Red blood cells may contribute to hypercoagulability in uraemia via enhanced surface exposure of phosphatidylserine

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Abstract

Background. The exposure of phosphatidylserine (PS) on the outer leaflet of the erythrocyte membrane may have several pathophysiological consequences, including the development of a procoagulant phenotype, a finding that seems relevant to the thrombotic risk seen in many disorders.

Methods. Because PS externalization increases in erythrocytes from patients suffering from chronic uraemia, which is frequently associated with a prothrombotic state, the possible relationship between erythrocyte PS exposure, erythrocyte procoagulant activity and plasma levels of several haemostatic markers was studied in a group of haemodialysed patients.

Results. Uraemic erythrocytes displayed increased procoagulant activity, which proved to be correlated directly with erythrocyte PS exposure. Pre-incubation of uraemic erythrocytes with annexin V, a protein with high affinity and specificity for PS, strongly inhibited in vitro thrombin generation induced by erythrocytes as compared with untreated red cells. Thrombin generation and activation of fibrinolysis were found to occur in uraemic patients, as substantiated by increased plasma levels of markers for thrombin generation (prothrombin fragment F1.2 and thrombin–antithrombin complex) and fibrinolysis (D-dimer and plasmin–antiplasmin complex), respectively. Significant correlations between prothrombin fragment F1.2 and D-dimer suggested that hyperfibrinolysis was secondary to thrombin generation. Correlations were also found between erythrocyte PS levels and plasma levels of haemostatic markers, including prothrombin fragment F1.2 ($P = 0.007$), thrombin–antithrombin complex ($P = 0.00009$), plasmin–antiplasmin complex ($P = 0.0009$) and D-dimer ($P = 0.005$).

Conclusions. Our study suggests that increased PS exposure may cause a pathological erythrocyte procoagulant phenotype, which may be a factor inducing a hypercoagulable state in uraemia.

Keywords: coagulation; erythrocyte; fibrinolysis; haemodialysis; hypercoagulability; phosphatidylserine; thrombin generation markers; uraemia

Introduction

Clinical experience indicates that both bleeding and thrombotic tendencies can be encountered in chronic uraemic patients undergoing haemodialysis (HD). The bleeding diathesis of uraemia seems mainly related to functional impairment of platelets [1]; the incidence of bleeding, however, is apparently declining at present. On the other hand, the tendency towards thrombosis (particularly of the arteriovenous shunt) is a frequent and major problem in dialysis [1]. Thrombotic complications have an increased incidence in patients suffering from uraemia [2] and may represent the predominant cause of mortality [3]. The existence of a hypercoagulable state in uraemia is suggested by several platelet and coagulation anomalies, including enhanced platelet reactivity, evidence of thrombin generation, increased levels of clotting factors and decreased levels of clotting inhibitors, hyperfibrinogenaemia and abnormal fibrinolytic activity [4–8].

Anionic phospholipids exposed on the outer leaflet of the cell plasma membrane may play an active role in the coagulation process by promoting the assembly of two coagulation factor complexes that lead to thrombin formation: the tenase complex and the prothrombinase complex [9]. In both complexes, negatively charged phosphatidylserine (PS) is the most effective phospholipid [10].
distributed in the cell plasma membrane, is normally confined to the inner membrane monolayer and maintenance of this asymmetry is of critical importance for the cell. Thus, loss of normal phospholipid asymmetry with PS appearance inside the outer leaflet of the plasma membrane is associated with many physiological and pathological phenomena, with particular regard to the processes of haemostasis, cell activation and cell–cell interaction [11,12]. Considerable evidence suggests that perturbations in red blood cell (RBC) PS exposure may contribute to the prothrombotic state of many disease conditions [13–17].

The cause of the hypercoagulable state in uraemia remains to be identified. We have recently shown an increased surface expression of PS in RBCs from end-stage renal disease (ESRD) patients [18], an abnormality that may have various pathophysiological implications [19,20]. It has not been assessed, however, whether the pathological RBC phenotype induced by PS externalization has any influence on the coagulation system balance in uraemia. In the present study, we determined the PS exposure and procoagulant activity of uraemic erythrocytes and, additionally, evaluated several haemostatic markers to look for a possible relationship between PS externalization and thrombin generation. Plasma prothrombin fragment F1.2, the prothrombin fragment cleaved by prothrombinase, and the thrombin–antithrombin complex were the parameters measured for in vivo thrombin generation. The main tests of fibrinolysis assessed included determination of D-dimer, a measure of both fibrin generation and degradation by plasmin, and of plasmin–antiplasmin, the irreversible complex between plasmin and its physiologic inhibitor α2-antiplasmin.

### Subjects and methods

#### Study population

Fifteen ESRD patients, 10 men and 5 women (mean age: 54 ± 15 years) undergoing maintenance HD for 4 h three times a week (mean time on dialysis: 97 months; range: 36–159 months) agreed to participate in this study. The aetiology of renal failure was nephroangiosclerosis (n = 6), chronic glomerulonephritis (n = 4), chronic interstitial nephritis (n = 3) and polycystic kidney disease (n = 2). All patients were on a stable anticoagulation regimen using unfractioned heparin at an infusion rate of 1000 U/h during the course of dialysis. Blood flow rate was 280 ml/min and the dialysate (bicarbonate) flow rate was 500 ml/min. Ultrafiltration varied according to the patient’s actual weight. First-use polyacrylonitrile (n = 4), polymethylmethacrylate (n = 4) and polysulphone (n = 6) dialyser membranes were used. Exclusion criteria included chronic or active infection; diabetes mellitus; malignant or systemic disease; a Kt/V ratio of <1.2; iron, folic acid or vitamin B12 deficiency; and use of antibiotics, corticosteroids, non-steroidal anti-inflammatory agents or any drug known to affect haemostasis (except for heparin during HD procedure). All patients were receiving erythropoietin therapy at a stabilized dosage and their haemoglobin levels were 11.3 ± 0.3 g/dl (range: 9.9–13 g/dl). In addition, none of the patients had received blood transfusions over the past 6 months. In parallel control experiments, blood from healthy blood donors (n = 15; 7 females and 8 males; mean age: 56 ± 2.5 years) was used.

The study was approved by the institutional review board of the hospital. All subjects were informed about the aim of the study and gave their consent.

#### Materials

Blood samples were obtained from ESRD patients before the start of treatment at the mid-week HD session. Blood was drawn into evacuated tubes containing ethylenediaminetetraacetic acid (EDTA). Erythrocytes were isolated by centrifugation at 400 g for 5 min, followed by three subsequent resuspensions and washing in saline (0.9% NaCl). The buffy coat was removed carefully. Erythrocytes were then resuspended in the appropriate buffer (see below). Annexin V, fluorescein isothiocyanate (FITC)-labelled annexin V and thrombin were from Sigma (St Louis, MO, USA). Purified prothrombin, factor Xa and the chromogenic substrate for thrombin CBS 34.47 (H-D-cyclohexylglycyl-z-aminobutyryl-arginyl-paranitroanilide) were purchased from Diagnostica Stago (Asnieres, France). Enzyme-linked immunosorbent assay (ELISA) kits for measurement of haemostatic markers were from Dade Behring (Marburg, Germany). All other chemicals were reagent grade.

#### Measurement of PS expression on the outer leaflet of RBC membrane

The exposure of PS at the extracellular face of the RBC plasma membrane was measured according to our previously reported flow-cytometric procedure based on the binding of FITC–annexin V [18]. Annexin V is a member of the annexin family of calcium-dependent phospholipid-binding proteins with a high affinity for PS. Isolated RBCs were diluted 1:100 (3–5 × 10^7 RBC/ml) to a final volume of 0.25 ml in a buffer containing 10 mmol/l HEPES–Na pH 7.4, 136 mmol/l NaCl, 2.7 mmol/l KCl, 2 mmol/l MgCl2, 1 mmol/l NaH2PO4, 5 mmol/l glucose, 5 mg/ml bovine serum albumin and 2.5 mmol/l CaCl2. Next, 100 nmol/l FITC–annexin V was added and the sample was incubated for 15 min at room temperature in the dark. After incubation, an aliquot was aspirated directly into the flow cytometer (Epics Elite; Coulter, Hialeah, FL, USA) for analysis. Fifty thousand events per sample were acquired. Fluorescence parameters were collected using a three-decade logarithmic amplification. The red-cell population was defined by size in forwards and side scatter plots; gated cells were counted as annexin-positive if they had a mean fluorescence of at least 1.0, a threshold value determined in previous studies [18]. The flow cytometer software was used to calculate the percentage of annexin-positive red cells.

#### RBC procoagulant activity

Procoagulant activity of erythrocytes was assessed by the prothrombinase assay performed according to Wilson et al. [13]. Isolated RBCs were resuspended at 0.1% haematocrit in Tris buffer (50 mM Tris/HCl, 120 mM NaCl, pH 7.4) containing 6 mM CaCl2, 0.33 U/ml factor V, 0.33 U/ml factor Xa and 1.3 U/ml prothrombin and incubated for 4 min at 37°C.
The reaction was stopped by adding EDTA at a final concentration of 15 mM. RBCs were sedimented by centrifugation and the amount of thrombin released during the incubation was measured in an aliquot of the supernatant (200 μl) using the chromogenic substrate CBS 34.47 (50 μM final concentration). The amount of thrombin released was measured by reference to a standard curve of authentic thrombin.

In some experiments, before being employed in the prothrombinase assay, uraemic RBCs were pre-incubated for 30 min with annexin V at a concentration (100 nmol/l) known to inhibit uraemic RBC PS-mediated processes [19,20].

Measurement of markers for thrombin generation and fibrinolysis

Plasma levels of human thrombin–antithrombin complexes, prothrombin fragment F1.2, D-dimer, plasmin–antiplasmin complexes, type 1 plasminogen activator inhibitor, α2-antiplasmin and plasminogen were measured using commercially available ELISA kits. All tests were performed according to the manufacturer’s instructions by the same person.

Statistical analysis

Data were analysed using the Sigmastat statistical package (Jandel Scientific, San Rafael, CA, USA). Because analyses of data revealed a non-parametric distribution, differences between controls and patients were analysed using the Mann-Whitney test. To assess the relationship between two variables, both Pearson and Spearman correlation tests were used. Both tests yielded similar results for the same pair of variables analysed. Results presented for correlation tests were obtained by the Pearson test on log-transformed data. All results are expressed as means ± SEM and a P-value of <0.05 was considered significant.

Results

RBCs from healthy subjects showed a very small fraction of red cells that had bound annexin V, with mean values ranging from 0.7% to 1.3% RBCs (Figure 1A). The mean percentage of annexin V-positive red cells in HD patients was significantly higher (P < 0.001) than in normal controls. The mean value for annexin V labelled RBCs in uraemic patients was 5.8 ± 0.5%, ranging from 3.8% to 11% (Figure 1A).

Procoagulant activity of RBCs from healthy individuals and HD patients was assessed by the prothrombinase assay, an established assay for the presence of PS on a cell membrane surface [10]. Indeed, several investigators have shown that when expressed on the external leaflet of cell membrane, PS provides the necessary lipid cofactor for the formation of the prothrombinase complex. Thrombin generation was significantly higher (P < 0.001) for samples containing RBCs from ESRD patients than for RBC samples from healthy control subjects (Figure 1B), indicating increased procoagulant properties for uraemic erythrocytes. Furthermore, a significant correlation (P = 0.001) was found in HD patients between annexin V-labelled RBCs (%) and thrombin generation (nM/min) induced by red cells (Figure 2). Pre-incubation of uraemic RBCs with annexin V to mask surface-exposed PS significantly reduced the amount of thrombin formation as compared with untreated red cells (2.9 ± 0.3 vs 7.03 ± 0.4 nM/min, respectively; P < 0.005; n = 6).

Plasma levels of markers of thrombin generation and fibrinolysis in normal controls and in patients on chronic HD are shown in Table 1. There were statistically significant differences between normal controls and HD patients for plasma levels of prothrombin fragment F1.2 and thrombin–antithrombin complexes (P < 0.001 and P = 0.008, respectively), suggesting ongoing thrombin generation in ESRD. Regarding markers of fibrinolysis, mean levels of D-dimer in the controls and patients with ESRD were 155 ± 9.9 and 335 ± 78 ng/ml, respectively (P = 0.006). Plasmin–antiplasmin complexes were also significantly increased in HD patients (P < 0.001), whereas plasma levels of α2-antiplasmin were reduced (P = 0.01). Plasma levels of both type 1 plasminogen activator
observed for type 1 plasminogen activator inhibitor, TAT (F1.2 (mmol/l) 0.72±0.04 1.03±0.05 <0.001 PAI-1 (AU/ml) 1.6±0.8 1.8±0.5 0.878 D-dimer (ng/ml) 155±9.9 335±78 0.006 PAP (ng/ml) 235±32 692±59 <0.001 correlation was for thrombin–antithrombin complexes and plasma prothrombin fragment F1.2, thrombin–antithrombin complexes; PAP, plasmin–antiplasmin complexes; PAI-1, type 1 plasminogen activator inhibitor; 2-AP, 2-antiplasmin.

Table 2. Relationships between levels of individual haemostatic markers and presence of PS-positive red cells in ESRD patients on chronic HD

<table>
<thead>
<tr>
<th>Marker</th>
<th>Controls (n = 15)</th>
<th>HD (n = 15)</th>
<th>P-value</th>
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</thead>
<tbody>
<tr>
<td>F1.2</td>
<td>0.72±0.04</td>
<td>1.03±0.05</td>
<td>&lt;0.001</td>
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<tr>
<td>TAT</td>
<td>2.9±0.1</td>
<td>6.8±1.3</td>
<td>0.008</td>
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<tr>
<td>PAI-1</td>
<td>1.6±0.8</td>
<td>1.8±0.5</td>
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</tr>
<tr>
<td>2-AP (%)</td>
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<td>70.5±4.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Plasminogen (%)</td>
<td>94.2±12.3</td>
<td>111.9±33.8</td>
<td>0.082</td>
</tr>
</tbody>
</table>

Values presented are means±SEM. The P-value was obtained by the Mann–Whitney test.

Table 2. Relationships between levels of individual haemostatic markers and presence of PS-positive red cells in ESRD patients on chronic HD

We next examined the relationships between plasma levels of individual haemostatic markers and the presence of PS-positive erythrocytes. Significant correlations were found between levels of prothrombin fragment F1.2 and D-dimer (r = 0.564, P < 0.05) and between D-dimer and plasmin–antiplasmin complexes (r = 0.541, P < 0.05).

We next examined the relationships between plasma levels of individual haemostatic markers and the presence of PS-positive erythrocytes. Significant correlations were found between levels of prothrombin fragment F1.2 and D-dimer (r = 0.564, P < 0.05) and between D-dimer and plasmin–antiplasmin complexes (r = 0.564, P < 0.05).

In the present study, we also examined the fibrinolytic system activity. Results on fibrinolysis in ESRD have been somewhat discrepant. Some studies have reported decreased fibrinolytic activity in uraemia, whether absolute [5] or relative to the extent of coagulation activation [8]. Other authors have documented hyperfibrinolysis [4,6,7], which seems to be secondary to fibrin deposition. Our results show activation of the fibrinolytic system in ESRD patients, as evidenced by raised levels of plasmin–antiplasmin complexes and of D-dimer and by diminished activity of 2-antiplasmin (an inhibitor of plasmin with which it forms an irreversible complex), most likely due to increased consumption. The significant correlation found between prothrombin fragment F1.2 and D-dimer (P < 0.05) supports the hypothesis that increased fibrinolytic activity and D-dimer formation in uraemia is secondary to the generation of thrombin.
and fibrin deposition. That hyperfibrinolysis in our patients might be unrelated to the effect of plasmin and attributable to white-cell proteolytic activity seems to be excluded by the absence of leukocytosis (data not shown) and by the correlation observed between D-dimer and plasmin-antiplasmin complex levels \( (P < 0.05) \), suggesting that fibrinolysis occurs because of plasmin generation.

In both sickle cell disease and beta-thalassaemia, an increase in red-cell procoagulant activity has been shown and this seems tied to exposure of PS on the erythrocyte surface \([14,15]\). The present study found not only increased RBC PS exposure, confirming our previous findings \([18–20]\), but also an RBC pathological procoagulant phenotype, which represents a new observation in uraemia. A role for surface-exposed PS in the increased procoagulant property displayed by uraemic erythrocytes is suggested by several observations. Uraemic RBCs were shown to enhance thrombin generation in a prothrombinase assay, which measures the conversion of prothrombin to thrombin induced by PS exposure in intact RBCs \([11]\). In addition, a highly significant correlation was found in uraemic RBCs between PS levels and prothrombinase activation. Strong evidence is provided by the significant inhibition in thrombin release by uraemic RBCs pre-incubated with annexin V, which has a propensity for binding to PS and rendering it unavailable for other PS-mediated processes. Thus, although our data do not exclude the possibility that other factors also may be important, they indicate a major role for externalized PS in the increased procoagulant activity of uraemic RBCs.

Increased PS exposure in a subpopulation of RBCs is seen as a pathogenic mechanism for the increased risk of thromboembolic complications witnessed in sickle cell disease, beta-thalassaemia and hereditary hydrocytosis \([15–17]\). Our study suggests that hypercoagulability in uraemia may, at least partly, result from increased RBC procoagulant activity linked to loss of lipid asymmetry. We found significant correlations between erythrocyte PS positivity and plasma levels of thrombin generation markers, such as prothrombin fragment F1.2 and thrombin–antithrombin complex, which suggests that the procoagulant effect of uraemic RBCs may amplify thrombin generation in vivo. A direct correlation was also found between erythrocyte PS levels and both D-dimer and plasmin–antiplasmin complex plasma levels. Similar correlations, previously noted in sickle cell disease, pointed to the sickle erythrocyte as being the cell responsible for the thrombophilia of that disease state \([15]\). Our data also show a positive link between erythrocyte PS levels and thrombotic events in uraemic patients. From these results, it is reasonable to assume a promoter effect on coagulation resulting from RBC PS exposure, which may be implicated in the high incidence of thrombotic diseases in uraemic patients \([2,3]\). Alternatively, RBC PS exposure could represent a new marker or a predictor of clinical thrombosis in uraemia.

In conclusion, the present study provides additional evidence for the existence of a chronic hypercoagulable state in patients with chronic uraemia and suggests that increased PS exposure on the outer RBC membrane surface may facilitate the activation of the haemostatic system by providing a permissive surface for assembly of the prothrombinase complex. Our data support \([19,20]\) the biological relevance of abnormal RBC phospholipid asymmetry in the pathophysiology of the uraemic syndrome.

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Conflict of interest statement. None declared.

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