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Mechanism of angiotensin converting enzyme inhibitor-related anemia in renal transplant recipients

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Mechanism of angiotensin converting enzyme inhibitor-related anemia in renal transplant recipients. To delineate the pathogenesis of the reduction in hemoglobin occurring in renal transplant patients treated with angiotensin converting enzyme inhibitors (ACEI) and azathioprine (AZA) a controlled, prospective trial of ACEI withdrawal was conducted. The ACEI was replaced by nifedipine or clonidine in 15 kidney transplant patients immunosuppressed with AZA and prednisone (enalapril in 14 and captopril in 1). Before and during 10 to 12 weeks after withdrawal of the ACEI, AZA metabolites, renal function parameters and hematological parameters including erythropoietin and reticulocytes were evaluated. Enalaprilat levels were measured and compared with 15 similar patients matched for transplant function and enalapril dosage immunosuppressed with cyclosporine and prednisone. AZA metabolites did not differ significantly in the presence or absence of the ACEI. Enalaprilat levels also showed no significant difference between the two patient groups treated with AZA or cyclosporine. Hematocrit and hemoglobin increased significantly from 37.5 ± 6.4 to 39.7 ± 3.6% (mean ± SD, $P = 0.02$) and 12.8 ± 2.2 to 13.5 ± 1.2 g/dl, $P = 0.04$, respectively, 10 to 12 weeks after ACEI treatment had been discontinued. Simultaneously numbers of reticulocytes and erythropoietin concentrations rose significantly after 2, 4 and 10 weeks, with a peak at two weeks (from 14.1 ± 3.8 to 20.6 ± 8.0‰, $P < 0.05$ and from 14.3 ± 12.4 to 29.3 ± 54.5 mU/ml, $P < 0.05$, respectively). In conclusion, ACEI-related anemia in renal transplant recipients seems to be due to the erythropoietin-lowering effect of this group of drugs. A pharmacokinetic interaction between AZA and enalapril is not likely since plasma enalaprilat levels were independent of the immunosuppressive regimen and AZA metabolite levels were unchanged in the presence and absence of the ACEI. Several mechanisms by which angiotensin converting enzyme blockade may cause a decrease in circulating erythropoietin are discussed.

Angiotensin converting enzyme inhibitors (ACEI) have been in use for years in the treatment of hypertension and cardiac failure and their actions and side effects have been reviewed extensively [1, 2]. But, apart from a letter in *The Lancet* [3] describing a decrease in hematocrit in a group of hypertensives and healthy volunteers treated with MK-421 (enalapril), anemia is not listed as a typical side effect of this group of drugs [1, 2, 4]. In renal transplant patients several groups, including our own [5–7], have shown that treatment with ACEI can lead to a significant decline in hematocrit and hemoglobin. ACEI have also been used successfully in the treatment of erythrocytosis in these patients [8, 9]. We [7] and others [10] have also shown that the concomitant use of AZA as immunosuppressant probably contributes to the ACEI related anemia in this population. Furthermore, it has been shown that AZA-treated patients are less likely than cyclosporine-treated patients to develop post-transplant erythrocytosis [11].

Studies on the mechanism of ACEI related anemia have yielded conflicting results. While some researchers reported a decline of erythropoietin (EPO) levels in ACEI treated patients [9, 12–14] and healthy volunteers [15], others did not see such an effect [16–18]. Since essentially all reports of ACEI related anemia were on hemodialysis or renal transplant patients, we hypothesized that abnormally high enalaprilat levels, either caused by impaired renal excretion or, in the case of a well functioning renal transplant, by an interaction with AZA could explain the relatively high incidence of this complication in that group of patients.

Since isolated cases of anemia have also been seen in patients treated with AZA alone [19, 20] it also seemed conceivable that enalapril may induce anemia by affecting AZA bioavailability or metabolite levels.

To clarify the mechanism leading to anemia in these patients and to find out if there was a pharmacokinetic interaction between AZA and enalapril, we undertook a prospective trial of withdrawal of the ACEI in a group of 15 AZA-treated renal transplant patients. Transplant function, a number of hematological parameters, including EPO plasma levels, enalaprilat concentrations in plasma and urine, and AZA metabolites in red blood cells, plasma and urine were determined before and several times during 10 to 12 weeks after discontinuation of the ACEI. As a control, enalaprilat plasma and urine levels were also determined in a group of 15 renal transplant patients taking cyclosporine as immunosuppressant.

Methods

The study protocol was approved by the local ethics committee and all patients gave written informed consent. From the records of our outpatient renal transplant clinic we identified 18 adult patients who were treated with AZA and enalapril and one patient who took AZA and captopril. The reason for the ACEI treatment was hypertension in all cases. Four patients in the former group were unwilling to participate in the study and were therefore excluded. No other exclusions were made.

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All patients took AZA once daily in doses ranging from 0.8 to 2.7 mg/kg/day and enalapril (or captopril) once or twice daily (dose range enalapril 0.06 to 0.28, captopril 1.02 mg/kg/day). Patients came to the clinic on two separate occasions before enalapril was replaced by clonidine or nifedipine. Two separate 12-hour urine samples were collected the day before the blood drawing. A first blood sample was drawn at 8.00 a.m. to determine trough levels of AZA and enalaprilat, followed by oral administration of both drugs. Three hours later, approximately coinciding with peak plasma concentrations of 6-mercaptopurine, the main plasma metabolite of AZA and with maximal plasma concentrations of enalaprilat, the active diacid metabolite of enalapril, another blood sample was drawn. Blood samples for determination of AZA metabolites were drawn using lithium-heparin containing tubes, and samples for enalaprilat plasma levels were collected into sodium-heparin containers.

The same day, the following parameters were determined by standard clinical laboratory methods: sodium, potassium, calcium, inorganic phosphate, creatinine, urea, complete blood count (CBC) including reticulocytes, ferritin, iron binding capacity, haptoglobin, LDH, vitamin B-12, and folate.

Two weeks and four weeks later blood samples were analyzed for renal function parameters, CBC and EPO. Ten to 12 weeks after withdrawal of enalapril, the analysis of AZA metabolites was repeated twice and the above mentioned laboratory parameters were again determined.

Analytical procedures

For determination of 6-thioguanine nucleotides in red blood cells (RBC) these were washed twice in Hank's balanced salt solution and stored frozen at −20°C as well as the remaining plasma. Sample preparation for red blood cells and plasma included a solid phase extraction using a mercurial cellulose resin modified after Lavi and Holcenberg [21]. Thereafter, 6-thioguanine nucleotides as well as thiouric acid and 6-mercaptopurine were measured by HPLC using 4-thiouridine-5-monophosphate for internal standard as described by Weller et al [22]. Recovery of 6-thioguanosin-di-phosphate after extraction came to 73.8% and to 93.8% for 6-thioguanosin-tri-phosphate with a coefficient of variation below 10%. The lower limit of detection of 6-thioguanine nucleotides was 30 pmol/100 μl RBC, the intraassay coefficient of variation over the range from 50 to 1000 pmol/100 μl RBC varied between 5.4 and 8.9%, the interassay variation came to 6.7 to 11.4%. The recovery of 6-mercaptopurine and thiouric acid was determined to 65% and 69%, respectively, with variation coefficients also below 10%. The lower limit of detection was 5 ng/ml for both, 6-mercaptopurine and thiouric acid. Inter- and intraassay coefficients of variation were below 12%.

Enalaprilat plasma and urine levels were analyzed by radioimmunoassay as described [23, 24] using material kindly provided by Merck, Sharp and Dohme (Rahway, NJ, USA). The lower limit of detection was 0.2 ng/ml, inter- and intraassay coefficients of variation were 10.7 and 7.6%, respectively, for an enalaprilat concentration of 25 ng/ml.

EPO was measured by ELISA (Medac, Hamburg, Germany). Creatinine clearance, not corrected for body surface area, and fractional sodium excretion were calculated for each of the two visits at the beginning and the end of the study and averaged.

To study the possible influence of AZA on enalaprilat concentrations, we analyzed enalaprilat plasma and urine levels in 15 control patients, matched for enalapril dosage and transplant function, who were treated with cyclosporine and enalapril.

Table 1. Characterization of 15 renal transplant patients who were concomitantly treated with azathioprine and an ACE inhibitor (ACEI)

Patient No.	Age	Sex	Time since transplant *months*	Creatinine *mg/dl*	Azathioprine dose *mg/kg/day*	ACEI dose *mg/kg/day*
1	65	M	157	1.00	0.86	E 0.23
2	60	F	127	1.30	1.53	C 1.02
3	39	M	63	1.95	2.40	E 0.14
4	29	F	56	1.10	2.08	E 0.10
5	45	M	88	2.20	2.47	E 0.28
6	53	F	66	1.05	1.27	E 0.25
7	43	M	47	1.20	1.97	E 0.13
8	53	M	172	1.95	1.58	E 0.11
9	52	F	135	1.15	2.73	E 0.18
10	33	M	159	1.30	1.60	E 0.26
11	32	M	39	2.15	2.19	E 0.06
12	35	M	78	5.55	0.81	E 0.08
13	42	M	68	1.35	1.77	E 0.24
14	53	F	62	1.25	1.88	E 0.13
15	59	M	109	3.15	2.27	E 0.06

Abbreviations are: E, enalapril; C, captopril.

Statistics

All data are given as mean ± SD. For statistical evaluation, the data from the two visits before withdrawal of enalapril were averaged and compared with the averaged values from the two visits 10 to 12 weeks later by Wilcoxon matched pairs test. Since trough plasma samples did not contain measurable concentrations of 6-mercaptopurine and thiouric acid, only peak concentrations were used. Due to the long half life of 6-thioguanine in red blood cells, concentrations pre and post-drug intake were almost identical at steady state. Therefore, concentrations from all four blood samples were averaged both before and after replacement of enalapril.

Correlation analyses were performed by Spearman's test for rank correlation.

Differences between the AZA group and the cyclosporine treated patients were evaluated by *U*-test of Mann/Whitney. All calculations were done with Instat 2.0 (Graph-Pad software, San Diego, CA, USA), and P values < 0.05 were considered significant.

Results

The basic characteristics of the study group are given in Table 1. A considerable number of long-term transplanted patients had excellent kidney function. Table 2 shows the laboratory parameters of the 15 patients at the beginning and end of the study. Nine patients had subnormal hematocrit values (lower level of normal 42% for men and 37% for women [25]), and six patients had no evidence of anemia. During the study period there was a significant increase in hematocrit (from 37.5 ± 6.4 to 39.7 ± 3.6%, $P = 0.02$) and hemoglobin (from 12.8 ± 2.2 to 13.5 ± 1.2 g/dl, $P = 0.04$, Table 2) that was largely confined to the anemic patients. This was accompanied by a significant decrease in ferritin (from 257.40 ± 196.86 to 178.60 ± 139.73 mg/liter, $P = 0.04$) and, paradoxically, by an increase in LDH (from 171.20 ± 30.20 to 194.67 ± 40.74 U/liter, $P = 0.0003$). Diastolic blood pressure rose

Table 2. Laboratory parameters of 15 renal transplant patients before and 10–12 weeks after the end of ACE inhibitor treatment

	Week 0	Weeks 10–12	P^a
Hematocrit %	37.54 ± 6.42[b]	39.72 ± 3.56	0.022
Hemoglobin *g/dl*	12.76 ± 2.20	13.49 ± 1.16	0.035
Erthrocytes/pl	3.64 ± 0.79	3.78 ± 0.51	NS
Leukocytes/nl	6010 ± 1774	6313 ± 1712	NS
Thrombocytes 10^3*/nl*	243 ± 53	238 ± 61	NS
MCV *fl*	104.66 ± 9.64	105.76 ± 8.13	NS
MCH *pg*	35.62 ± 3.47	35.96 ± 2.96	NS
Ferritin *µg/liter*	257.40 ± 196.86	178.60 ± 139.73	0.041
Haptoglobin *mg/dl*	172.50 ± 102.04	155.00 ± 80.84	NS
LDH *U/liter*	171.20 ± 30.20	194.64 ± 40.74	0.0003
Vitamin B-12 *pg/ml*	277.47 ± 158.72	282.67 ± 182.24	NS
Folic acid *ng/ml*	7.35 ± 2.04	6.86 ± 2.41	NS
Creatinine *mg/dl*	1.84 ± 1.19	1.88 ± 1.11	NS
Systolic BP *mm Hg*	143 ± 19	153 ± 19	NS
Diastolic BP *mm Hg*	87 ± 12	94 ± 12	0.048
Fractional sodium excretion	1.58 ± 0.97	1.82 ± 1.48	NS

[a] Determined by Wilcoxon matched-pairs test
[b] Data are mean ± SD

from 87 ± 12 to 94 ± 12 mm Hg ($P = 0.05$). The other laboratory parameters showed no significant differences.

Parallel to the rise in hematocrit and hemoglobin EPO levels and numbers of reticulocytes increased significantly, reaching a peak value at two weeks after withdrawal of the ACEI (Fig. 1A). The large standard variation in the EPO levels shown is due mainly to one patient (Fig. 1B has the individual values). This was the patient with the worst renal function (patient 12 in Table 1), the highest enalaprilat level (46 ng/ml) and the most marked increase in both EPO levels (which increased by 333.79% after 2 weeks) and hematocrit (increasing by 72.33% at the end of the study). Exclusion of this patient from the analysis did not affect the significance of our findings. There was a strong positive correlation between enalaprilat trough concentrations measured before withdrawal of the drug and the percentage of increase in hematocrit ($r = 0.78$, $P < 0.01$, $N = 14$), suggesting a dose dependency of this effect (Fig. 2).

6-Thioguanine-nucleotides in red blood cells showed a large inter-subject variability ranging from 39 to 654 pmol/100 µl RBC; however, intraindividual variability during the observation period was below 20%. In contrast to the findings of Lennard et al [26], we found no correlation between the AZA dose per kg body wt and 6-thioguanine concentrations in RBC ($r = -0.396$, $P > 0.05$; not shown). 6-Mercaptopurine and thiouric acid in plasma, and 6-thioguanine nucleotides in erythrocytes remained almost unchanged in the presence or absence of enalapril, although urinary excretion of 6-mercaptopurine and thiouric acid decreased significantly from 141 ± 71 to 112 ± 97 µg/24 hr ($P < 0.05$) and from 1778 ± 912 to 1328 ± 748 µg/24 hr ($P < 0.05$), respectively (Table 3).

We considered it ethically and practically not feasible to change the immunosuppressive medication of the patients in the study group in order to assess the possible influence of AZA on enalaprilat concentrations. Therefore we used a group of enalapril-treated renal transplant patients with cyclosporine-based immunosuppression as controls. These were matched to the study group as to creatinine clearance and enalapril dosage. The average enalapril dose in AZA treated patients was 0.15 ± 0.07 mg/kg and in cyclosporine treated patients 0.18 ± 0.07 mg/kg, creatinine clearance was 71.05 ± 27.80 ml/min in AZA treated patients and 80.56 ± 38.35 ml/min in the cyclosporine group ($P > 0.05$). We found no statistically significant difference of enalaprilat levels in plasma and urine between the 14 patients on AZA compared with the group of 15 patients using cyclosporine (Table 4).

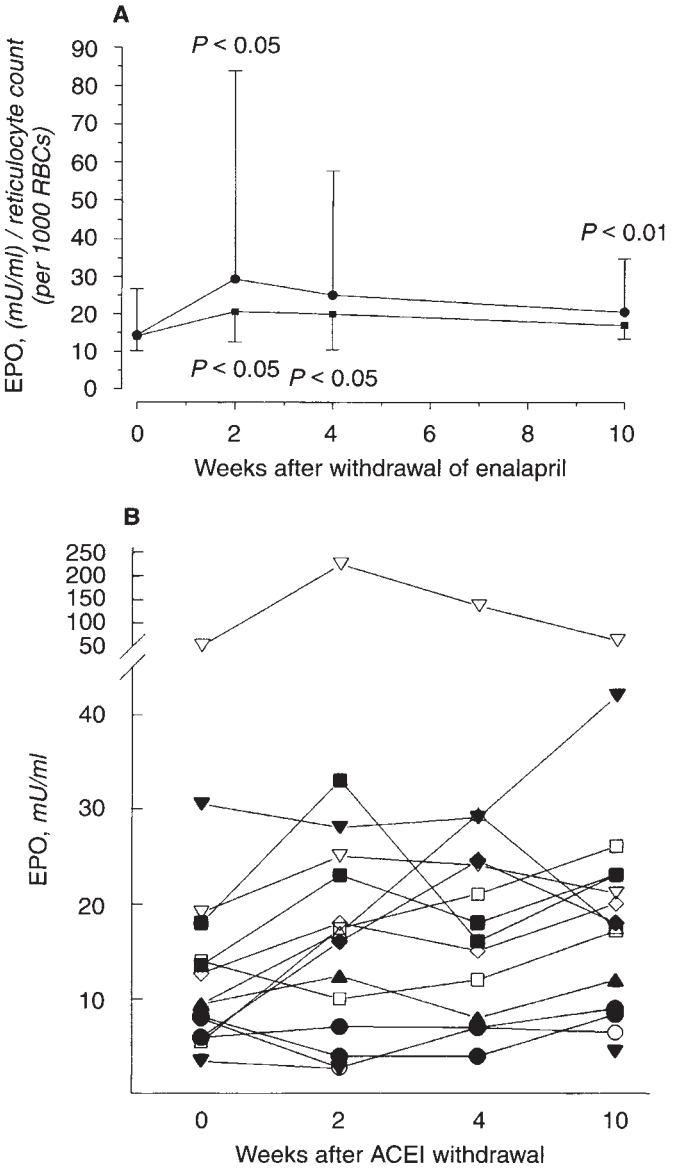

Fig. 1. *Erythropoietin (EPO) concentrations and reticulocyte counts of 15 renal transplant patients 2,4, and 10 to 12 weeks after discontinuation of the ACEI.* **A.** Mean values ± SD. *P* values were calculated with Wilcoxon matched pairs statistic. Symbols are: (●) EPO, (■) reticulocytes. **B.** Individual values of erythropoietin concentrations in the 15 patients.

Discussion

Our data support the view that ACEI related anemia, which has been described mainly in hemodialysis [13, 18] and renal transplant patients [5–7, 27, 28], is caused by a decrease in erythropoietin plasma concentration. A decrease of erythropoietin levels

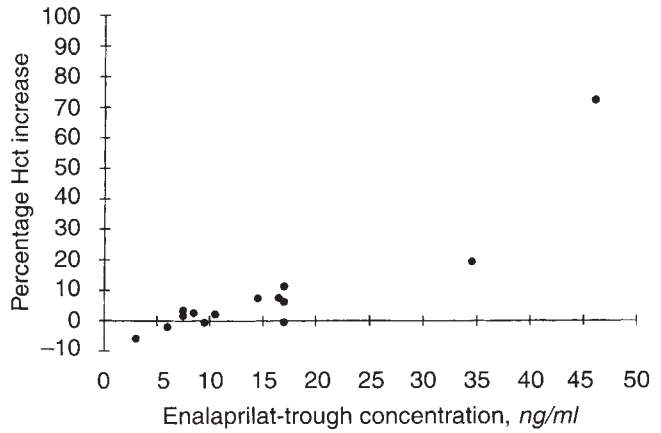

Fig. 2. *Correlation between the increase in hematocrit (Hct) after withdrawal of enalapril and plasma enalaprilat trough concentration at the beginning of the study.* Spearman rank correlation analysis, $r = 0.78$, $P < 0.01$, $N = 14$.

Table 3. 6-Thioguanine nucleotide (TGN) levels in red blood cells (RBCs), 6-mercaptopurine (MP) and thiouric acid (TUA) levels in plasma (p) and 24 hour urine (u) before and after withdrawal of the ACE inhibitor (ACEI)

	TGN *pmol/100 µl RBCs*	MP (p)	TUA (p)	MP (u)	TUA (u)
		ng/ml		*µg/day*	
With ACEI	277 ± 171	14.8 ± 9.9	398 ± 262	141 ± 71	1778 ± 912
Without ACEI	304 ± 194	15.3 ± 9.1	451 ± 253	112 ± 97	1328 ± 748
P value	NS	NS	NS	< 0.05	< 0.05

Data are mean ± SD. *P* values were determined by Wilcoxon matched-pairs test.

Table 4. Peak and trough concentrations in plasma and 12-hour urinary excretion of enalaprilat in renal transplant patients taking azathioprine (AZA) or cyclosporine (CS)

	Trough	Peak	Urine
	ng/ml		*mg/12 hr*
AZA group ($N = 14$)	14.53 ± 11.31	34.81 ± 16.13	1.16 ± 0.59
CS group ($N = 15$)	22.23 ± 15.31	36.70 ± 20.71	1.36 ± 1.13
P value	NS	NS	NS

Data are mean ± SD. *P* values were determined by the Mann/Whitney U-test.

following ACEI therapy has also been found in patients with renal insufficiency [12], hemodialysis patients [13], patients with congestive heart failure [14] and normal volunteers [15]. Several of the studies on the mechanism mediating the hematocrit lowering effects of ACEI in patients with post-transplant erythrocytosis seem to point in the same direction. All authors report a hematocrit-lowering effect of ACEI treatment in these patients, but only some of them found a significant concomitant reduction of erythropoietin levels [9, 29, 30]. Others found a decrease in the hormone that failed to reach statistical significance [16, 17].

The reason why an ACEI should lower erythropoietin production or increase its degradation is not understood. One hypothesis put forward by several authors [28, 31] is that ACEIs may lower EPO production by increasing renal blood flow. This hypothesis is supported by the findings of Tan and Ratcliffe [32], who showed that the production of EPO mRNA was not only dependent on the partial pressure of oxygen but could be enhanced by a decrease in flow rate in isolated perfused rat kidneys. These experimental data are in conflict, however, with the results of Pagel, Jelkmann and Weiss [33] and Kokot and Wiecek [34], who did not find a significant effect of renal artery stenosis on EPO production *in vivo*. Another explanation could be a decrease in renal oxygen consumption, which is largely determined by sodium absorption in the proximal tubules [35] that, among other mechanisms, is regulated by angiotensin II. Since sodium reabsorption is decreased by ACEI [36], a decrease in renal oxygen consumption may lead to an increased oxygen availability for the EPO producing cells. Support for this possibility comes from the experimental data of Eckhardt, Kurtz and Bauer [37], who described decreased EPO production after inhibition of proximal tubular sodium absorption by acetazolamide. We were, however, unable to detect a significant difference of the fractional excretion of sodium in the presence or absence of ACEI (Table 2), which may be due to concomitant diuretic medication. A third possibility is a direct influence of angiotensin II on EPO production, which has been shown in experimental animals [38–40]. Interestingly, suppression of angiotensin II levels has been found in dialysis patients treated with recombinant EPO for renal anemia, suggesting a feedback loop between EPO and the renin-angiotensin system [41].

AZA has been described to increase the amount of ineffective erythropoiesis [42] that may lead to a greater demand for EPO, rendering these patients more vulnerable than others for the EPO-lowering effect of ACEIs. Indeed, in patients with congestive heart failure and normal renal function [14] and in healthy volunteers [15] it has been shown that, despite a significant decline in erythropoietin concentrations following ACEI treatment, there was no measurable effect on hematocrit. In a prospective study [43] including 12 hypertensive patients with normal renal function treated with ACEI (the only formal study on this problem in non-renal patients known to us) it took 18 months until a decrease of hematocrit and hemoglobin, by 6.1% and 7.6%, respectively, became detectable. In our original observation [7] the ACEI related anemia developed in patients whose immunosuppressive regimen was converted from cyclosporine to AZA and who continued to take enalapril. Therefore, we suspected a pharmacokinetic interaction between the two drugs. Since this kind of anemia has been seen predominantly in patients with some degree of renal insufficiency and because most ACEIs are excreted almost exclusively by the kidney [44], we thought that inappropriately high enalaprilat plasma concentrations might be involved that, in our patients, might have been caused not by renal insufficiency but by the concomitant AZA medication. This possibility now seems to be largely excluded by our finding of similar enalaprilat levels in patients on AZA as compared to patients taking cyclosporine. To our knowledge this is the first study reporting enalaprilat plasma levels in renal transplant patients. Our data correspond well to the data on enalaprilat levels in patients with different degrees of renal insufficiency. In accordance with the findings published by Fruncillo and co-workers

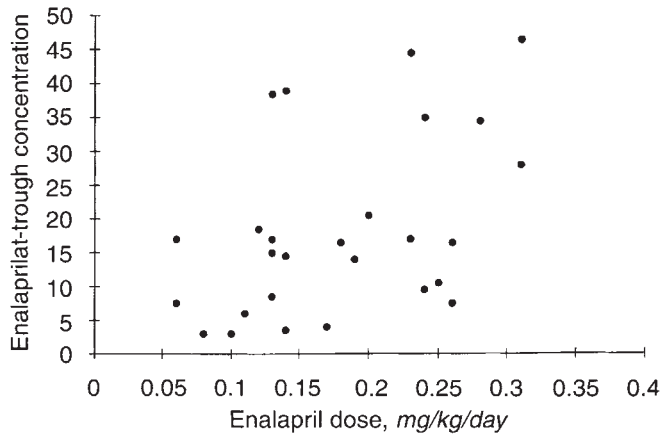

Fig. 3. *Correlation between enalapril dosage and plasma enalaprilat trough concentration in patients from the AZA and the cyclosporine group with a creatinine clearance above 30 ml/min.* Spearman rank correlation analysis, $r = 0.41$, $P = 0.03$, $N = 29$.

[45], a significant correlation of enalaprilat dose and plasma level was only found in patients with a creatinine clearance above 30 ml/min ($r = 0.41$, $P = 0.03$, $N = 27$, Fig. 3); in patients with lower clearances we found no correlation between dosage and plasma level.

Since AZA itself has also been implicated in rare cases of isolated anemia without evidence for bone marrow suppression [19, 20], and in one published case was found to be due to elevated thionucleotide levels in red blood cells caused by abnormal AZA metabolism [46], we also tested the possibility that enalaprilat might alter AZA bioavailability or metabolism. However, no significant differences in AZA metabolites with or without coadministration of enalapril were detected. The increased incidence of ACEI related anemia in patients taking AZA may simply be due to its antiproliferative action. In the face of the relative rarity of this side effect of ACEI in patients with normal renal function it appears that, for their effect on erythropoietin concentrations to become clinically relevant, another prerequisite must be fulfilled. This might be uremia or concomitant therapy with AZA.

We conclude that ACEI related anemia is causally linked to the erythropoietin-lowering effect of this group of drugs. A pharmacokinetic interaction between AZA and enalaprilat is unlikely to be the culprit in the face of essentially unchanged plasma levels of the major AZA metabolites in the plasma with or without enalapril comedication and comparable enalaprilat levels in AZA and cyclosporine treated patients. A likely mechanism explaining the influence of an ACEI on EPO levels is a feedback between EPO and the renin-angiotensin system.

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