Nephrol Dial Transplant (1999) 14: 2173–2177

Nephrology
Dialysis
Transplantation

Original Article

Serum thrombopoietin levels in haemodialysis patients: involvement of arteriovenous fistula

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Abstract

Background. Thrombopoietin (Tpo) is a recently cloned growth factor which plays a critical role in the regulation of thrombopoiesis. Tpo has also been shown to stimulate *in vitro and in vivo* erythroid cell growth. Although Tpo transcripts were detected in hepatocytes, proximal tubules and endothelium, mechanisms regulating the level of circulating Tpo have not been fully delineated. Changes in the vessel wall and blood flow in arteriovenous fistula (AVF) might alter Tpo activity.

Methods. Serum thrombopoietin levels and serum erythropoietin levels in samples concurrently obtained from venous returns of AVF and contralateral peripheral veins in 31 haemodialysis patients were determined and compared with 12 healthy controls. Levels were also compared between 14 haemodialysis patients (group I) treated with recombinant human erythropoietin (rHu-Epo) and 17 haemodialysis patients (group II) not requiring rHu-Epo.

Results. Serum Tpo levels (44.8 ± 23.9 pg/ml, *vs* 129.9 ± 113.6 pg/ml, $P < 0.05$) and platelet counts (194 ± 55. 10^6/ml *vs* 273 ± 94. 10^6/ml, $P < 0.05$) of haemodialysis patients were lower than healthy controls. Serum Tpo levels were inversely correlated with platelet counts in the control group ($R = -0.61$, $P < 0.05$), but not in haemodialysis patients. Tpo concentrations of AVF samples were lower than peripheral venous samples (31.6 ± 17.7 pg/ml *vs* 44.8 ± 23.9 pg/ml, $P = 0.001$). No significant difference was present between the serum Tpo concentrations of haemodialysis patients in group I and group II. Serum Tpo levels were not correlated with haemoglobin levels or serum erythropoietin levels in haemodialysis patients.

Conclusion. Decreased serum Tpo levels despite low platelet counts in haemodialysis patients suggest that the proposed feedback mechanism of platelet uptake of Tpo is not fully operative in these patients. Moreover, AVF might affect the local production and/or catabolism of this growth factor.

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Key words: arteriovenous fistula; erythropoietin; haemodialysis; thrombopoietin

Introduction

Thrombopoietin (Tpo- cMpl ligand) is a recently isolated haematopoietic growth factor which plays a regulatory role in megakaryopoiesis and thrombopoiesis [1]. It acts *via* a cell surface receptor (Mpl -R) present on pluripotent stem cells, megakaryocyte progenitors, megakaryocytes, platelets, and endothelium [2,3]. Although Tpo transcripts were detected in hepatocytes, proximal tubules and endothelium, mechanisms regulating circulating Tpo levels appear to be complicated and are controversial [4,5]. Platelets have been shown to bind and internalize circulating Tpo *via* Mpl -R expressed on its surface [6].

Erythropoietin and thrombopoietin, two major regulators of erythroid and megakaryocyte development, have been shown to have a high degree of amino acid sequence homology [7]. Consistent with this finding, progenitor cell cloning studies have suggested a common bi-potent progenitor for erythrocytes and megakaryocytes [8]. Given the closeness of their cell origin and sequence similiarity of the two growth factors, the issue of whether Tpo might play a role in erythropoeisis was raised. In several studies, Tpo has been shown to stimulate *in vitro* and *in vivo* erythroid cell growth [9–11]. Inhibiton of eythroid cell apoptosis by Tpo has recently been reported [12].

End-stage renal failure patients are at risk for haematological complications. Hypercoagulability, and paradoxically haemorrhagic tendency, have been described in uraemic patients [13]. Haemorrhagic complications are primarily due to uraemic platelet dysfunction and intermittent anticoagulation therapy used in haemodialysis [13–15]. Recently it has been proposed that changes in vascular wall morphology and blood flow in the arteriovenous fistula (AVF) might lead to both local and systemic alterations in coagulation and fibrinolysis in haemodialysis patients [16]. Shear stress

generated by the turbulent flow in AVF affects the gene expression and production of endothelial cell mediators and may cause cellular injury in endothelium [17]. Tpo production and expression of its receptor have been shown in endothelial cells [3,18]. Therefore AVF might influence Tpo activity in haemodialysis patients by altering its production and uptake by injured endothelium.

The aim of this study was to evaluate the regulation of Tpo levels in end-stage renal failure and assess the contribution of AVF on the activity of this multifunctional growth factor.Tpo levels were determined in samples concurrently obtained from the venous return of arteriovenous fistulae and contralateral peripheral veins in haemodialysis patients. Moreover, serum Tpo concentrations of haemodialysis patients were compared after they were stratified according to their recombinant human erythropoietin (rHu-Epo) requirement in order to clarify the role of this cytokine in the pathogenesis of the impaired erythropoiesis observed in uraemia.

Subjects and methods

Study protocol

Thirty-one ESRD patients (11 female, 20 male, mean age 42 ± 13 years, range 17–67 years) on maintenance haemodialysis three times a week utilizing cuprophane membrane were included in this study with their informed consent. Patients with a history of thrombosis (including thrombosis of a fistula, myocardial infarction, or deep venous thrombosis) or a haemorrhagic event within the previous 6 months and those with a history of malignancy, autoimmune disease, or a documented infection were not included in this study. In all patients, the haemodialysis procedure was performed by means of a Brescio–Cimino arteriovenous fistula created in one of the upper extremities, maintaining a blood flow of at least 200 ml/min. The mean duration of dialysis was 71 ± 28 months (range 12–121 months). The causes of end-stage renal failure were chronic glomerulonephritis in 13 patients, chronic pyelonephritis in nine patients, polycystic kidney disease in four patients, and unkown in five. All the patients were negative for hepatitis B antigen (HBsAg) and for hepatitis C virus RNA (HCV-RNA). Serial serum alanine aminotransferase and aspartate aminotransferase measurements were within normal limits in all patients for the previous 6 months. None of the patients received medication known to interfere with haemostasis (i.e oral contraceptives, anticoagulants) except for heparin administered during haemodialysis and rHu-Epo. All the patients received calcium carbonate as phosphate binder, iron and essential amino acid supplement, and antihypertensive therapy when indicated. None of the patients were administered angiotensin-converting enzyme inhibitor or angiotensin II-receptor antagonist as an antihypertensive drug. Fourteen of the haemodialysis patients were treated with recombinant erythropoietin at a dose of 205 ± 45 U/kg/s.c. per week (group I). Seventeen of the patients were only treated with oral iron supplementation (group II). Twelve age and gender-matched healthy volunteers (four female, eight male, mean age 37 ± 10 years, range 18–60 years) served as a control group. They were non-smokers and had no evidence of systemic disease. No control subject was suffering from infection and receiving any kind of medication.

Laboratory procedures

Blood specimens were obtained from the patients just before the haemodialysis session in the morning between 8:00 and 8:30 a.m and from the control subjects at the same time of the day. Blood samples of the patients were drawn both from the venous return of the AVF and the large veins of the contralateral upper extremity in haemodialysis patients and from large antecubital veins of healthy volunteers using a 19-G needle connected with a plastic syringe without interruption of the venous flow. Each blood sample was transferred to glass tubes containing no additives or anticoagulant. The clotted blood was centrifuged at 1500 r.p.m. for 15 min at 20–22°C and the obtained serum was stored at −20°C until assayed. Assays were carried out on duplicate serum samples thawed only once. Total blood cell count was also measured in haemodialysis patients and the control group.

Serum Tpo levels were determined by a solid-phase enzyme-linked immunosorbent assay (Quantikine R & D). Briefly, a murine monoclonal antibody specific for thrombopoietin was precoated onto a microplate. Assay diluent composed of a buffered protein base with preservative was added to each well. Standards and samples were pipetted into wells and incubated for 3 h at 2–8°C. After aspiration and washing four times with a buffer, monoclonal antibody against Tpo conjugated with horseradish peroxidase was added to each well and incubated for 1 h at 2–8°C. Aspiration and washing were repeated four times, and a mixture of hydrogen peroxide and tetramethylbenzidine was added to the wells and incubated for 30 min at room temperature. The reaction was stopped by addition of 2N sulphuric acid. Optical density of each well was determined within 30 min by using a microplate reader set at 450 nm. Tpo concentrations were extrapolated from a standard curve. The minimum detectable dose of Tpo was less than 15 pg/ml.

Serum Epo levels were measured by a solid-phase enzyme-linked immunosorbent assay (Quantikine R & D) based on the double-antibody sandwich method. Microtitre wells were precoated with a murine monoclonal antibody against rHu-Epo. Assay diluent was pipetted into each well. Standards and samples were added into wells and incubated for 2 h at room temperature. Polyclonal antibody against rHu-Epo conjugated to horseradish peroxidase was added to each well after aspiration and incubated for 2 h. Aspiration and washing were repeated four times and mixture of hydrogen peroxide and tetramethylbenzidine was added to wells and incubated for 20 min at room temperature. To stop the reaction, 2N sulphuric acid was added and optical density was determined within 30 min using a microplate reader set at 450 nm. Epo concentrations were extrapolated from a standard curve. The sensitivity of the test was determined to be 0.6 mIU/ml.

Statistics

To avoid a covariance effect, we performed multivarite analysis of the parameters using each preassay characteristic (age, gender, duration of dialysis, hypertension, haemoglobin, haematocrit platelet count) as a covariate in the model. Mann–Whitney U test was utilized to evaluate the differences between control group and haemodialysis patients and also for the comparison of parameters of two subgroup

of haemodialysis patients treated with or without rHu-Epo. The differences between peripheral vein samples and arteriovenous fistula samples of haemodialysis were analysed by Wilcoxon matched pairs signed-rank test. Correlation between parameters of haemodialysis patients were measured by Pearson's correlation test. Positive results obtained from correlation analysis were tested to fit in a linear model by regression analysis. Results were expressed as mean $\pm$ SD and differences were considered significant when P values were <0.05. SPSS (Statistical Package Program for Social Sciences) v. 5.01 for windows was utilized to analyse the data.

Results

Haemoglobin levels ($P<0.01$), haematocrit levels ($P=0.001$) and platelet counts ($P<0.05$) of haemodialysis patients were lower than normal controls (Table 1). Haemoglobin and haematocrit levels of patients treated with rHu-Epo (group I) were higher than the patients not requiring this therapy (group II) ($P=0.008$). No significant difference was present between the platelet counts of the two groups.

Serum Tpo concentrations in peripheral veins of haemodialysis patients were lower than controls ($P=0.03$) (Table 2). Circulating Tpo levels and platelet counts were inversely correlated in healthy volunteers ($R=-0.61$, $P=0.03$) (Figure 1), but not in haemodialysis patients. Tpo concentrations in AVF samples were lower than the peripheral values in haemodialysis patients (31.6 ± 17.7 pg/ml *vs* 44.8 ± 23.9 pg/ml, $P=0.001$) (Figure 2).

Serum Epo levels of haemodialyis patients both in group I ($P=0.001$) and in group II ($P=0.002$) were higher than in the control group (Table 2). Serum Tpo concentrations were similiar in group I and group II of haemodialysis patients ($P>0.05$) (Table 2). Serum Tpo concentrations were not correlated with Epo levels nor haemoglobin levels in the patient group and in the control group.

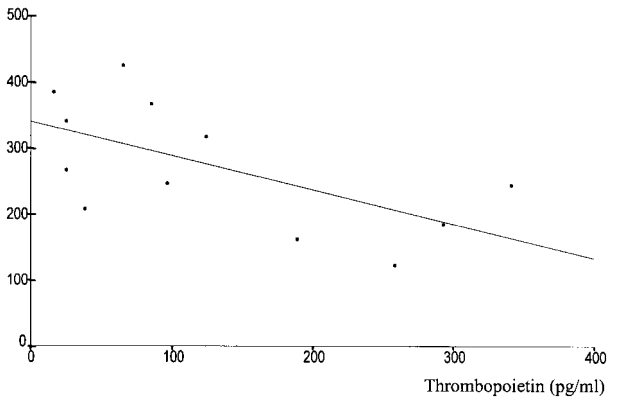

Fig. 1. Correlation of platelet counts and serum thrombopoietin levels in the control group ($R=-0.61$, $P=0.03$).

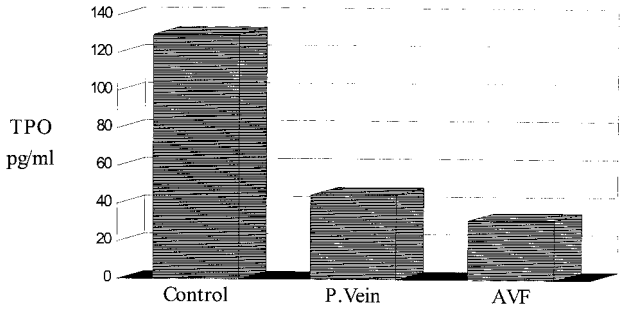

Fig. 2. Thrombopoietin concentrations of peripheral vein samples (P.vein) and arteriovenous fistula (AVF) samples of the haemodialysis patients and the control group (Control *vs* P.vein, $P<0.05$; P.vein *vs* AVF $P=0.001$).

Discussion

In addition to the well-known acquired qualitative platelet defects associated with uraemia, haemodialysis itself is frequently accompanied by transient platelet

Table 1. Haemoglobin levels, haematocrit levels and platelet counts of the haemodialysis patients and control group

	Control ($n=12$)	Group I ($n=14$)	Group II ($n=17$)	All patients ($n=31$)
Haemoglobin (g/dl)	14.1 ± 1.2	10.7 ± 1.3^{c}	9.5 ± 0.9	10.1 ± 1.3^{b}
Haematocrit (%)	44.2 ± 3.3	32.0 ± 3.4^{c}	28.4 ± 2.7	30.5 ± 4.6^{b}
Platelet (10^6/ml)	273 ± 94	203 ± 53	183 ± 58	194 ± 55^{a}

[a]$P<0.05$ haemodialysis patients *vs* control group. [b]$P<0.01$ haemodialysis patients *vs* control group. [c]$P<0.01$ group I *vs* group II.

Table 2. Serum thrombopoietin and serum erythropoietin levels of the haemodialysis patients and control group

	Control ($n=12$)	Group 1 ($n=14$)	Group II ($n=17$)	All patients ($n=31$)
Thrombopoietin (pg/ml)	129.9 ± 113.6	40.2 ± 23.3	48.5 ± 24.5	44.8 ± 23.9^{a}
Erythropoietin (mIU/ml)	3.7 ± 1.3	8.9 ± 5.9	6.2 ± 2.4	7.4 ± 4.4^{b}

[a]$P<0.05$ haemodialysis patients *vs* control group. [b] $P<0.01$ haemodialysis patients *vs* control group.

activation [19]. Moreover, decreased platelet counts and increased platelet turnover during haemodialysis have been reported [20,21]. Despite accumulated evidence regarding the effects of haemodialysis on platelets, the key regulator of thrombopoiesis, thrombopoietin, has not been studied in haemodialysis patients yet. In this study, serum Tpo levels and platelet counts of the haemodialysis patients were found to be lower than healthy volunteers. Although Kuter and Rosernberg [22] have postulated that Tpo activity is regulated by its binding to platelets and degradation, the mechanism of regulation of Tpo levels is still controversial. Both a decrease in serum Tpo levels with platelet transfusions and an inverse correlation between serum Tpo activity and platelet numbers have been shown in thrombocytopenic animals and patients [23–25]. In contrast to thrombocytopenic conditions, serum Tpo levels are elevated in reactive and primary thrombocytosis, despite the high platelet mass [26]. In this study, inverse correlation was found between serum Tpo levels and platelet counts in healthy volunteers, but not in haemodialysis patients. These findings suggest that the proposed feedback mechanism of Tpo by platelets is not fully operative or is not the only mechanism for regulation of Tpo in haemodialysis patients.

No change in Tpo mRNA in the liver and kidney of thrombocytopenic mice suggested constitutive production of Tpo rather than transcriptional regulation [27]. An 85% and 50% decrease in platelet counts in homozygous and heterozygous Tpo knockout mice respectively provide further support for constitutive production of Tpo [28]. As the kidney and liver are the major sites of constant production, the low Tpo activity in haemodialysis patients may be the decreased production of this growth factor. In cirrhotic patients activity of this haematopoietic growth factor was found to be well correlated with prolonged activated partial thromboplastin time and prothrombin time, indicating the capacity of the liver to synthesize the proteins [29]. Moreover, an increase in Tpo levels after liver transplantation has been recently reported [30]. Impaired kidney Tpo production might have a role in the low levels of Tpo observed in haemodialysis patients.

Recently, active research has focused on the role of arteriovenous fistulae in the abnormalities of haemostasis observed in chronic renal failure patients. It has been suggested that AVF might be an independent factor modulating both fibrinolysis and coagulation. In this study, the lower Tpo levels found in the arteriovenous fistulae compared to peripheral veins suggest that AVF might contribute to the metabolism of this growth factor in haemodialysis patients. Several mechanisms causing low levels of Tpo in AVF may be envisaged. An alteration in the production and expression of endothelial cell mediators such as endothelin, platelet-derived growth factor, and nitric oxide has been reported in endothelial cells exposed to shear stress *in vitro* [17]. Since secretion of Tpo has been shown in endothelial cell cultures [5], decreased local production in AVF by the endothelium under shear stress might play a role in the difference observed between the samples of AVF and peripheral vein. *In vitro* expression of Tpo receptors on vascular endothelium [18] and stimulation of growth and repair of liver endothelial cells by Tpo through its receptor have been recently reported [3]. Therefore, it might be hypothesized that increased uptake of Tpo by the injured endothelium under shear stress might contribute to lower levels of Tpo in arteriovenous fistulae in than peripheral veins. Alternatively, increased catabolism of Tpo due to the activated platelets within the AVF may be the other possible mechanism. In the vascular access, changes in blood flow, have been reported to activate platelets in haemodialysis patients [31,32]. On this basis, Tpo might bind to activated platelets but may then soon be consumed by aggregation of the platelets.

Considerable *in vitro* evidence suggests that Tpo has a stimulatory effect on erythropoiesis [9,10]. Moreover, recombinant Tpo administration has been shown to expand the erythroid progenitors and to augment the recovery of the erythroid cell lineage in a variety of myelosuppressed animal models [33,34]. However, no difference was observed in Tpo levels when haemodialysis patients were stratified according to their rHu-Epo requirement. In addition the serum levels of two growth factors, Epo and Tpo did not correlate in patients treated only with oral iron supplementation. Although these results suggest no role for Tpo in impaired erythropoiesis observed in renal failure, it is noteworthy that overall Tpo levels of haemodialysis patients were lower than those in healthy individuals.

In conclusion, serum Tpo levels were found to be decreased in haemodialysis patients despite the low platelet counts. Although the exact reason for this decline remains unknown, it might be related to the kidney's role in Tpo production. On the other hand, low levels of Tpo in AVF compared to peripheral venous samples suggest possible involvement of the fistula in the production and/or catabolism of this growth factor. These findings serve as a starting point for further studies to determine the regulatory mechanisms of Tpo levels and its role in end-stage renal disease.

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Received for publication: 31.8.98
Accepted in revised form: 14.5.99