Plasma vascular endothelial growth factor and angiogenin are positively related to erythropoietin dose in hemodialysis patients

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ABSTRACT

Purpose: Experimental data confirmed that erythropoietin (EPO) administration alters the course of various pathological situations such as heart failure and tumor growth by inducing vascular endothelial growth factor-A (VEGF-A) expression. The effect of EPO dose on plasma VEGF-A level in hemodialysis (HD) patients was evaluated. The effect of EPO dose on plasma angiogenin level in HD patients was also evaluated, since angiogenin is necessary for angiogenesis induced by VEGF-A.

Methods: Thirty two HD patients (10 diabetics) enrolled into the study. Patients were iron replete and did not suffer from infections, autoimmune diseases or malignancies. Plasma VEGF-A and angiogenin, as well as serum interleukin-6 and tumor necrosis factor-α were measured by means of ELISA.

Results: Weekly EPO dose per kg of dry body weight was positively related to both VEGF-A and angiogenin, whereas no relation was detected among VEGF-A or angiogenin and hemoglobin, inflammation or presence of diabetes mellitus. These relations among EPO dose and VEGF-A or angiogenin remained after adjustment for hemoglobin concentration or inflammation or presence of diabetes mellitus.

Conclusions: EPO dose may affect plasma VEGF-A and angiogenin concentrations in HD patients.

Key words: erythropoietin, vascular endothelial growth factor, angiogenin, hemoglobin, inflammation, diabetes mellitus, hemodialysis

INTRODUCTION

The introduction of recombinant Human erythropoietin (EPO) in every day clinical practice has revolutionized the treatment of anemia in hemodialysis (HD) patients [1]. However, besides fetal liver and adult kidney, EPO is also produced by various extra-renal tissues. Additionally, the expression of its receptor in non-erythroid tissues such as the brain, retina, heart, kidney, smooth muscle cells, myoblasts and vascular endothelium has been associated with the discovery of novel biological functions of EPO in non-hematopoietic tissues and the ability of exogenous EPO to modulate organ function and cellular responses [2].

Such a function of EPO is the induction of neoangiogenesis. EPO administration promotes vascular endothelial growth factor-A (VEGF-A) production predominantly in cardiomyocytes, which in turn stimulates myocardial endothelial proliferation and incorporation of endothelial progenitor cells increasing the capillary density and improving cardiac performance in a rat model of heart failure [3]. Another elegant experimental study showed that vascular EPO receptors play an important role in neoangiogenesis in response to hindlimb ischemia through upregulation

of VEGF-A and its receptor, both directly by enhancing neovascularization and indirectly by recruiting endothelial progenitor cells [4]. However, sometimes EPO-induced neoangiogenesis could be deleterious, for example in the case of growth of tumors. It has been established experimentally that EPO administration increases neoangiogenesis and promotes the growth of tumors lacking its receptor [5].

Considering that EPO administration induces neoangiogenesis in various experimental models, the association of EPO dose with the plasma levels of VEGF-A and angiogenin in HD patients was evaluated in this study. VEGF-A is one of the most potent and most studied angiogenic factors. It stimulates extracellular matrix degradation, proliferation and migration of endothelial cells and tube formation by them. Also it facilitates incorporation of endothelial progenitor cells. In addition, VEGF-A regulates vascular permeability by interfering with nitric oxide metabolism [6]. Angiogenin is a member of the ribonuclease (RNase) superfamily and is much less studied in HD patients. It is a normal constituent of the circulation and contained in vasculature that rarely undergoes proliferation, but in some physiological and pathological conditions its levels increase in blood, promoting neovascularization. Angiogenin binds membrane actin and induces basement membrane degradation. It affects endothelial cell proliferation by binding to a cell membrane receptor and entering into the nucleus of the target cells enhancing ribosomal RNA transcription and protein synthesis [7, 8]. Importantly, through the last mechanism angiogenin is necessary for angiogenesis induced by other angiogenic factors, the VEGF-A included [9].

Because EPO is used for the treatment of anemia in HD patients, the possible interference of hemoglobin (Hb) level with the effect of EPO dose on plasma VEGF-A and angiogenin was assessed.

The dose of EPO may need to be increased in case of inflammation [10, 11], which is common in HD patients [12, 13]. In addition, population based studies have shown that both circulating VEGF-A and angiogenin levels are related to serum markers of inflammation [14, 15]. There is also direct experimental data that angiogenin is regulated in vivo as an acute phase protein [16]. To our knowledge, in HD patients there are no data about the impact of inflammation on angiogenin level, whereas regarding VEGF-A the available studies are inconclusive. Some studies showed that serum VEGF-A level is associated with markers of inflammation [17, 18], whereas other studies did not find relation [19, 20]. Thus, the possible interference of inflammation with the effect of EPO dose on VEGF-A and angiogenin levels was evaluated as well.

VEGF-A is increased in children with diabetes mellitus (DM) type I and it is further increased in case of diabetic retinopathy, which is characterized by neovascularization [21]. On the other hand, serum angiogenin has been found decreased in patients with DM type II. The last authors suggest that low angiogenin levels could contribute to the known in DM reduced collateralization in ischemic tissues, which causes impaired wound healing, exacerbation of peripheral limb ischemia, and a three- to fourfold increase in cardiac mortality in comparison with non-diabetics [22]. Thus both neovasularization and impaired angiogenesis co-exist and play role in DM pathology. Therefore, the possible interference of DM with the effect of EPO dose on VEGF-A and angiogenin levels in HD patients was also assessed.

MATHERIAL AND METHODS

Patients
Thirty two HD patients (mean age 60.6±12.7 years, 20 men) participated into the study. All patients were anuric and the cause of end stage renal disease was DM in 10 patients, primary glomerulonephritis in 9 patients, hypertension in 2 patients, autosomal dominant polycystic kidney disease in 2 patients, obstructive nephropathy in 1 patient, analgesic nephropathy in 1 patient, Alport syndrome in 1 patient and unknown in 6 patients.

Patients underwent regular HD with polysulfone low-flux dialyzers and bicarbonate buffer for 4 hour sessions, 3 times a week and for at least 1 year prior to the study. The mean urea reduction ratio was 65.06±7.24%. Tinzaparin (Innohep; Leo Pharmaceuticals, Ballerup, Denmark) was used as anticoagulant in all patients. None of the patients had received blood transfusions at least during the 6 months interval prior to the study. With the exception of two patients, who did not need EPO therapy, all patients were receiving epoetin alpha (EPREX; Janssen Biologics B.V., Leiden, The Netherlands) intravenously at the end of each HD session.

Patients were selected to be iron replete with ferritin greater than 200ng/mL and transferrin saturation higher than 20%. Mean serum intact parathyroid hormone was 261.73±217.57pg/mL. Serum albumin was 3.98±0.29g/dL, body mass index 24.48±5.26Kg/m^2 and normalized protein catabolic rate 1.23±0.17g/Kg/day. None of the patients was hepatitis-B, hepatitis-C or Human Immunodeficiency Virus carrier or suffered from acute infection, malignancy or autoimmune disease. Finally, none of the patients was receiving cytotoxic drugs, corticosteroids or non-steroidal anti-inflammatory drugs.

An informed consent was obtained from each individual enrolled into the study and the hospital ethics committee gave its approval to the study protocol.

Methods
Blood samples were drawn just before the start of the second dialysis session of the week. The samples were centrifuged and the harvested platelet poor plasma and serum were stored at -80^{0}C.

Plasma angiogenin and VEGF-A were measured by means of the commercially available ELISA kits Quantikine Human Angiogenin Immunoassay and Quantikine Human VEGF-A Immunoassay respectively (R&D Systems Europe, Abington, UK). Platelet poor plasma instead of serum was preferred for these measurements because a significant and highly variable platelet-mediated secretion of VEGF-A during the clotting process has been reported [23].

The proinflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured in the serum by means of ELISA (Biosource Europe S.A., Nivelles, Belgium).

Statistical analysis

One-sample Kolmogorov–Smirnov test confirmed the normality of the evaluated variables. For evaluating relations Pearson's r was calculated. Because of the small size of the sample, multivariate linear regression analysis was performed three times. For evaluating the combined effect of EPO dose and Hb concentration on VEGF-A or angiogenin levels. For evaluating the combined effect of EPO dose and inflammation on VEGF-A or angiogenin levels. Finally, for evaluating the combined effect of EPO dose and DM on VEGF-A or angiogenin levels. For comparison of means unpaired t-test was used. Results were expressed as mean±SD and a p<0.05 was considered statistically significant.

RESULTS

Relations of VEGF-A and angiogenin with EPO dose, Hb and markers of inflammation and the impact of DM

Plasma VEGF-A concentration was 75.83±60.15pg/mL and plasma angiogenin concentration was 485.13±193.56ng/mL.

Serum IL-6 concentration was 14.43±12.90pg/mL and serum TNF-α concentration was 15.01±5.47pg/mL. Mean Hb concentration was 11.24±1.06g/dL, whereas mean weekly EPO dose per Kg of dry body weight was 198.61±128.28U/Kg.
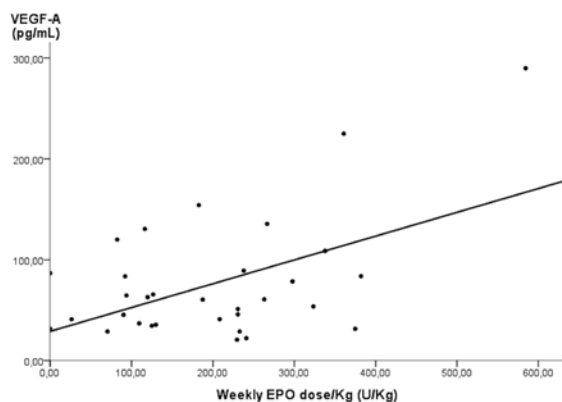
VEGF-A was positively related to weekly EPO dose per Kg of dry body weight (r=0.504, p=0.003), but not to Hb (r=0.105, p=0.569), IL-6 (r=0.066, p=0.721) and TNF-α (r=-0.132, p=0.473) (*Fig. 1*). Angiogenin was positively related to weekly EPO dose per Kg of dry body weight (r=0.467, p=0.007), but not to Hb (r=-0.195, p=0.286), IL-6 (r=-0.080, p=0.665) and TNF-α (r=0.330, p=0.065) (*Fig. 2*). Interestingly, VEGF-A was not related to angiogenin (r=0.012, p=0.949).

Because almost one third of the HD patients suffered from DM the impact of DM on plasma VEGF-A and angiogenin levels was evaluated. Plasma VEGF-A concentration was 67.60±40.10pg/mL in diabetics, and did not differ significantly with its concentration in non-diabetics, which was 79.56±67.86pg/mL (p=0.610). Similarly, plasma angiogenin concentration was 488.60±215.20ng/mL in diabetics, and did not differ significantly with its concentration in non-diabetics, which was 483.55±188.27ng/mL (p=0.947).

The interference of Hb or inflammation or DM with the effect of EPO dose on VEGF-A and angiogenin levels
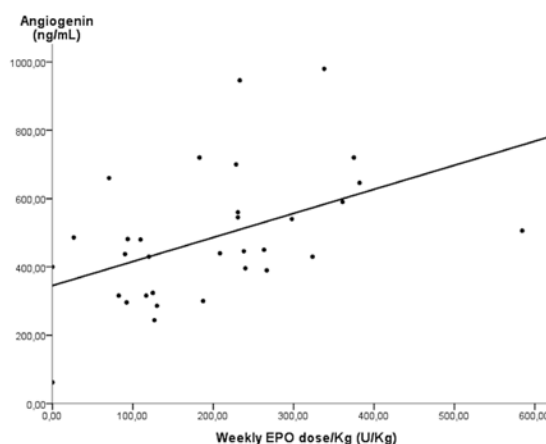
Multivariate linear regression revealed that EPO dose affects VEGF-A level independently of Hb concentration. Adjusted R^2 was 0.243 with a p=0.007. Standardized b was 0.538 with a p=0.002 for weekly EPO dose per Kg of dry body weight, whereas for Hb standardized b was 0.198 with a p=0.222 (Tab. 1a). Similarly, EPO dose affects angiogenin level independently of Hb concentration. Adjusted R^2 was 0.178 with a p=0.022. Standardized b was 0.447 with a p=0.011 for weekly EPO dose per Kg of dry body weight, whereas for Hb standardized b was -0.117 with a p=0.485 (Tab. 1b).

Figure 1. **Relation between erythropoeitin dose and VEGF-A.**

Weekly EPO dose per Kg of dry body weight is positively related to plasma VEGF-A concentration (r=0.504, p=0.003)

Figure 2. **Relation between erythropoeitin dose and angiogenin.**

Weekly EPO dose per Kg of dry body weight is positively related to plasma angiogenin concentration (r=0.467, p=0.007)

Multivariate linear regression revealed that EPO dose affects VEGF-A level independently of inflammation, assessed by IL-6 and TNF-α levels. Adjusted R^2 was 0.273 with a p=0.007. Standardized b was 0.595 with a p=0.001 for weekly EPO dose per Kg of dry body weight, whereas for IL-6 standardized b was 0.040 with a p=0.797 and for TNF-α standardized b was -0.315 and p=0.066 (Tab. 2a). Similarly, EPO dose affects angiogenin level independently of IL-6 and TNF-α levels. Adjusted R^2 was 0.198 with a p=0.027. Standardized b was 0.447 with a p=0.011 for weekly EPO dose per Kg of dry body weight, whereas for IL-6 standardized b was -0.135 with a p=0.411 and for TNF-α standardized b was -0.217 and p=0.210 (Tab. 2b).

Finally, multivariate linear regression analysis revealed that EPO dose affects VEGF-A level independently of the presence of DM. Adjusted R^2 was 0.219 with a p=0.011. Standardized b was 0.512 with a p=0.003 for weekly EPO dose per Kg of dry body weight, whereas for the presence of DM standardized b was -0.126 with a p=0.436 (Tab. 3a). Similarly, EPO dose affects angiogenin level independently of the presence of DM. Adjusted R^2 was 0.164 with a p=0.028. Standardized b was 0.468 with a p=0.008 for weekly EPO dose per Kg of dry body weight, whereas for the presence of DM standardized b was -0.017 with a p=0.919 (Tab. 3b).

DISCUSSION

In the light of experimental data which confirmed that EPO administration alters the course of various pathological situations such as heart failure [3], neoangiogenesis in response to hindlimb ischemia [4] and tumor growth [5] by inducing VEGF-A expression, we evaluated the effect of EPO

dose on plasma VEGF-A levels in HD patients. The effect of EPO dose on plasma angiogenin levels in HD patients was also evaluated, since angiogenin is necessary for angiogenesis induced by VEGF-A [9].

Both plasma VEGF-A and angiogenin levels were related to weekly EPO dose per Kg of dry body weight, whereas no relation was detected among these two angiogenic factors and Hb concentration, inflammation, assessed by IL-6 and TNF-α levels or the existence of DM. To our knowledge no other data

Table 1. The combined effect of weekly EPO dose per Kg of dry body weight and hemoglobin concentration on plasma VEGF-A (a) or angiogenin (b) levels.

(a)	VEGF-A	
	Standardized b	p
Weekly EPO/Kg	0.538	0.002
Hb	0.198	0.222
Adjusted R^2=0.243, p=0.007		

(b)	Angiogenin	
	Standardized b	p
Weekly EPO/Kg	0.447	0.011
Hb	-0.117	0.485
Adjusted R^2=0.178, p=0.022		

Table 2. The combined effect of weekly EPO dose per Kg of dry body weight, IL-6 and TNF-α levels on plasma VEGF-A (a) or angiogenin (b) concentrations.

(a)	VEGF-A	
	Standardized b	p
Weekly EPO/Kg	0.595	0.001
IL-6	0.040	0.797
TNF-α	-0.315	0.066
Adjusted R^2=0.273, p=0.007		

(b)	Angiogenin	
	Standardized b	p
Weekly EPO/Kg	0.447	0.011
IL-6	-0.135	0.411
TNF-α	-0.217	0.210
Adjusted R^2=0.198, p=0.027		

Table 3. The combined effect of weekly EPO dose per Kg of dry body weight and the presence or not of diabetes mellitus on plasma VEGF-A (a) or angiogenin (b) concentrations.

(a)	VEGF-A	
	Standardized b	p
Weekly EPO/Kg	0.512	0.003
DM	-0.126	0.436
Adjusted R^2=0.219 , p=0.011		

(b)	Angiogenin	
	Standardized b	p
Weekly EPO/Kg	0.468	0.008
DM	-0.017	0.919
Adjusted R^2=0.164 , p=0.028		

are available about the effect of EPO dose on angiogenin level in HD patients, whereas serum VEGF-A level was increased in peritoneal dialysis patients treated with EPO [24].

Hemodynamic and rheological changes can be induced by differences in Hb level. It is confirmed experimentally that hemodynamic changes can induce VEGF-A expression and neoangiogenesis in the heart in an EPO dependent way [25]. However, no relation among plasma VEGF-A or angiogenin levels and Hb concentration was detected in the present study. It should be noted that in the referred experimental study [25] the hemodynamic changes were severe since they were induced by   transverse aortic constriction. Certainly, changes in Hb concentration cannot induce such a dramatic change in the hemodynamic state.

In HD patients there are no data about the impact of inflammation on angiogenin levels. Plasma angiogenin level was not related to either serum IL-6 or TNF-α levels. Regarding VEGF-A, some studies showed that serum VEGF-A level is associated with markers of inflammation [17, 18], whereas other studies did not find relation [19, 20]. Our results are in agreement with the last studies, since we failed to detect relation between plasma VEGF-A level and serum IL-6 or TNF-α levels. It should be noted that patients suffered from infections, autoimmune diseases or malignancies were excluded from the study, decreasing the inflammatory burden in the cohort of the participated to the study patients. However, it is known that renal failure and HD procedure per se should be considered as inflammatory conditions [12].

Both neovasularization and impaired angiogenesis co-exist and play role in DM pathology [21, 22]. However, in the cohort of our patients, both plasma VEGF-A and angiogenin levels did not differ between HD patients with DM and HD patients without DM.

Because EPO is used for the treatment of anemia in HD patients, multiple linear regression analysis was performed with plasma VEGF-A or angiogenin as dependent variables and EPO dose and Hb as independent variables. It was shown that EPO dose affects both plasma VEGF-A and angiogenin levels independently of Hb levels.

Inflammation is common in HD patients [12, 13] and is a frequent cause of resistance to EPO treatment [10, 11], leading to the need for increased EPO doses. However, multivariate regression analysis with VEGF-A or angiogenin as dependent variables and EPO dose, IL-6 and TNF-α as independent variables showed that EPO dose affects both plasma VEGF-A and angiogenin levels independently of inflammation. It should be noted that in order to exclude the most common cause of resistance to EPO treatment, iron depleted patients were excluded from the study. Similarly, multivariate regression analysis revealed that that EPO dose affects both plasma VEGF-A and angiogenin levels independently of the presence or not of DM.

A limitation of the present study is the small number of the participated patients. However, if our results confirmed by larger studies, and considering the available experimental data, it is tempting to assume that increased neoangiogenesis because of EPO treatment could play a role in both beneficial and deleterious effects observed in clinical studies, such as the improvement in cardiovascular markers detected in patients with chronic heart failure [26], or the increased mortality in patients with chronic kidney disease, DM type 2, anemia and history of cancer detected in the TREAT study [27]. However, large randomized prospective clinical trials are required in order to elucidate if the observed non-hematopoietic effects of chronic EPO treatment are at least in part related to its effect on neoangiogenesis.

Two other factors have also to be elucidated. There are data suggesting that VEGF-A modulates erythropoiesis through inhibition of adult hepatic erythropoietin synthesis [28]. Because of the dramatically reduced functional renal mass in HD patients, it is possible that the contribution of the liver on endogenous EPO level in HD patients is increased, and could affect the dose of the required exogenous EPO. This aspect has to be further evaluated.

Levels of VEGF-A and angiogenin have been found to be increased in HD patients [29, 30]. Recently we showed that plasma angiogenin and VEGF-A levels are about twofold increased in anuric HD patients [31]. This could in part be attributed to renal failure per se. Indeed, in predialysis patients, plasma VEGF-A level showed a strong inverse association with estimated glomerular filtration rate [32]. In peritoneal dialysis patients a significant negative correlation between serum VEGF-A and residual renal function was detected. However, peritoneal dialysis patients treated with EPO showed significantly higher serum levels of VEGF-A than non-treated patients, although they had similar residual renal function [24]. The relative participation of renal failure and of EPO dose in VEGF-A and angiogenin levels has to be evaluated as well.

CONCLUSIONS

Plasma VEGF-A and angiogenin levels are positively related to EPO dose in HD patients. These relations remain after adjustment for Hb concentration or inflammation or presence of DM. The clinical significance of the above results remains to be elucidated.

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