Removal of uraemic plasma factor(s) using different dialysis modalities reduces phosphatidylserine exposure in red blood cells

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Abstract

Background. Solute(s) retained during uraemia cause increased exposure of aminophospholipid phosphatidylserine (PS) on the outer surface of erythrocyte membranes, and this phenomenon may be involved in the pathophysiology of uraemia by promoting abnormal erythrocyte interactions.

Methods. We examined in a prospective randomized cross-over fashion the ability of various dialysis modalities to remove the circulating uraemic factor(s) causing increased PS externalization in red cells. Each patient was treated with haemodialysis (HD) and with on-line haemodiafiltration (HDF) using standard high-flux polysulphone membranes or with the new polisulphone-based Helixone membrane to compare the effects of dialysis technique and membrane type on PS exposure. Removal of PS was assessed indirectly by measuring PS-expressing normal erythrocytes exposed to uraemic plasma or to ultrafiltrate obtained at various time points during the extracorporeal session.

Results. Removal of the uraemic plasma factor(s) causing PS exposure was demonstrated by the reduced ability of uraemic plasma at the end of dialysis to induce PS externalization in red cells. Each patient was treated with haemodialysis (HD) and with on-line haemodiafiltration (HDF) using standard high-flux polysulphone membranes or with the new polisulphone-based Helixone membrane to compare the effects of dialysis technique and membrane type on PS exposure. Removal of PS was assessed indirectly by measuring PS-expressing normal erythrocytes exposed to uraemic plasma or to ultrafiltrate obtained at various time points during the extracorporeal session.

Conclusions. Uraemia is associated with retention of compound(s) that are lipophilic, possibly protein-bound and which cause an abnormal exposure of PS in erythrocytes. Our findings, that such compound(s) can be removed during dialysis and at higher rates with convection techniques, indicate a potential benefit for uraemic patients. The present results also seem to confirm the marked ability of high-flux Helixone membranes to eliminate high molecular weight solutes.

Keywords: erythrocyte; haemodialysis; helixone; high molecular weight solutes; on-line haemodiafiltration; phosphatidylserine

Introduction

The phospholipids of the human red blood cell (RBC) are distributed asymmetrically in the bilayer of the red cell membrane. The outer leaflet of the RBC plasma membrane is formed predominantly of choline-containing phospholipids (phosphatidylcholine and sphingomyelin), whereas the amine-containing phospholipids are localized mainly (phosphatidylethanolamine) or exclusively (phosphatidylserine) in the membrane’s inner leaflet. Maintenance of this asymmetry is an energy-requiring process of major physiological importance for the cell. Indeed, loss of normal phospholipid asymmetry, especially the appearance of phosphatidylserine (PS) at the outer leaflet of the erythrocyte membrane, may have several pathophysiological implications [1,2]. The exposure of PS on the surface of RBCs represents a well-defined signal for recognition by macrophages and subsequent
elimination from circulation [3]. Patients with sickle cell disease have an increased PS exposure on erythrocytes [2,4], and surface-exposed PS has been implicated in the decreased RBC survival time characteristic of the disorder [4]. PS-expressing RBCs also show a propensity for adhesion to the endothelium [5], a pathophysiological cell–cell interaction that may explain coincident PS exposure on RBCs and vascular damage in disorders such as sickle cell anaemia, diabetes and thalassaemia [2,4]. The presence of PS at the cell surface may also affect haemostatic balance [1].

A number of abnormalities in the structure and function of erythrocyte membranes have been found in patients suffering from chronic renal failure. Recently, we observed a significant increase in PS-exposing erythrocytes in uraemic patients compared with healthy controls [6]. This altered phospholipid asymmetry is most likely caused by inhibition of PS transport from the outer to the inner leaflet of RBC plasma membrane [6], and may be of relevance for the pathophysiology of the uraemic syndrome by promoting abnormal RBC interactions [7,8]. We have also demonstrated that increased erythrocyte PS externalization occurs upon exposure to uraemic plasma. Preliminary characterization of putative uraemic plasma compound(s) causing increased PS exposure points to heat unstable substances having a molecular weight between 10 000 and 20 000 Da [6]. However, little is known about whether and to what extent such factor(s) are removed during dialysis.

The present prospective cross-over study was performed to determine whether various dialysis modalities are able to remove the circulating uraemic factor(s) that cause increased PS exposure on RBCs. Patients were treated with haemodialysis (HD) and with on-line haemodiafiltration (HDF) using a conventional polysulphone membrane or the new Helixone membrane to compare the effects of both membranes and techniques (diffusive vs convective) on PS exposure. Helixone is a polysulphone-based high-flux membrane characterized by nanoscale modulation of the innermost surface structures and was designed specifically for removal of uraemic toxins in the size range of β2-microglobulin (11 800 Da) while maintaining a low albumin sieving coefficient [9,10]. The membrane structure characteristics and permeation-related parameters of Helixone have been described recently [10].

**Subjects and methods**

**Patient population**

After obtaining informed consent, we enlisted eight end-stage renal disease patients (two women and six men) who were on chronic maintenance haemodialysis (4 h thrice weekly) for >6 months. The mean age of patients was 48 years (range 35–68 years) and the mean time on dialysis was 41 months (range 21–97 months). The cause of end-stage renal disease was chronic glomerulonephritis in five patients and chronic interstitial nephritis in three patients. Clinical examinations and routine laboratory tests confirmed that patients were in a good state of health. All patients were stable and free from intercurrent illnesses.

**Study design**

Each patient was treated with HD and with on-line HDF sessions utilizing dialysis membranes equipped with ultrafiltration control (4008S haemodialysis machine; Fresenius Medical Care, Bad Homburg, Germany). Extracorporeal sessions were carried out with dialyzers containing either standard high-flux polysulphone membranes (Hemoflow F70S, 1.6 m² surface area, steam sterilized) or Helixone membranes (FX80 dialyzer, 1.8 m² surface area, steam sterilized). Each treatment was used for 1 week in each patient, and the order of treatment was based on a Latin Square model. Samples (blood and ultrafiltrate) were collected for analysis during the third treatment. They were taken after calibration of the blood pump at 300 ml/min during a dialysate flow rate of 500 ml/min at 5 min (as beginning of dialysis), at 120 min and at the end of the dialysis session.

**Methods**

Blood was drawn from the afferent blood line into evacuated tubes containing ethylenediaminetetra-acetic acid (EDTA), and was centrifuged at 400 g for 5 min at 4°C to separate the plasma and the buffy coat. The erythrocyte pellet and plasma were used immediately. Ultrafiltrate (10 ml) collected from the dialysate side of the membrane was poured into floatable dialysis tubes (Spectra/Port® Float-A-Lyzer; Spectrum, Rancho Dominguez, CA) with a molecular weight cut-off of 1000 Da, and left to clear overnight in deionized water to allow removal of small molecular weight contaminants and desalting through a concentration gradient. After 24 h dialysis, the dialysis tubing containing the sample was removed and coated with a polycrylate–polyalcohol gel (Spectra/Gel™ Absorbent; Spectrum) for 30 min in order to concentrate the sample by drawing out water and other small molecules. To assure the reproducibility of the method, the same concentration factor was always used.

Plasma or ultrafiltrate samples were resuspended at a haematocrit of 50% with isolated RBCs from healthy subjects having normal renal function. In all reconstitution experiments, care was taken to ensure ABO and Rh blood group compatibility. For some experiments, the ultrafiltrate specimen was previously passed over a filter (Spectrum) with a nominal molecular weight cut-off of 10 or 20 kDa. Normal isolated RBCs were also resuspended at a haematocrit of 50% in autologous plasma. After an incubation time of 4 h at room temperature, aliquots of the suspension were removed and processed for determination of PS exposure in RBCs. Erythrocyte PS exposure was measured by a flow-cytometric assay making use of fluorescein isothiocyanate-labelled annexin V (FITC-AnV; Sigma, St Louis, MO). Annexin V is a member of the annexin family of calcium-dependent phospholipid binding proteins with a high affinity for PS. The flow cytometric assay has been previously validated and described in detail [6].


To exclude the possibility that FITC-AnV was actually binding to PS on the internal membrane leaflet after gaining entry into the interior of red cells, rather than to the outer membrane leaflet, a validated method was used [11]. Erythrocytes were incubated in 7 vol of Tris-buffered saline (TBS; 144 mmol/l NaCl, 10 mmol/l Tris, pH 7.40) solution containing 1 mmol/l CaCl₂ and 2% bovine serum albumin (BSA) for 15 min at 37°C. Cells were then incubated with FITC-AnV, and after incubation were divided into two equal portions. Cells of one portion were pelleted by centrifugation, resuspended in TBS with 1.2 mmol/l CaCl₂, and analysed by flow cytometry. The other portion was centrifuged to pellet erythrocytes, which were then resuspended in TBS solution with 2 mmol/l EDTA, incubated at room temperature for 15 min, washed twice and then resuspended in TBS containing 2 mmol/l EDTA, and analysed by flow cytometry. Since binding of annexin V requires calcium, this incubation accomplishes removal of any FITC-conjugated AnV bound to the outside of red cells without affecting that bound intracellularly [11].

Gel-filtration experiments were performed at 25°C using a Superdex 75 HR/10/30 (Amersham Pharmacia Biotech). Chromatographic separations were achieved using a HPLC system equipped with a Rhodyne injector and a photodiode variable-wavelength detector (Kontron). Samples (200 µl) of uraemic ultrafiltrate obtained after 5 min of online HDF using Helixone were injected into the column, pre-equilibrated and eluted isocratically at a flow rate of 0.4 ml/min with 50 mM Tris–HCl buffer (pH 7.75) containing 150 mM NaCl and 10 mM EDTA. The elution profile was monitored at 280 nm, and 400 µl fractions were collected throughout the separation and tested for specific ability to promote RBC PS exposure. Calibration of the eluted fractions was performed by using proteins with known molecular masses as standards: BSA (67 kDa), ovalbumin (43 kDa), chymotripsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

In further studies, ultrafiltrate samples obtained as above were processed according to an established method for the separation of less polar biological compounds (e.g. lipids) from more polar constituents [12]. Briefly, 0.8 ml H₂O, 2 ml methanol and 2 ml chloroform were sequentially added to a tube containing 1 ml of ultrafiltrate. After mixing and centrifugation at 2500 r.p.m. for 10 min at room temperature, two distinct phases were obtained. The lower phase was transferred to another tube, and the upper phase was washed twice with chloroform. The combined lower and upper phases were separately evaporated to dryness. The upper (polar) and lower (apolar) phases were then resuspended with either 0.5 ml PBS or BSA 1% in PBS (0.5 ml). Samples were then incubated for 30 min with RBCs from healthy subjects for flow cytometric analysis of PS exposure.

Data analyses

Before statistical analysis, data obtained with polysulphone membranes (1.6 m²) were normalized to the surface of the Helixone membrane (1.8 m²) by multiplying each value by the correction factor 1.125 (= 1.8/1.6).

We used a cross-over study design which required a two factor repeated measures analysis of variance (ANOVA). Because each patient in this study underwent four different dialytic treatments, the first factor consisted of on-line HDF with polysulphone membrane, on-line HDF with Helixone membrane, haemodialysis with polysulphone membrane, and haemodialysis with Helixone membrane. The second factor consisted of three measurements for each treatment at 5, 120 and 240 min of dialysis. For statistical analysis, the differences between the filters and each of the four treatment groups were calculated at each time point. One-way ANOVAs for repeated measurements were applied within each treatment group to evaluate interactions between membranes and time. Student’s t-tests for paired data were applied to test differences between membranes for each of the four treatments and to compare treatments at the same time points. Results obtained from in vitro experiments were also analyzed by Student’s t-tests. Data are presented as means ± SEM, and P-values < 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS® Advanced Statistics™ 7.5 software (1997; SPSS, Chicago, IL).

Results

The present cross-over study was undertaken to evaluate effects of removal by dialysis of substance(s) pathologically retained in the uraemic organism which may cause increased exposure of PS on the outer leaflet of erythrocyte membrane. Since the exact nature of the factor(s) is unknown, removal was assessed indirectly by measuring PS-expressing normal RBCs exposed to plasma or ultrafiltrate obtained at various time points during the dialytic session. The percentage of PS-expressing erythrocytes was measured by a flow cytometric assay based on FITC-AnV labelling (6). That FITC-AnV was indeed bound to PS exposed on the outer RBC membrane leaflet rather than to PS on the internal membrane leaflet was confirmed in experiments that incubated red cells in EDTA-containing buffer before flow cytometric analysis. This step effectively removes any annexin V bound to the external face of RBC membranes without affecting intracellular annexin V [11]. Under these conditions, fluorescence dropped to background levels (fluorescence determined by incubating erythrocytes with FITC-AnV in the absence of calcium), indicating that annexin V was binding to the outer surface of erythrocyte membrane (data not shown).

Figure 1 shows effects of uraemic plasma from the different experimental conditions on PS exposure in erythrocytes from healthy subjects. Incubation with uraemic plasma caused increased PS exposure compared with RBC incubation with autologous normal plasma (1.0 ± 0.06 annexin V-positive red cells; n = 8) at all time points (Figure 1). PS-positive erythrocytes (%) at 5, 120 and 240 min were 3.5 ± 0.1, 3.45 ± 0.1 and 3.24 ± 0.1, respectively, for HD using polysulphone; 3.47 ± 0.1, 3.16 ± 0.1 and 3.0 ± 0.09 for HD using Helixone; 3.37 ± 0.2, 3.21 ± 0.17 and 3.0 ± 0.18 for on-line HDF using polysulphone; and 3.36 ± 0.14, 2.95 ± 0.19 and 2.52 ± 0.13 for on-line HDF using Helixone. Dialysis for 4 h decreased the capacity of plasma to cause exposure of PS in normal RBCs. After 120 min, and especially at the end of the dialysis
session (whether carried out as high-flux HD or on-line HDF), there was a reduction in the capacity of uraemic plasma to cause PS exposure in normal RBCs, although exposure remained elevated. This decrease in PS exposure indicates the removal from plasma of the uraemic fraction causing RBC PS exposure, and varied depending on both the dialyzer type and dialysis technique. Our results indicate that on-line HDF using the Helixone-containing dialyzer provided the highest removal from plasma of uraemic compound(s) promoting RBC PS exposure (Figure 1). There were significant differences between Helixone and polysulphone during on-line HDF, and between on-line HDF and HD when using both polysulphone and Helixone membranes (Figure 1).

Figure 2 shows effects of uraemic ultrafiltrate from in vivo HD and on-line HDF with conventional polysulphone membranes or Helixone membranes on PS exposure on the outer face of red cell membranes from healthy subjects. The results are reported as x-fold increases in PS-positive RBCs over control values, which were obtained by incubating red cells with autologous plasma. Importantly for these findings, and unlike the results in Figure 1, higher x-fold increases in PS-positive RBCs corresponded to higher levels of uraemic factor(s) affecting RBC PS exposure in the ultrafiltrate, and indicated higher removal of plasma from plasma during the session. Ultrafiltrate collected during on-line HDF with the Helixone membrane caused the highest PS exposure at all time points (Figure 2). Results obtained with on-line HDF with each of the membranes were significantly different \((P < 0.001)\) from results with HD using the same membranes. In addition, Helixone-containing dialyzers produced results that were significantly different from those with high-flux polysulphone during both HD and on-line HDF \((P < 0.005 \text{ and } P < 0.001, \text{ respectively}; \text{ Figure 2})\). Absolute values of PS-exposing erythrocytes (%) at 5, 120 and 240 min were 17 ± 0.68, 14.8 ± 0.46 and 12.1 ± 0.65, respectively, with HD using polysulphone; 17.3 ± 0.63, 15.2 ± 0.47 and 13.4 ± 0.47 with HD using Helixone; 19 ± 0.77, 16 ± 0.5 and 13.45 ± 0.53 with on-line HDF using polysulphone; and 21.5 ± 0.9, 18.8 ± 0.73 and 15.9 ± 0.77 with on-line HDF using Helixone.

The kinetics of each curve illustrated in Figure 2 show that PS exposure in normal RBCs was much higher with ultrafiltrate samples obtained at 5 min than at the end of the treatment, suggesting that the bulk of PS-inducing toxin(s) is removed in the early phase of treatment. The decrease in removal efficiency over treatment time, a feature of all dialysis treatments, may be explained by increased adsorption of proteins and other blood substances on the surface of the membrane, which reduces ultrafiltration rate and permeability [13].

Our results show that during each dialysis session RBC PS exposure caused by uraemic plasma was maximally attenuated at 240 min, and the greatest capacity of uraemic plasma to cause RBC PS exposure occurred at 5 min. At the same time points, we also examined uraemic plasma factor(s) removal efficiency during haemodialysis \((n = 5)\) of other high-flux dialyzers such as polymethylmethacrylate (PMMA) (Filtryzer BK-F, 1.6 m², γ-ray sterilized; Toray, Japan) and cellulose triacetate (CTA) (Sureflux 210 E, 2.1 m², γ-ray sterilized; Nipro, Japan). Values obtained with each dialyzer were normalized to 1.8 m² (the surface of Helixone membrane) by the correction factor 1.125 \((= 1.8/1.6; \text{ PMMA}) \) or 0.85 \((= 1.8/2.1; \text{ CTA})\). After 240 min of HD, the ability of uraemic plasma to cause RBC PS exposure (expressed as x-fold increase over PS-positive normal erythrocytes incubated with autologous plasma) was 3.08 ± 0.11 for PMMA and 3.25 ± 0.08 for CTA. The increase with CTA was significantly larger than with Helixone (2.9 ± 0.06) \((P < 0.02)\). The x-fold increase in PS-expressing RBCs caused by uraemic ultrafiltrate collected after 5 min of
dialysis was 15.9 ± 0.3 with PMMA and 14.5 ± 0.4 with CTA. The increases with the CTA membrane were significantly smaller ($P < 0.001$) than with PMMA, Helixone (16.5 ± 0.1) and polysulphone (16.2 ± 0.1).

In additional studies ($n = 5$), we examined ultrafiltrate obtained after 5 min of on-line HDF using Helixone membrane. Erythrocytes isolated from healthy subjects were incubated with ultrafiltrate (control sample) or a fraction thereof obtained from either 20 or 10 kDa cut-off filters. The 20 kDa ultrafiltrate sample caused a significant PS exposure ($20 ± 0.6$ annexin V-positive red cells) that was similar to exposure with the untreated uraemic ultrafiltrate sample ($20.3 ± 0.7$ annexin V-positive red cells). However, PS exposure after incubation with the 10 kDa ultrafiltrate was much lower ($4.5 ± 0.2$ annexin V-positive RBCs; $P < 0.001$), which suggests that PS-inducing substance(s) were $>10$ kDa. There was also a marked inhibition of PS exposure increases when uraemic ultrafiltrate had been repeatedly frozen (at $–80^\circ$C) and thawed (at $25^\circ$C) before its incubation with normal RBCs ($5 ± 0.2$% annexin V-positive red cells; $P < 0.001$ vs untreated ultrafiltrate sample).

We also performed gel filtration experiments to more accurately define the hydrodynamic molecular weight of the compound(s) under investigation. The chromatographic profile was monitored by both UV trace and by activity profile (promotion of RBC PS exposure) using cytofluorimetric assay (data not shown). An increased PS exposure activity in red blood cells was observed in two collected fractions. These were spanning the hydrodynamic mass range between 10 800 and 9200 Da.

To further define the chemical nature of the putative uraemic compound causing PS exposure, ultrafiltrate samples ($n = 3$) obtained as above were further processed according to the method of Bligh and Dyer [12]. This allows the rapid separation of polar from less polar compounds, which are then collected in aqueous and chloroformic phases, respectively. The results of these experiments are reported in Table 1. The aqueous phase of ultrafiltrate specimens (untreated, ultrafiltered down to a 20000 or 10000 molecular weight cut-off) did not cause PS exposure in RBCs, whereas the chloroformic phase of both untreated and 20 kDa ultrafiltrate specimens resuspended in PBS alone caused marked increases in PS-positive RBCs compared with RBCs incubated with autologous plasma ($P < 0.001$; Table 1). RBC PS exposure was more than doubled when the chloroformic phase from the ultrafiltrate specimens were resuspended in PBS containing 1% BSA ($28.9 ± 0.5$% and $26.2 ± 0.4$% PS-positive erythrocytes for untreated ultrafiltrate and 20 kDa ultrafiltrate, respectively; $P < 0.001$ vs values obtained in PBS resuspension). Neither phases (aqueous or chloroformic) of ultrafiltrates obtained through a 10 kDa cutoff filter affected RBC PS exposure when resuspended in PBS or in PBS + BSA (Table 1). Taken together, these data suggest that the putative uraemic factor causing PS exposure may be highly lipophilic.

**Discussion**

Increased erythrocyte PS exposure is thought to contribute to the pathophysiology of several human diseases [1] through possible effects on blood coagulation, cell adhesion and cell clearance [1,2]. In addition, the increased surface-exposed PS in uraemic RBCs [6] may be of significant pathophysiological importance. For example, abnormal exposure of outer-leaflet PS in uraemic RBCs significantly affects their propensity for recognition by human monocyte-derived macrophages [7], which may partly explain the shortened erythrocyte life span characterizing uraemia [14]. Increased PS exposure may promote enhanced uraemic erythrocyte adhesion to vascular endothelial cells [8], a mechanism that may play a role in the accelerated pathogenesis of vascular disease commonly encountered in chronic renal failure patients. Because uraemic plasma strongly

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**Fig. 2.** Ability of uraemic ultrafiltrate collected on the dialysate side of the membrane to increase the exposure of phosphatidylserine in erythrocytes from healthy subjects. Results are expressed as an $x$-fold increase in PS-positive erythrocytes over values obtained by incubating normal RBCs with autologous plasma. *$P < 0.001$ vs HD FX80; † $P < 0.01$ vs HDF F70S; ‡ $P < 0.001$ vs HD F70S; § $P < 0.05$ vs HD F70S. Abbreviations as in Figure 1.
Uraemic compounds causing RBC phosphatidylserine exposure

Table 1. Ability of uraemic ultrafiltrate to stimulate PS exposure on normal erythrocytes according to polarity and molecular weight fractions of the ultrafiltrate

<table>
<thead>
<tr>
<th>Polarity</th>
<th>Molecular weight cut-off</th>
<th>BSA</th>
<th>PS-expressing erythrocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (chloroformic phase)</td>
<td>None</td>
<td>−</td>
<td>13.5 ± 0.5\textsuperscript{a}</td>
</tr>
<tr>
<td>Low (chloroformic phase)</td>
<td>None</td>
<td>+</td>
<td>28.9 ± 0.5\textsuperscript{b}</td>
</tr>
<tr>
<td>Low (chloroformic phase)</td>
<td>20 kDa</td>
<td>+</td>
<td>12.1 ± 0.5\textsuperscript{a}</td>
</tr>
<tr>
<td>Low (chloroformic phase)</td>
<td>20 kDa</td>
<td>−</td>
<td>26.2 ± 0.4\textsuperscript{a,b}</td>
</tr>
<tr>
<td>Low (chloroformic phase)</td>
<td>10 kDa</td>
<td>−</td>
<td>1.30 ± 0.1</td>
</tr>
<tr>
<td>Low (chloroformic phase)</td>
<td>10 kDa</td>
<td>+</td>
<td>1.56 ± 0.17</td>
</tr>
<tr>
<td>High (aqueous phase)</td>
<td>None</td>
<td>−</td>
<td>0.96 ± 0.08</td>
</tr>
<tr>
<td>High (aqueous phase)</td>
<td>None</td>
<td>+</td>
<td>1.01 ± 0.09</td>
</tr>
<tr>
<td>High (aqueous phase)</td>
<td>20 kDa</td>
<td>−</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>High (aqueous phase)</td>
<td>20 kDa</td>
<td>+</td>
<td>0.96 ± 0.12</td>
</tr>
<tr>
<td>High (aqueous phase)</td>
<td>10 kDa</td>
<td>−</td>
<td>0.90 ± 0.02</td>
</tr>
<tr>
<td>High (aqueous phase)</td>
<td>10 kDa</td>
<td>+</td>
<td>0.91 ± 0.07</td>
</tr>
</tbody>
</table>

Uraemic ultrafiltrate samples (n = 3) were obtained after 5 min of on-line HDF using the Helixone membrane, processed as described in Subjects and methods, and incubated with erythrocytes from healthy subjects. Results were compared with those obtained by incubating normal erythrocytes with autologous plasma (1.1 ± 0.05% PS-positive erythrocytes; n = 3).

\textsuperscript{a}P < 0.001 vs normal erythrocytes incubated with autologous plasma.

\textsuperscript{b}P < 0.001 vs similar ultrafiltrate specimen without BSA.

Uraemic factors affecting PS externalization in RBCs [6], it is possible that removal of the putative soluble uraemic compound(s) causing RBC PS exposure may benefit uraemic patients.

Our data indicate that removal of the uraemic plasma fraction causing RBC PS exposure occurred under the selected dialytic conditions of the present study (HD and on-line HDF using high flux polysulphone or Helixone-containing dialyzers). That removal occurred is indicated by the reduced ability of uraemic plasma at the end of each dialysis session to induce PS exposure in normal RBCs compared with plasma obtained at the beginning (5 min) of the sessions. In addition, the ultrafiltrate collected from the dialysate side of the dialyzer membrane markedly increased PS exposure on RBCs. However, the degree of removal varied with the different experimental conditions of the study. Thus, removal was greater for on-line HDF using the Helixone membrane, intermediate with on-line HDF using standard polysulphone and with HD using Helixone, and lower with HD using standard polysulphone membrane.

The present results obtained with ultrafiltrate specimens confirm that uraemic compound(s) having a molecular weight between 10 and 20 kDa are able to increase PS exposure in RBCs. Incubation of normal RBCs with the 10 kDa ultrafiltrate samples caused a marked decrease in RBC PS exposure compared with the 20 kDa samples or with untreated ultrafiltrate samples. The ability of uraemic ultrafiltrate to cause PS exposure in RBCs was also greatly inhibited by repeated freezing and thawing of samples before incubation with red cells, suggesting that the putative uraemic factor could be a plasma protein in the molecular weight range of 10–20 kDa. Gel permeation experiments further characterized the uraemic factor as having a hydrodynamic mass between 10 800 and 9200 Da. Altogether, these data suggest that the uraemic factor causing RBC PS exposure is probably in the molecular weight range between 10 and 10.8 kDa. Furthermore, the 20 kDa ultrafiltrate extract indicated that the putative uraemic factor was retained in the less polar rather than in the more polar phase. Thus the factor may be a strong lipophilic compound in uraemic plasma that is bound to a protein with an apparent molecular weight in the range indicated previously. Lipophilic and/or protein-bound solutes are thought to have an important biological impact in uraemia [15]. Further studies are clearly warranted to investigate the precise chemical nature of the uraemic factor(s) identified in the present experiment.

Results from the present study indicate that the Helixone membrane has a marked ability to eliminate high molecular weight solutes. This new polysulphone-based high-flux membrane, developed through application of nanotechnology principles, has highly refined inner-surface features that permit the controlled elimination of larger uraemic toxins within a narrow size range [9]. The elimination of β2-microglobulin, the yardstick for showing the ability and efficiency of a membrane to remove large-solutes [10], was increased by 23% by Helixone compared with conventional high-flux polysulphone membranes. We found that the Helixone-containing dialyzer caused a greater removal during both HD and on-line HDF of the putative uraemic factor(s) (molecular weight between 10 and 20 kDa) that cause PS exposure than can be obtained with conventional polysulphone. Many of the currently known biological effects of uraemia are attributable to relatively large-sized solutes [16]. Helixone appears to meet the present-day requirements of dialysis membranes, which are to eliminate larger uraemic substances with high efficiency but without causing excessive leakage of useful proteins.

Convective treatment modalities, such as high-flux HD and HDF, which in conjunction with high-flux
membranes enhance the removal of larger uraemic solutes, are increasingly recognized for providing improved therapy over standard dialysis [17]. Several studies have shown that these treatment modes may significantly improve patient outcome. On-line HDF is a dialysis technique that combines diffusion with higher convection than standard HDF, and the dialysis fluid with on-line HDF (free of toxins and pyrogens) is used as substitution fluid. We found that on-line HDF with both standard polysulphone and Helixone membranes was more effective than haemodialysis using the same membranes for causing removal of the uraemic factor(s) that induce PS exposure. Our results are therefore in keeping with the finding that convection plays a major role in the transport mechanism of solutes having high molecular weights [17].

Many [18,19], but not all [20], recent studies have indicated that on-line HDF may more strongly correct anaemia than conventional treatments or standard HDF techniques. On-line treatments may be more effective in achieving higher haematocrits because they reduce pyrogenic and microbiological contamination of the dialysate and because they produce higher removal of medium and large molecules (possibly including bone marrow inhibitors) [17]. The present results point to an additional mechanism by which on-line HDF may positively affect the anaemic state of ESRD patients compared with haemodialysis. Enhanced elimination of the uraemic factor(s) promoting PS exposure may lead to diminished PS externalization in the uraemic RBC population to result in prolonged red cell life span, thereby improving anaemia. A determination of whether this concept holds true will require further investigation.

Conflict of interest statement. None declared.

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