Monomeric and dimeric $\beta_2$-microglobulin may be extracted from amyloid deposits \textit{in vitro}

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Abstract

\textbf{Background.} There is a controversy as to whether $\beta_2$-microglobulin ($\beta_2$M) amyloid deposits may be degraded resulting in regression and cure of amyloidosis. We have recently reported a long-term clinical study involving transplanted patients suggesting that there is no resorption of amyloid deposits \textit{in vivo}, even after correction of the primary cause of amyloidosis. To progress in the study of the solubility of amyloid fibrils we performed an \textit{in vitro} study with the intent to remove protein constituents from amyloid fibrils and amyloid deposits.

\textbf{Methods.} Amyloid fibrils were prepurified from three amyloid deposits surgically obtained from carpal tunnel. They were incubated for 2 h with a phosphate-buffered saline (PBS) solution containing trypsin, collagenase, kallikrein, the three of them, or PBS alone. The experiments were repeated in the presence of the antiprotease $\alpha_2$-macroglobulin ($\alpha_2$M).

\textbf{Results.} Several bands were observed when the supernatants were run through SDS-PAGE. Western blotting identified in these bands the presence of $\alpha_2$M, light chains of immunoglobulins and $\beta_2$M in monomeric and dimeric form. The same proteins were solubilized with PBS alone. Equivalent results were obtained with crude amyloid deposits; however, $\beta_2$M presented almost exclusively in monomeric form.

\textbf{Conclusions.} These results show that the protein constituents may be recovered from amyloid fibrils \textit{in vitro}. They also show that even the more insoluble $\beta_2$M dimers are resuspended by the action of PBS, with no need for proteases to cleave their attachment to the amyloid deposits.

\textbf{Key words:} amyloid resorption; proteases; $\alpha_2$-macroglobulin; $\beta_2$-microglobulin; light chains of immunoglobulins amyloid resorption; proteases; $\alpha_2$-macroglobulin; $\beta_2$-microglobulin; light chains of immunoglobulins

Introduction

Amyloidosis is a disease caused by the presence of tissue deposits fulfilling the following criteria: amorphous material, mainly of extracellular distribution, positive Congo red staining showing green birefringence under polarized light, fibrillar appearance in electron microscopy and a typical X-ray diffraction pattern suggesting a $\beta$ pleated sheet structure [1]. Dialysis associated amyloidosis (DRA) is a new type of amyloidosis whose main protein component is $\beta_2$-microglobulin ($\beta_2$M) [2] and which is mainly observed in patients treated with dialysis for long periods [3]. There is agreement in that the serum levels of $\beta_2$M are not the main driving mechanism for amyloid fibril formation [4] and several modifications in the structure of the protein have been proposed as being responsible for amyloidogenesis [5–7]. However, none of these modifications has proven to be necessary or specific for $\beta_2$M amyloidosis [8]. Several proteins have been reported to be present in amyloid deposits in DRA in addition to $\beta_2$M [9] and it could well be that these other protein constituents participate in the genesis of amyloid fibrils [reviewed in 9].

Although a few clinical reports suggest that amyloid deposits may regress [10–14], the majority of the studies on $\beta_2$M amyloidosis show that once the amyloid deposits are formed, there is no degradation \textit{in vivo}, even after the primary cause of amyloidosis disappears [15–19]. We have recently reported the persistence of amyloid deposits as well as an accelerated course of dialysis-related amyloidosis when resuming haemodialysis, after long-term satisfactory transplant function [20].

Two single studies have assessed the ability for proteases to resorb amyloid fibrils \textit{in vitro} [21,22]. Skogen and Natvig showed that amyloid fibrils from AA type amyloidosis in suspension can be degraded by serine proteases and that this degradation is greater when the amyloid proteins, purified from the prepared amyloid fibrils, are incubated with serine proteases [21]. The degradation of amyloid fibrils in AA and Alzheimer’s disease amyloidosis has recently been reported [22].
**In vitro degradation of β2-M amyloid deposits**

Based on Skogen and Natvig’s work [21], we wanted to extend the study of *in vitro* degradation of amyloid fibrils to β2-M amyloidosis. Our aim was to assess *in vitro* the ability of different proteases to degrade DRA deposits and amyloid fibrils. We sought to identify the proteins removed from the amyloid deposits by different proteases in order to establish a hypothetical solubility gradient. This should provide information on the stability of the attachment for a given protein which influences the non-degradation of the amyloid deposits. Finally, we wanted to test the influence of z2-M on the protease-induced resorption of proteins from the amyloid deposits.

**Subjects and Methods**

**Patients and sample preparation**

Three patients maintained on haemodialysis for more than 15 years were included in the study; they had undergone surgery for a carpal-tunnel syndrome. The surgery obtained the relief of symptoms and the material removed was amyloid of β2-microglobulin type. The clinical characteristics of the patients are depicted in Table 1.

β2-Microglobulin fibrils were obtained from the amyloid deposits surgically obtained, following a modification of Gorevic *et al*’s method [23]. Briefly, after three freeze–thaw cycles the deposits were homogenized with a Polytron (Kinematica, GmbH, Luzern, Switzerland) in 0.1 M phosphate-buffered saline (PBS) and centrifugated at 13000 r.p.m. for 10 min at 4°C in a Sorvall RC-5B centrifuge. This step was repeated discarding the supernatant until it was free of protein (Bradford assay, Bio-Rad reagents). The pellets were enriched in amyloid fibrils. Each amyloid deposit was studied separately.

**Protease incubations**

Protease incubations were performed based on the work by Skogen and Natvig [21] with some modifications. Trypsin and collagenase proteases were purchased from Boehringer (Mannheim, Germany) and kallikrein was purchased from Sigma (St Louis, USA). The proteases were dissolved in PBS 0.1 M, pH 7.4 at a concentration of 10 mg/ml immediately before use. Both, amyloid deposit fragments and amyloid fibril preparations were incubated separately with each protease and also treated with the three proteases together. We did the same experiments in the presence of z2-macroglobulin (Sigma, St Louis, USA) at 2.7×10^-7 M. The incubations were done at 25°C for 2 h shaking gently. The enzymatic reactions were stopped with ethylene diamine tetracetic acid (EDTA) 0.2 M. After protease incubation, the supernatants and pellets were separately used for protein analysis. Controls were done with the omission of proteases in PBS, both with and without z2-M. The experiments of protease incubations were repeated twice with different aliquots of the amyloid deposits from the three patients.

**Protein preparation**

Proteins were extracted by precipitation with trichloroacetic acid (TCA) as previously described [24]. Briefly, the amyloid deposits and the fibril preparations after protease incubations were crushed under liquid nitrogen at −80°C. A 10% TCA solution was added to precipitate proteins. Ethanol–ether solutions with an increasing ether concentration (90, 95 and 100%), were used after thawing to eliminate TCA and lipid constituents. Washes and centrifugations were done twice for each ethanol–ether solution. Supernatants were discarded and pellets dried under nitrogen and stored at −70°C until protein analysis.

**Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

TCA precipitated pellets and supernatants were solubilized in 6 M urea, 5 mM dithiothreitol (DTT), 1% SDS and 50 mM Tris-HCl, pH 6.8 and centrifuged at 10000 r.p.m. for 2 min. The supernatants were withdrawn for 5–20% (w/v) SDS-PAGE performed using a discontinuous buffer system as described by Laemmli [25]. The molecular weight markers used were z-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase b (94 kDa). Gels were stained with Coomassie blue.

**Western bloting**

Protein transfer from polyacrylamide to nitrocellulose sheets (0.45 μm) was performed according to Burnette *et al*’s method [26]. Polyclonal antibodies against β2-M, z2-M, λ and κ light chains of immunoglobulin were purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). The second antibodies were goat anti-rabbit and rabbit anti-goat immunoglobulin G conjugated with horseradish peroxidase (Nordic Immunological Laboratories). Horseradish peroxidase was visualized with an enhanced chemiluminescence detection kit (Amersham CEA, AB, Sweden) and exposed to Hyperfilm ECL (Amersham).

**Ultrastructural studies by electron-microscopy**

The morphology and β2-M content of amyloid deposits was assessed in the pellets of the different experiments after being submitted to the respective treatments, by electron microscopy and gold immunolabelling.

**Resin Embedding**

Small blocks (1 mm³) of amyloid deposits were fixed in 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde at 4°C, progressively dehydrated in graded ethanol and finally

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**Table 1. Patients’ descriptions**

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Renal disease</th>
<th>Age at dialysis onset (in years)</th>
<th>Time on dialysis (in years)</th>
<th>Dialysis membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Interstitial Nephritis</td>
<td>34</td>
<td>24</td>
<td>22-C/2-PS</td>
</tr>
<tr>
<td>2</td>
<td>Polycystic</td>
<td>48</td>
<td>19</td>
<td>3-C/16-HF</td>
</tr>
<tr>
<td>3</td>
<td>Chronic GN</td>
<td>56</td>
<td>19</td>
<td>15-C/4-PS</td>
</tr>
</tbody>
</table>

C, cuprophane; PS, polysulphone; HF, high flux (including polyamide and PS). The figures are the years treated with each membrane.
embedded in Lowicryl K4M (Chemische Werke Lowi, Wald-Kraiburg, Germany). Ultrathin sections (40–60 nm) were mounted on formvar coated and etched gold grids.

**Gold immunolabelling**

The immunogold protocol included the following incubation and wash steps, which were performed in phosphate-buffered saline (PBS) at room temperature in a wet chamber: (1) two blocking rinses in 100 mM glycine-PBS, (2) 30 min incubation with 2% ovalbumin to block non-specific binding, (3) 2 h incubation with a polyclonal anti-human β2-microglobulin antibody (Dakopatts, Glostrup, Denmark) diluted 1:500 in 1% ovalbumin, (4) three rinses in glycine-PBS, (5) one-hour incubation with gold-conjugated protein A diluted in 1% ovalbumin, (6) two rinses with 0.1 M PBS, and (7) distilled water jet wash. Controls were performed with the omission of the primary antibody. The sections were finally counterstained with aqueous 2% uranyl acetate and examined in a Hitachi H-600 AB electron-microscope.

The content of β2M in the pellets was estimated by stereological methods according to previous reports [27] as the number of gold particles per area. Five repeats were performed for each sample and the results given are the mean ± standard deviation of gold particles/μm².

**Results**

**Amyloid deposits**

 Supernatants of protease treated deposits contained several proteins as demonstrated by SDS-PAGE analysis. Several bands were observed, including those migrating at 12, 23 and 180 kDa mol wt range. Western blotting showed that these bands contained β2M, κ light chains of immunoglobulins, and κ2M respectively. The addition of κ2M in coinoculation experiments of the deposits with proteases had no evident effect on the amount of protein released from the deposits nor on the SDS-PAGE pattern of the supernatants. Figure 1 shows an example of Western blotting with anti κ light chains antibody of the supernatants of amyloid deposits incubated with proteases, in the absence (Figure 1-A) and in presence (Figure 1-B) of κ2M: there was a positive band containing the κ light chains which was not modified by coinoculation with κ2M. Similar results were obtained with β2M antibodies.

κ Light chains of immunoglobulins were only partially extracted from amyloid deposits as the Western blotting was clearly positive in the pellets (Figure 2-A) while only a faint positive band was observed in the supernatants (Figure 2-B). Figure 3 shows a Western blotting with a positive reaction with anti-κ2M antibody demonstrating that κ2M may also be resuspended from the amyloid deposits.

Incubation of the amyloid deposits with PBS alone resulted in a similar pattern of proteins in the supernatants, suggesting that β2M, κ light chains, and κ2M may be extracted from amyloid deposits in the absence of proteases (lanes 5 of Figures 1-A, 1-B, 2-B and 3-A).

**Prepurified amyloid fibrils**

β2M was observed in the supernatants of treated amyloid fibrils. The Western blotting showed one
positive band at 12 kDa mol wt and a second one at 24 kDa mol wt, suggesting that dimers of $\beta_2$M can be extracted from the amyloid fibrils in addition to monomers of the protein (Figure 4-A). The presence of $\alpha_2$M did not modify significantly this pattern (Figure 4-B). A faint positive reaction was observed in the Western blot-tings of the supernatants using anti-$\kappa$ light chains of immunoglobulins antibody; $\kappa$ light chains were observed in the supernatants of the amyloid fibrils (Figure 5-A) and this reaction was much stronger in the presence of $\alpha_2$M (Figure 5-B).

The SDS-PAGE patterns obtained in the experiments containing proteases were similar to those obtained in the absence of them, with PBS incubations; again suggesting that the proteins contained in amyloid deposits, and particularly $\beta_2$M, can be released only by the action of ionic forces, even in prepurified amyloid fibrils. It is to be stressed that $\beta_2$M could also be solubilized in these experiments.

**Morphological studies**

Electron-microscopy and immunolabelling with anti-$\beta_2$-microglobulin antibodies, showed that $\beta_2$-micro-

globulin was almost exclusively located within amyloid fibrils. Very seldom were gold particles observed distant from amyloid fibrils. The amyloid deposits treated with trypsin displayed a decrease in the non-amyloid matrix when compared to the PBS treated samples. However, the counts of labelled $\beta_2$M did not differ when comparing PBS and trypsin treated samples (respectively $250 \pm 20$ and $242 \pm 31$ gold particles/$\mu$m$^2$, NS). An example of these preparations is given in Figure 6-A and 6-B.

The morphological analysis of amyloid fibrils showed a decrease in the non-amyloid matrix in both samples, PBS and trypsin treated, when compared to the amyloid deposits without prepurification. However, the trypsin treatment in prepurified amyloid fibrils did not enhance the removal of non-amyloid matrix when compared to the PBS-treated samples. The density of gold particles was similar in both PBS and trypsin-treated conditions and they were not significantly different from the unprepared amyloid deposits (respectively $232 \pm 21$ and $227 \pm 12$ gold particles/$\mu$m$^2$, NS) (Figure 6-C and 6-D).

**Discussion**

The pathogenesis of DRA remains unclear. Biochemical studies have suggested N-terminal proteolysis [5], 17Asn deamination [6] and advanced glycation end-product modification (AGEP-$\beta_2$M) [7] as key modifications which would render $\beta_2$-microglobulin amyloidogenic. Further studies identified other proteins present in the amyloid deposits, such as $\alpha_2$M [28], light chains of immunoglobulins [24,29], amyloid P substance [23,30], and glucosaminoglycans [31,32]. From the clinical side, a lack of correlation between serum levels of $\beta_2$M and the prevalence of DRA was reported very early [4]. Further, we have recently reported that the modifications in $\beta_2$M, which were previously proposed as specific for amyloidosis, can be found in other non-amyloid situations [8,33]. Taken together, these data suggest that the reasons for amyloid deposit formation cannot be found only in the changes in serum levels of $\beta_2$M nor in the known alterations of the protein itself.

Since a participation of the other protein components of $\beta_2$M amyloid deposits cannot be ruled out at present, we wanted to study their possible role. We decided to study the ability of recovering the different proteins from the amyloid deposits. By doing these experiments, we aimed to establish a sort of solubilization gradient and to evaluate the differential strength of linkage between each protein and the amyloid deposit.

Skogen and Natvig used a similar approach in their study of human secondary amyloidosis [21]. They found that in this kind of amyloidosis, which has AA as the major protein component, the fibrils could be partially released by the action of several serine proteases. Our study first confirms the presence of the light chains of immunoglobulins as well as $\alpha_2$M in the
Amyloid deposits of $\beta_2$-M amyloidosis, as previously reported [18,34]. Second, and more importantly, it shows that these proteins can be extracted from the amyloid deposits in vitro. Further, and to our surprise, the proteins were recovered in the supernatants of the amyloid deposits and prepared fibrils, incubated with PBS alone, showing that there is no need for proteases to break their attachment with the amyloid deposits. Our morphology studies showed that the modifications induced by trypsin treatment on crude amyloid deposits were very similar to those observed with the accepted method for amyloid fibril preparation. Namely, there was a decrease in the extracellular matrix-like structures of the amyloid deposits on which the amyloid fibrils are observed. Interestingly, the morphology studies of amyloid fibrils showed that trypsin did not enhance the purity of the preparation, and it was unable to extract more $\beta_2$-M from the pellets of amyloid fibrils, suggesting that the solubilization of the amyloid proteins depends on ionic forces, rather than on a proteolytic activity provided by the protease added. Thus, the amyloid proteins in the amyloid deposits would be resistant to proteases, either because of their unique space conformation, because of the presence of antiproteases or because of the participation of other unknown factors [28,35]. We have recently reported that resuspended amyloid fibrils obtained following a modification of Pras et al.’s [36] method, are degradable in vitro by the action of trypsin, and that $\beta_2$-M protects some of the amyloid proteins from degradation [37]. These observations suggest that the factors responsible for the undegradability of amyloid proteins do not exist anymore when guanidine solubilization and liophylization are preferred to TCA precipitation in preparing amyloid fibrils. Whether these factors are conformational or some amyloid constituents retained only with TCA precipitation has not been elucidated by our studies.

The present data again stress the importance of the technical approach used in studying amyloid deposits, as we have previously reported [38]. After the final homogenization step of the amyloid deposits with PBS, the absence of protein in the supernatants was confirmed by the very sensitive Bradford assay; however, after 2- and 3-h incubation with PBS, the supernatants contained again significant amounts of proteins, and importantly, these proteins were not merely contaminants. They included $\beta_2$-M and light chains of immunoglobulins which have both been located within the amyloid fibrils by ultrastructural immunogold labelling. Therefore the incubation time is likely to be an important factor in resuspending the proteins contained in the prepurified amyloid fibrils with PBS in vitro. Although in a different situation from that of amyloid deposits, Clark et al. [39] were able to recover $\beta_2$-microglobulin adsorbed in dialysis membranes by using PBS alone when measuring the adsorptive capacities of $\beta_2$-microglobulin for different dialysis membranes [39].

Another new finding of the present work, was the
In vitro degradation of \( \beta_2 \)-M amyloid deposits

presence of dimers of \( \beta_2 \)-M in the supernatants of PBS-treated amyloid fibrils. It has been suggested by Gorevic \textit{et al.} that the dimerization of \( \beta_2 \)-M would represent an important step in the formation of amyloid fibrils [23]. Vincent \textit{et al.} have made progress in the study of the dimers of \( \beta_2 \)-M from peritoneal fluid and serum [40]. They have reported that the isoform with a higher avidity to form dimers is the 5.3 pI. Our experiments show that, at least not all the dimers of \( \beta_2 \)-M are strongly linked to the fibrils, suggesting that the dimers are not necessarily trapped in the core of the fibril and dimerization could also take place at a later time (protein analysis of serum from patients having \( \beta_2 \)-M amyloidosis support this view (unpublished observations)).

In summary, our study confirms the presence of the several proteins in the deposits and shows that contrasting with the \textit{in vivo} situation, the protein components of amyloid deposits may be recovered fairly easily \textit{in vitro}. Many factors may participate in the maintenance of amyloid fibrils \textit{in vivo}, which do not exist anymore in the \textit{in vitro} situation. Among them the perfectly maintained composition of the extracellular milieu seems very much relevant. The proteins contained in amyloid fibrils seem to be quite resistant to proteolytic degradation, because of its conformational characteristics or because of the presence of antiproteases which would maintain a skewed protease–anti-protease imbalance. The absence of modification of the protein pattern in the presence of proteases supports this hypothesis. Finally, the resistance of amyloid fibrils to protease degradation is in keeping with our recent observation showing that intralysosomal \( \beta_2 \)-M from amyloid fibrils is not processed by macrophages, while other proteins are normally degraded by the lysosomal enzymes [41].

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