher when the polycythemic mouse assay [35] and the fetal mouse liver cell assay [24] were used.

The observation that an additional hypoxic influence enables the diseased kidney to increase compensatorily its production of erythropoietin suggests that even in patients with terminal renal failure the regulatory feedback between hypoxia and S_{EP} concentration is sustained, probably operating at a lower level. The decrease, however, of S_{EP} concentration following the onset of dialysis treatment, in addition to the improvement of tissue oxygenation, may have been caused, at least in part, by a further reduction of renal mass with progress of renal disease, which is most likely accompanied by a further impairment of endocrine renal function. In 20 of our patients, in whom creatinine clearances were measured together with S_{EP} concentrations, it declined from a mean of 5.7 to 1.4 ml/min/1.73 m^2 ($P < 0.001$), indicating further loss of functioning renal parenchyma.

On the basis of the present data, we conclude that an increase of renal or extrarenal erythropoietin production can be excluded as a cause of the rise of peripheral hemoglobin concentrations following introduction of maintenance hemodialysis treatment in patients with end-stage renal failure, because in all patients the hematocrits increased in spite of a significant decrease of S_{EP} concentrations.

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