Filtration of native and glycated $\beta_2$-microglobulin by charged and neutral dialysis membranes

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Background. It has been postulated that protein glycation and formation of advanced glycation end products (AGE) are among toxic factors in chronic uremia, whether the renal disease is of diabetic or nondiabetic origin. In this setting, AGE-modified $\beta_2$-microglobulin ($\beta_2m$) may favor dialysis $\beta_2m$-related dialysis amyloidosis. Consequently, efficient removal of modified $\beta_2m$ by highly permeable dialysis membranes is as important as removal of native $\beta_2m$ to postpone the development of dialysis amyloidosis.

Methods. To define the role of dialysis membrane surface electronegativity on plasma protein transfer, an in vitro model was used to test the interactions of native and glycated $\beta_2m$ with various highly permeable dialysis membranes. An experimental circuit with minidialyzers was used. The neutral high-flux polysulfone membrane (PS), the electronegative polymethylmetacrylate membrane (PMMA), the electronegative AN69 membrane and a modified AN69 membrane, the surface of which was neutralized with polyethyleneimine (AN69-PEI), were tested using both native $\beta_2m$ and the more acidic glycated $\beta_2m$. Protein mass transfer and binding to the membrane were measured.

Results. Mass transfer of glycated $\beta_2m$ was significantly decreased through all membranes tested when compared with native $\beta_2m$. This result was due to the increased molecular weight of $\beta_2m$, which became less permeable to porous membranes, whereas adsorption of both native and glycated $\beta_2m$ to membranes, due to ionic interactions, decreased similarly with AN69 and AN69-PEI, but remained unchanged with PS and PMMA. Moreover, surface neutralization of AN69 membrane did not alter its core binding capacity, since $\beta_2m$ absorption accounted for 98 and 97% and glycated $\beta_2m$ for 83.7 and 81.4% of the protein removed with AN69 and AN69-PEI, respectively.

Conclusion. Clearance of glycated $\beta_2m$ through highly permeable and negatively charged membranes was lower than that of native $\beta_2m$, reflecting a decreased sieving coefficient for the neoformed higher molecular weight and conformationally altered molecule. The binding capacity of the neutral PS was roughly half that of the charged membranes. Neutralizing surface electronegativity of the AN69 membrane with PEI did not alter its binding capacity. These results suggest that it would be useful for dialysis protocols to include comparative studies of both serum native and modified $\beta_2m$ in order to prevent $\beta_2m$-amyloidosis.

Chronic renal failure induces the production of various compounds that may have toxic effects [1]. The pathogenic role of these compounds in metabolic and structural changes leading to many disorders, such as dialysis-related amyloidosis, encountered in long-term hemodialyzed patients is postulated. Recent biochemical and clinical studies have suggested that modified $\beta_2$-microglobulin ($\beta_2m$) favors polymerization of the protein and induces amyloid deposits [2]. Critical biotransformation of $\beta_2m$ is associated with the serum increase of its main acidic isoform with pI 5.1, while the native form has a pI of 5.7. More precisely, the acidic isoforms of $\beta_2m$, which represent a few percent of total serum $\beta_2m$, in addition to various breakdown fragments, contain several $\beta_2$ adducts due to oxidation and advanced glycation end products (AGE) [3–5]. AGEs have been extensively studied and are increased in patients with diabetes mellitus as well as patients with end-stage renal failure not associated with diabetes and in the older population (abstract; Miyata T et al, J Am Soc Nephrol 6:552, 1995) [6–12].

Removal of $\beta_2m$, including AGE-modified $\beta_2m$, using highly permeable membranes, has been documented [13–15]. The constant use of highly permeable synthetic dialysis membranes, from dialysis onset in long-term hemodialyzed patients, has been shown to postpone the occurrence of the clinical and radiological expression of juxta-articular bone and synovial amyloid deposits [16–18]. The choice of the dialysis membrane is therefore of major concern to limit morbidity due to $\beta_2m$-related amyloidosis.

Several dialysis membranes are sufficiently permeable to native $\beta_2m$ for removing large amounts of the protein. In this setting, the hemofiltration technique appears
slightly more efficient than hemodialysis [19]. However, membrane performances and particularly the sieving coefficient for \( \beta_m \) are not identical [20]. The removal of AGE-modified \( \beta_m \) has been tested by measuring the clearance of AGE-bearing proteins detected, for example, with anti-pentosidine or anti-carboxymethyllysine antibodies [13, 14, 21–23]. Synthetic dialysis membranes may be classified according to physicochemical criteria that govern interactions with blood components, such as porosity, electrical charges at the surface and in the bulk of the membrane, and adsorption properties [24]. For example, the polymethylmetacrylate (PMMA) membrane was specially designed to adsorb proteins including \( \beta_m \) in an attempt to prevent the development of dialysis amyloidosis [25], while the AN69 membrane exhibits a high adsorption capacity for C3a, C5a anaphylatoxins [26, 27] and complement factor D [28]. The polysulfone membrane is neutral with an \( \text{in vitro} \) zeta potential near −5 mV, whereas the polyacrylonitrile AN69 is negatively charged with a potential of −70 mV. For the latter membrane, surface electronegativity accounts for activation of the kallikrein-kinin system in the presence of citrated plasma (pH 7.1) but not heparinized plasma (pH 7.4). Partial neutralization of these charges at the membrane surface using polyethyleneimine (PEI) decreases high-molecular-weight kininogen adsorption and the contact phase activation of coagulation [29, 30]. It has been observed that PEI, which has a molecular weight higher than 10⁵ kDa, reacts at the membrane surface and does not penetrate into the pores of the membrane [29]. The purpose of this study was to investigate in \( \text{vitro} \) the interaction of dialysis synthetic membranes that were either neutral or negatively charged with native and glycated \( \beta_m \), as a reflection of AGE-modified proteins observed not only in patients with diabetes mellitus but also in patients on chronic hemodialysis for non-diabetic nephropathies.

**METHODS**

**In \( \text{vitro} \) \( \beta_m \) glycation**

Five hundred micrograms of human \( \beta_m \) (Sigma, St. Louis, MO, USA) were incubated in 0.15 mol/L phosphate buffer, 0.02 % (m/v) sodium azide, pH 7.4, with or without 1 mol/L glucose (Merck, Darmstadt, Germany) during 26 days at 37°C, in sterile conditions (filtration on 0.22 μm Millipore filter, under laminatory flow). After incubation, the \( \beta_m \) solution was dialyzed overnight at 4°C against isotonic saline solution. Five hundred microliter aliquots of native \( \beta_m \) (incubation with phosphate buffer alone) and glycated \( \beta_m \) (incubation with phosphate buffer and glucose) were stored at −20°C until utilization. The intensity of \( \beta_m \) glycation was evaluated by the alkaline reduction of nitroblue tetrizolium or fructosamine test [31] expressed as μmol of deoxymorpholinofructose (DMF)/mg \( \beta_m \) and by fluorescence measurement of AGEs [32, 33]. Fluorescence was measured with a Perkin-Elmer LS50B spectrofluorometer (Beaconsfield, Burks, UK) at two different ranges of wavelengths (λ\text{ex} 335 nm to λ\text{em} 385 nm; λ\text{ex} 370 nm to λ\text{em} 340 nm). Glycated \( \beta_m \) showed 15.9 and 6.0 times (56.4 vs. 3.6 and 23.3 vs. 3.9 arbitrary units/mg \( \beta_m \), respectively) increase in fluorescence at the two sets of wavelengths, as well as 6.1 times increase in the content of protein-bound sugars evaluated by the nitroblue tetrazolium test (1.64 vs. 0.27 μmol DMF/mg \( \beta_m \)). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, and isoelectric focusing showed that in vitro glycation induced the formation of higher molecular weight and more acidic forms of \( \beta_m \), as previously described [34].

**Hemofiltration circuit**

The experimental model consisted of a closed circuit with a tank containing 50 mL of the test solution and a pump (Vial Medical VM 8000, France) flowing the solution into the minidialyzer at 10 mL/min. The test solution was a mixture of 49.5 mL of 4 % human albumin (wt/vol) from healthy donors (Blood Transfusion Centre, Reims, France) and 50 μg of glycated or nonglycated \( \beta_m \) in 0.5 mL phosphate-buffered solution. Albumin was chosen as the carrier to limit \( \beta_m \) or glycated \( \beta_m \) adsorption on the circuit walls. At beginning of experiment, the concentration in the tank solution was 40 mg/L for albumin and 1 mg/L for \( \beta_m \) or glycated \( \beta_m \). These concentrations were selected to mimic normal serum values in humans. A second pump (VM 09) set at 0.2 mL/min was used for filtrate recollection throughout the experiment. The \( \beta_m \) concentration was measured by microparticular enzyme immunoassay using IMX analyzer (Abbott Diagnostics, Rungis, France) in the tank solution and in the ultrafiltrate every 15 minutes for two hours. Mass transfer and adsorption on dialysis membrane were measured under similar hydrodynamic conditions, taking into account the regular increase of protein concentration in the circuit. All the samples were measured in duplicate, and every membrane was tested five to seven times under similar experimental conditions.

**Membranes and minidialyzers**

Four commercially available dialysis membranes were tested: polysulfone (PS, Fresenius, Germany), polyacrylonitrile AN69, polyacrylonitrile AN69-PEI with reduced surface electronegativity by polyethyleneimine (AN69-ST, Hospal-Cobe, France), and polymethylmetacrylate (PMMA BK, Meditor, France). The hollow fiber minidialyzers used in the experiments were developed as a small scale model (1/50) of a standard hollow fiber dia-
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Table 1. Structural characteristics of the membranes equipping minidialyzers used in the in vitro model of hemofiltration

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AN69</th>
<th>AN69-PEI</th>
<th>PS</th>
<th>PMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective length cm</td>
<td>12</td>
<td>12</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>Number of fibers</td>
<td>170</td>
<td>170</td>
<td>170</td>
<td>60</td>
</tr>
<tr>
<td>Effective surface area cm²</td>
<td>230</td>
<td>230</td>
<td>90</td>
<td>57</td>
</tr>
<tr>
<td>Zeta potential mV</td>
<td>−70 ± 5</td>
<td>−3 ± 1</td>
<td>−5 ± 1</td>
<td>−25 ± 2</td>
</tr>
</tbody>
</table>

Abbreviations are: AN69, electronegative AN69 membrane; AN69-PEI, modified AN69 membrane with polyethyleneimine; PS, neutral high-flux polysulfone membrane; PMMA, polymethylmethacrylate membrane.

Table 2. Protein mass transfer

<table>
<thead>
<tr>
<th>Membrane</th>
<th>β₂m</th>
<th>Glycated β₂m</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN69</td>
<td>0.081 ± 0.032</td>
<td>0.032 ± 0.010</td>
</tr>
<tr>
<td>AN69-PEI</td>
<td>0.068 ± 0.017</td>
<td>0.028 ± 0.010</td>
</tr>
<tr>
<td>PS</td>
<td>0.069 ± 0.033</td>
<td>0.048 ± 0.014</td>
</tr>
<tr>
<td>PMMA</td>
<td>0.080 ± 0.011</td>
<td>0.034 ± 0.004</td>
</tr>
</tbody>
</table>

Abbreviations are: AN69, electronegative AN69 membrane; AN69-PEI, modified AN69 membrane with polyethyleneimine; PS, neutral high-flux polysulfone membrane; PMMA, polymethylmethacrylate membrane.

RESULTS

Mass transfer

The results obtained after 90 minutes of filtration are summarized in Table 2. β₂m mass transfer was in the same range for the four dialysis membranes tested, and glycation decreased its mass transfer significantly (P < 0.05) for AN69, AN69-PEI, and PMMA, respectively, but not for PS. Protein binding to native membrane was correlated with their respective zeta potential, as shown in Table 3 and Figure 1: The higher the membrane’s surface density of negative charges, the higher its adsorption of β₂m whether native or glycated. In the present experimental conditions, protein binding to the membrane accounted for more than 90 % of removal of native β₂m and 80% of glycated β₂m with the exception of PS, where it contributed to 55.1 and 57.7%, respectively.

Protein binding to AN69-PEI and to AN69 was similar, indicating that neutralizing the negative charges on the surface of the membrane with PEI did not reduce the bulk of charges acting in the core of the gel membrane.

β₂m kinetics

Using polyacrylonitrile membranes, native β₂m concentration decreased exponentially in the circuit, whereas its concentration in the filtrate increased slowly, indicating β₂m binding to dialysis membrane up to a plateau concentration, which suggested a saturable process. The curves shown in Figure 2 indicate the quite similar binding affinity of both the AN69 membrane and the modified
AN69-PEI membrane. Neutralization of surface electrical charges by PEI slightly decreased, but not significantly, the adsorption of $\beta_2$m on AN69, the AN69-PEI membrane retaining the same maximal binding capacity as AN69, reached after some 90 minutes. At 120 minutes of dialysis, approximately 80% of $\beta_2$m was removed. Fifty percent of the $\beta_2$m removed was due to membrane binding and occurred within 15 to 30 minutes of dialysis onset. Polysulphone membrane exhibited a different behavior since its binding capacity was low; it slowly increased with time and reached saturation by 60 minutes (Fig. 3). Saturation of the membrane binding capacity was reached at the $\beta_2$m plateau concentration of approximately 15 to 20% of the initial tank concentration, which was four times less than that of AN69. At 120 minutes of dialysis, 20% of $\beta_2$m was removed by adsorption. Near 100% of native $\beta_2$m was removed by membrane binding with the PMMA membrane, as seen in Figure 3.

Glycation of $\beta_2$m decreased its binding capacity to both polyacrylonitrile membranes. Using ANF69, 50% of glycated $\beta_2$m was removed by adsorption at 60 minutes. The same binding proportion was slightly deleted at 90 minutes with the AN69-PEI membrane. At 120 minutes, 60.5 and 58.8% of glycated $\beta_2$m removal resulted from binding to the AN69 and the AN69-PEI membrane, respectively. No gross difference was noticed between $\beta_2$m and glycated $\beta_2$m binding capacity for PS and PMMA membranes, respectively.

DISCUSSION

In chronically hemodialyzed patients, the use of a synthetic, highly permeable dialysis membrane is recommended to prevent the increase of $\beta_2$m body burden and to lessen the formation of $\beta_2$m-associated amyloid deposits, a very characteristic complication of chronic uremia. The critical role of dialysis membrane permeability and adsorption in removing $\beta_2$m was documented by kinetics studies using radiiodinated $\beta_2$m [19]. As a consequence of the high sieving coefficient for $\beta_2$m and better biocompatibility, highly permeable synthetic membranes postpone the occurrence of clinical signs of dialysis-related amyloidosis [16–18]. However, even daily hemofiltration sessions were unable to balance net $\beta_2$m generation and prevent a rise in the $\beta_2$m storage pool [35, 36]. Dialysis membrane interactions with plasma proteins are governed by physical forces, and among them electrical charges play a prominent role. This role was investigated in the present in vitro study.

Native $\beta_2$m was submitted to in vitro glycation in order to induce synthesis of AGEs and then tested in minidialyzers. It has already been documented that these modifications increase molecular weight and render $\beta_2$m more acidic [9]. Membrane permeability and adsorption were compared for both native and glycated $\beta_2$m using the neutral PS membrane and the electronegative AN69 and PMMA membranes. Protein adsorption was expressed as $\mu g/cm^2$ and not as $\mu g/cm^3$, since membrane thickness was not constant, precluding accurate calculation of membrane volume.
Native $\beta_{1m}$ mass transfer was not significantly different between membranes. However, $\beta_{1m}$ adsorption accounted for more than 90% of protein removal using AN69, AN69-PEI, or PMMA, whereas it was near 50% for PS.

Glycation of $\beta_{1m}$ decreased its mass transfer through the four membranes tested. As compared with native $\beta_{1m}$, mass transfer decrease was $-47.7\%$ with AN69 and $-43.8\%$ with AN69-PEI. In parallel, the membrane binding capacity decreased by $-14.6\%$ and $-16.1\%$, respectively. In contrast, glycation did not alter the $\beta_{1m}$ binding capacity of PS. This difference may be explained by the electric charges acting in the bulk of AN69 membranes as compared with the PS electroneutrality. The linear correlation documented between the zeta potential and the binding capacity (expressed as mg/cm$^2$) of the three non-modified membranes (AN69, PMMA, and PS) for both proteins illustrates the crucial role of electrical charge interactions between membrane and blood proteins. The demonstration that the acidic (pH 5.1) glycated $\beta_{1m}$ binding was less than that of the native, less acidic, $\beta_{1m}$ (pH 5.7) supports this hypothesis [37].

The role of membrane electronegativity was tested using a unique modification of the AN69 membrane, whose cationic charges were neutralized by the polyanionic compound PEI. Native $\beta_{1m}$ mass transfer was similar through AN69 and AN69-PEI membranes, as was the fraction of native $\beta_{1m}$ binding to the membrane. However, the kinetic pattern of glycated $\beta_{1m}$ filtration also was similar for both membranes but delayed when compared with native $\beta_{1m}$, indicating that decreasing membrane surface electronegativity by PEI did not change significantly both the porosity and core binding capacity of AN69 for glycated $\beta_{1m}$.

One of the key points for understanding the selective removal of plasma glycated proteins by hemodialysis is to take into account the extreme diversity of modified proteins resulting from the irreversible steps of the Maillard reaction. Friedlander et al have studied kinetics of early and advanced glycation end products in end-stage renal patients treated either by peritoneal dialysis or intermittent hemodialysis [21, 22]. Fractionation by gel filtration of serum proteins from patients on hemodialysis revealed that one of the best studied AGEs, pentosidine, was linked to proteins of various molecular weights. Up to 95% of the pentosidine was associated with $>10,000$ molecular weight proteins, $<1\%$ with $<10,000$ molecular weight proteins, and $<1\%$ was free. These authors documented the selective removal of free and low molecular weight bound pentosidine with reused PS membranes, whereas the concentration of $>10,000$ molecular weight AGE-modified proteins did not change significantly. Similar results were also obtained in hemodialyzed diabetic patients, where postdialysis AGEs levels decreased significantly with the high-flux PS membrane but not with low permeability conventional membranes [36, 38]. In contrast to hemodialysis, patients on peritoneal dialysis had lower but still elevated steady-state AGE levels. Whatever the dialysis technique, it has been suggested that AGEs are products of the combined process of glycation and oxidation, since there is a good evidence of a sustained oxidative stress in chronic uremia [39, 40].

In conclusion, among the factors that govern the in
vitro filtration of native and glycated $\beta$-m through highly permeable dialysis membranes, we have documented that changes in $\beta$-m molecular weight and electrical charges rather than dialysis membrane surface electric potential must be taken into account. Glycated $\beta$-m is more acidic and has a higher molecular weight than native $\beta$-m, and consequently crosses both charged and noncharged membranes less well. Decreasing the electronegativity of the AN69 membrane surface demonstrated that these physical modifications do not alter its binding capacity for both native and glycated $\beta$-m. This paradoxal result could be due to the superficial effect of PEI, which does not neutralize the negatively charged sulfonates of the copolymer core that remain unaltered 17. Binding capacity for both native and glycated proteins, such as cytochrome c and lysozyme [41, 42].

Although these results were obtained in vitro, they may be useful for designing clinical protocols focusing on the prevention of dialysis amyloidosis.

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