Inverse relationships between haemoglobin and ristocetin-induced platelet aggregation in haemodialysis patients under erythropoietin therapy

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Abstract
Background. Amelioration of the anaemia of chronic renal failure and subsequent improved haemorheology result in correction of bleeding diathesis as evidenced by shortening of the skin bleeding time (BT). However, the relationship between the haematocrit and platelet-vessel wall interactions in haemodialysis (HD) patients under recombinant human erythropoietin (rHuEpo) therapy, assessed by platelet aggregation in response to ristocetin is more complex and somewhat inconsistent.

Methods. We investigated the relationship between haemoglobin (Hb) levels and whole blood ristocetin-induced platelet aggregation (electric impedance method) in 28 HD patients treated with rHuEpo, and with normal BT. The measurements were repeated in 16 subjects after having reduced platelet aggregability with orally administered ketanserin.

Results. Ristocetin-induced platelet aggregation in the whole group was comparable to those found in 21 age-matched healthy subjects (normals) and in 25 HD patients not treated with rHuEpo (uraemics). Interestingly, a significant inverse correlation between this aggregation and Hb concentration was found (r = -0.392, P < 0.05). In the group of 16 patients, the pre-ketanserin aggregation was more intensive than in the normals and uraemics (P < 0.05). Ketanserin produced a fall in ristocetin-induced platelet aggregation (P < 0.02), prolongation of the BT (P < 0.02) and, unexpectedly, a decrease in serum Epo concentration (P < 0.0002) and the Hb level (P < 0.001). Again, an inverse correlation between depressed ristocetin-induced platelet aggregation and lowered Hb concentration was found (r = -0.590, P < 0.02). Moreover, a strong positive correlation between the extent of pre-ketanserin platelet aggregation and the decrease in the intensity of this process that followed the trial was observed (r = 0.919, P < 0.00005). There were no changes in other haematological parameters or arterial blood pressure.

Conclusions. Considering the role of von Willebrand factor and fibrinogen in mediating ristocetin-induced platelet aggregation, and enhanced synthesis and/or release of these macromolecules in response to uraemia or inflammation, we suggest that exaggerated whole-blood platelet aggregability to ristocetin points to blunted erythropoiesis in HD patients on rHuEpo therapy.

Key words: erythropoietin; ketanserin; platelet aggregation; ristocetin; uraemia

Introduction
Prolongation of the skin bleeding time (BT) reflects defective formation of the primary haemostatic plug, and is a well-established estimate of uraemic bleeding tendency [1]. Decreased erythrocyte numbers result in less efficient radial transport of platelets towards the vessel wall, which is at least partially responsible for impaired platelet-vessel wall interactions. The crucial role of raising haematocrit in correcting bleeding diathesis in uraemia was evidenced by shortening of the BT that followed transfusion of packed red blood cells [2, 3], or amelioration of the anaemia by recombinant human erythropoietin (rHuEpo) without any evidence of activation of intravascular coagulation [4-7]. Another reliable measure of platelet-vessel wall interactions is their aggregation in response to ristocetin. The operative mechanism of this process involves complex interactions between platelet membrane glycoprotein (GP) Ib-IX, its ligand von Willebrand factor (vWF), fibrinogen, and subendothelial tissue [8-10], and has been reported to improve parallel with the rising haemoglobin (Hb) levels during rHuEpo therapy [11-13].

Herein we report an unexpected inverse correlation between Hb concentration and whole-blood ristocetin-induced platelet aggregation found in rHuEpo-treated haemodialysis (HD) patients. The study also describes...
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some consequences of pharmacological inhibition of ristocetin-induced platelet aggregation by ketanserin, which is an antagonist of platelet and vascular smooth muscle serotonin (5-hydroxytryptamine, 5-HT) 5-HT<sub>2</sub> receptors [14].

Subjects and methods

Patients

We studied 28 non-diabetic chronic HD patients. Dialysis dose was adjusted to Kt/V > 1.3. They all were treated with rHuEpo (Eprex<sup>®</sup>, Cilag AG International, Switzerland) injected subcutaneously. Relevant clinical data of these patients are outlined in Table 1. In sixteen of them, BP was stable with normal iron stores, and had no evidence of functional iron deficiency. Although measurements of blood PTH were not available, there was no biochemical or clinical evidence of severe secondary hyperparathyroidism. No patient was receiving any medication known to affect haemostasis, except for unfractionated heparin during HD. Dialysis was always performed using the double-needle technique, with cuprophane capillary dialysers, and with bicarbonate as buffer in the dialysate. Vascular access was in all cases a Cimino–Brescia arteriovenous fistula.

Within this group of patients, there was a subgroup of 16 subjects (Table 1) to whom ketanserin (Sufrex<sup>®</sup>, Janssen Pharmaceutica, Belgium) at a oral dose of 10–20 mg twice daily was administered. The studies were repeated after a 2-week (n = 12) and a 4-week (n = 4) ketanserin treatment. The rationale of such a design and some haematopoietic results of this trial have already been described in detail [15]. The research protocol was approved by our institutional ethical committee. All patients gave their informed consent.

Study design

A complete blood count and whole-blood platelet aggregation in response to ristocetin were measured in fasting patients in the morning before HD session. Bleeding time was measured and blood was taken by free flow through a 19-G butterfly needle at the onset of HD, and anticoagulated with 3.8% sodium citrate for platelet function studies. In the ketanserin-treated patients the measurements were repeated after the drug was withdrawn. Serum for Epo determination was prepared conventionally and stored at −40°C until assayed.

For platelet aggregation tests where standard reference ranges were not available, studies were also performed in 21 age-matched non-uraemic healthy individuals (normals), and in 25 HD patients not treated with rHuEpo (uraemics), for comparison with the patients studied.

Methods

Haematological parameters were determined by standard laboratory techniques. Bleeding time was measured using the method of Ivy modified by Mielke et al. [16]. Whole-blood platelet aggregation in response to ristocetin (0.6 g/l, Sigma, USA) was monitored by measuring electric impedance using Chronolog aggregometer (Chrono-Log Corp., Havertown, PA, USA) according to the method of Wilsoncroft et al. [17]. The extent of the aggregation was evaluated by measuring the maximal extension of the aggregation curve at 6 min after the addition of the agonist and expressed in ohms (Ω). Serum Epo concentration was determined using an enzyme-linked immunosorbent assay kit (EPO-ELISA, Boehringer Mannheim, Germany; normal range 0.4–9.0 U/l).

Statistical analysis

All data are mean ± SD. For pairwise comparisons, statistical analysis was performed by means of Student’s t test for paired and unpaired data with significance level set at P<0.05. Regression analysis after log–log transformation as well as linear regression analysis were employed to evaluate the significance of relationship between variables.

Results

The Hb concentration in the group of 28 patients was 10.1 ± 1.4 g/dl and BT was 6.1 ± 2.0 min. Ristocetin-induced platelet aggregation (15.2 ± 14.4 Ω) was not different from the value found either in the normals (11.6 ± 2.5 Ω) or the uraemics (11.4 ± 2.8 Ω). The regression line between ristocetin-induced platelet aggregation and Hb concentration showed a significant inverse logarithmic trend (r = −0.392, P<0.05) (Figure 1).

Ketanserin administration produced a significant fall in serum immunoreactive Epo concentration (21.8 ± 10.0 U/l pre, and 13.5 ± 8.4 U/l post, P<0.0002), a fall in the Hb level (10.2 ± 1.5 g/dl pre, and 9.4 ± 1.7 g/dl post, P<0.001), and prolongation of the BT (5.8 ± 1.9 min pre, and 11.2 ± 8.0 min post, P<0.02). Ristocetin-induced platelet aggregation value of 20.4 ± 17.1 Ω was significantly more intensive (P<0.05) than in the normals and uraemics, and decreased to 10.4 ± 7.1 Ω (P<0.02) following the ketanserin trial. Once more an inverse correlation between depressed ristocetin-induced platelet aggregation and lowered Hb concentration (r = −0.590, P<0.02) was found (Figure 2).

| Table 1. Clinical characteristics of the rHuEpo-treated HD patients and the subgroup of these subjects treated with ketanserin |
|-----------------|-----------------|
| Patients (n)    | 28              |
| Male vs. female | 18/10           |
| Age (years)     | 42.8 ± 15.3     |
| CRF causes      | 12/4            |
| primary glomerulonephritis (n) | 18            |
| interstitial nephritis (n) | 6             |
| ADPKD (n)       | 2               |
| unknown (n)     | 2               |
| HD duration (months) | 20.7 ± 13.7 |
| rHuEpo therapy duration (weeks) | 26.2 ± 10.2 |
| rHuEpo dose (IU week) | 4714 ± 1462 |

Data are mean ± SD.
ADPKD, adult dominant polycystic kidney disease.
As shown in Figure 3, a strong positive linear correlation between the extent of pre-ketanserin platelet aggregation and the decrease in the intensity of aggregation that followed ketanserin treatment was observed ($r = 0.919, P < 0.000005$).

The platelet count remained within normal limits in both groups of patients. Furthermore, neither the platelet count nor BP were affected by ketanserin (data not shown).

**Discussion**

The inverse correlations between the values of ristocetin-induced platelet aggregation and the concurrent Hb levels found in this study suggest that some of the factors involved in this complex aggregation process exert an inhibitory effect on erythropoiesis and therefore blunt the therapeutic response to rHuEpo.

Considering the elements involved in platelet aggregation in response to ristocetin, the level of GP Ib antigen in the platelets was found to be significantly reduced in uraemia [18]. Interestingly, an increase of expression of these molecules during rHuEpo therapy was reported [19]. If vWF antigen and ristocetin cofactor activity are considered, both elevated [12,19–21] or stable [11,22,23] values assessed in different stages of rHuEpo therapy were found. There is also a large body of clinical studies on changes of fibrinogen during rHuEpo therapy. Stable [5,11,23] as well as rising [24,25] plasma levels of these platelet-bridging molecules were found. Recently it has been shown [26] that, in contrast to platelet aggregation in response to other agonists, ristocetin-induced clumping is dependent on an influx of Ca$^{2+}$ via GP IIb/IIIa (the integrin $\alpha_{IIb} \beta_3$) rather than on a discharge from intraplatelet Ca$^{2+}$ stores. Inability of this integrin that functions as a fibrinogen receptor to undergo a conformational change during platelet activation has been reported in uraemia [27].

High plasma fibrinogen and vWF levels are well-established markers of acute phase reactions. More specifically, elevated levels of endothelium-derived vWF reflect a state of chronic endothelial cell injury often accompanying uraemia [28,29], or their activation during rHuEpo treatment [21,30]. Considering the above-described mechanisms of ristocetin-induced platelet aggregation, augmentation of this process may also reflect the presence of inflammation. This, often subclinical and not easily diagnosed state is usually followed by development of anaemia due to increased generation of numerous inflammatory cytokines which may either suppress Epo synthesis or induce a resistance of erythroid progenitors to the hormone [31].

Normal BT values in the whole group and improved ristocetin-induced platelet clumping encountered pre-treatment in ketanserin-treated patients give evidence of effective platelet-vessel wall interactions under rHuEpo therapy. The fact of significantly more intensive platelet aggregability in this subgroup of patients in comparison to the whole group could have been due to its greater homogeneity in regard to rHuEpo
treatment duration. In this population, 13 of 17 patients included were treated for as long as 8 months, which further confirms our findings that ristocetin-induced platelet aggregation is especially exaggerated in long-term rHuEpo patients (unpublished data). The BT was prolonged and ristocetin-induced platelet aggregation became depressed following ketanserin treatment. While ketanserin was shown to prolong BT by antagonising vascular alpha-adrenergic receptors or by preventing interaction between 5-HT and catecholamines in the vascular bed [32], the precise mechanism by which this agent reduces platelet aggregation remains speculative. In view of the stable platelet count, a 25% fall in fibrinogen concentration that induced platelet aggregation is especially exaggerated in these already anaemic patients included were treated for as long as 8 months, versus its use as an antiplatelet agent in these already anaemic patients. Although no relationships between rHuEpo dose and Hb levels, or between the former and platelet aggregability to ristocetin were found, based on this report, further studies should be undertaken to determine if enhanced ristocetin-induced platelet aggregation is an indicator of a state of hyporesponsiveness to rHuEpo in uraemic patients. It would also be of value to determine if this parameter can serve as a predictor of erythropoietic response in patients in whom the hormone is started. This would allow avoidance of dose escalation and would improve the cost-effectiveness of rHuEpo therapy.

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