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α_2-Macroglobulin Protects Some of the Protein Constituents of Dialysis-Associated Amyloidosis from Protease Degradation

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A crucial point to know in the prevention and treatment of β2-microglobulin (β2-m) amyloidosis is the putative resorption of amyloid fibrils in vivo. Although still controversial, long term clinical studies suggest that there is no resorption of amyloid fibrils in vivo, even after the suppression of the primary cause of amyloidosis. Two in vitro studies on murine and human AA amyloidosis as well as Alzheimer's disease suggest that protein constituents of amyloid fibrils may be resorbed. Protein resorption can be inhibited by the antiprotease amyloid P component. We extended these in vitro studies on β2-m amyloidosis, and assessed the effect of α2-macroglobulin (α2-M), a serum antiprotease previously found in this type of amyloidosis, on the putative protease induced protein resorption. Here, we show that amyloid proteins, β2-m and light chains of immunoglobulins, were degraded by trypsin. Preincubation of the amyloid proteins with α2-M significantly inhibited the trypsin induced protein degradation of λ chains. These data add further support to the hypothesis proposing a role for α2-M and other antiproteases in the formation and/or persistance of β2-m amyloidosis.

Dialysis related amyloidosis (DRA) is a recognised complication frequently observed in long-term dialysis patients. The incidence of this type of amyloidosis increases with time on dialysis, reaching more than 80% in patients dialysed for over 15 years (recently reviewed in 1). The main clinical manifestations are carpal tunnel syndrome, chronic synovitis and bone cysts (1). It is widely accepted that once the amyloid deposits are formed, there is no degradation in vivo even after the primary cause of amyloidosis disappears. In vitro, 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 submitted to the action of serine proteases (2). Tennent *et al* have recently reported that amyloid fibrils from AA amyloidosis and Alzheimer's disease may be degraded in vitro by proteases, and that this degradation is inhibited by the serum amyloid P component (SAP) (3), a Ca binding protein with antiprotease activity which is a universal constituent of most types of amyloidosis (4).

The main protein constituent of DRA has been identified as β2-m (5). However, a number of observations show that β2-m accumulation and or modification do not fully explain the appearance of amyloid fibrils suggesting that other factors participate in the formation and/or persistance of amyloid fibrils. α2-M (6), κ and λ chains of immunoglobulins (7), glucosaminoglycans (8), ubiquitin (9), apoE (10) are among the amyloid constituents which may play a role in the pathogenesis of DRA.

α2-M is a widely distributed anti-protease whose physiological role is not completely understood. By trapping proteases, α2-M might protect amyloid from degradation in vivo, this favouring amyloid protein deposition and precipitation (11). In this study, we investigated the putative degradation of amyloid fibrils from dialysis related amyloidosis by proteases, and assessed the ability of α2-M in inhibiting this degradation.

MATERIALS AND METHODS

Amyloid fibril preparation. Amyloid fibrils were purified from amyloid deposits surgically obtained from the carpal tunnel of dialysed patients following a modification of Gorevic et al's method (12). Briefly, after three freeze-thaw cycles, the deposits were homogenised with a polytron (kinematico, GmbH, Luzern, Switzerland) and centrifuged at 13,000 rpm for 30 minutes at 4°C in a Sorval 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.

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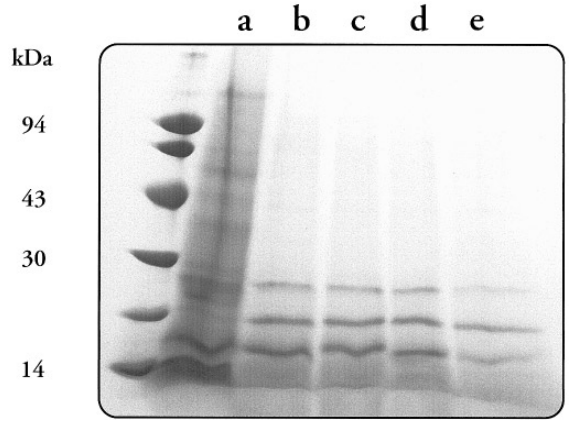

FIG. 1. 5-20% gradient SDS-PAGE of amyloid fibrils, and the effect of trypsin. Purified amyloid fibrils from DRA deposits were run directly (lane a) or after incubation with trypsin (100 μg/ml) for 1, 4, 6 and 18 hours (lanes b, c, d and e respectively. It can be observed the presence of several proteins in amyloid deposits (lane a), which are degraded by the action of trypsin in vitro (lanes b to e).

β2-m purification. Both urinary and serum human β2-m were used. They were purified following Nissen et al's method (13), as previously described (14). Briefly, after lyophylisation, prepurified proteins were solubilised in 25 mM imidazole/HCl pH 7,41 buffer and run through a G75 sephadex 1m $\times$ 4cm column using the same buffer. The tubes containing mainly B2M were pooled, freezed-dried and run through a polybuffer Exchanger 94 (Pharmacia Fine chemicals, Uppsala, Sweden) 60 $\times$ 1.5 cm column equilibrated with imidazole buffer and eluted with 1:8 polybuffer 74 Hcl pH 4. Pure β2-m was then precipitated with 100% ammonium sulfate and centrifuged at 30 000 rpm for 30 minutes. The pellet was resolubilised in PBS and desalted with a G25 P10 column (Pharmacia Fine chemicals, Uppsala, Sweden).

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. 5-20% gradient SDS-PAGE was performed according to Laemli's method (15). The molecular weight markers were alpha-lactalbumin (14kDa), Soybean trypsin inhibitor (20kDa), Carbonic anhydrase (30kda), ovalbumin (40 kDa), bovine serum albumin (67 kDa) and phosphorylase B (94kDa). Gels were stained with Coomassie blue. Protein transfer from polyacrylamide gel to nitrocellulose sheet was performed according to Burnette et al's method (16). Polyclonal antibodies against β2-m, α2-M, λ and κ light chains of immunoglobulins were purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). The second antibodies were Goat anti-Rabbit and Rabbit anti-Goat immumoglobulin G conjugated with horseradish peroxidase (Nordic Immunological Laboratories). Horseradish peroxidase was visualised with an enhanced chemiluminescence detection kit (Amersham CEA, AB, Sweden) and exposed to hyperfilm ECL (Amersham).

Human α2-M was purchased from SIGMA and resuspended in 67 mM phosphate buffer when used. Trypsin and Soybean trypsin inhibitor were purchased from SIGMA and used at a concentration of 1mg/ml in a posphate buffer (67 mM).

RESULTS

I. Digestion of Amyloid Fibrils

SDS-PAGE analysis confirmed the presence of several proteins in purified DRA amyloid fibrils (Fig. 1, lane a). Western blotting analysis showed that the bands migrating at 12, 24 and 180 kDa molecular weight contained β2-m, κ and λ light chains of immunoglobulins and α2-M respectively. Indeed, incubation of amyloid fibrils in vitro with pancreatic proteinase trypsin (1-18 hours at 37°C) resulted in the disappearance of these bands as observed in Fig 1 lane b,c d and e, showing that the proteins contained in amyloid fibrils are susceptible to proteolysis, and are digested by trypsin.

II. α2-M–β2-m Interaction

As internal control for α2-M/β2-m binding studies, we first assessed α2-M/trypsin interaction. As shown in Fig. 2 treatment of α2-M with trypsin resulted in a cleavage of the bite region of α2-M and increasing amounts of the *activated* form of α2-M were observed when the incubation time was prolonged (Fig. 2, lanes b through e).

α2-M/β2-m interaction is represented in Fig. 3, lane a. Western blotting with anti-β2-m antibody after denaturing SDS-PAGE of a mixture of α2-M and β2-m maintained for 24h at room temperature, showed four positive bands at ~12, ~24, ~90 and ~190 kDa mol wt. These mol wt correspond to the known mol wt of monomeric and dimeric forms of β2-m, *activated* α2-M plus β2-m and native α2-M plus β2-m, respectively.

Competitive binding studies were also performed. Fig. 3 shows the effect of adding trypsin into a mixture of α2-M/β2-m (lanes a and b); kinetics of this reaction were obtained by adding exces soybean trypsin inhibitor at different time points (Fig. 3, lanes b, c, d). It can be observed that α2-M bound β2-m was shifted by trypsin, as the positive bands with anti-β2-m antibody diseappeared from the first time point assessed (1 hour). Fig. 3 (right hand side) shows the Western blotting with anti-α2-M antibody of the same experiment. It can be observed that the α2-M positive bands

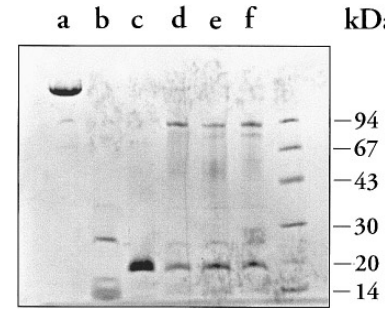

FIG. 2. α2-macroglobulin/trypsin interactions. α2-macroglobulin (lane a) was allowed to react (10/1 concentration ratio) with trypsin (lane b); soybean trypsin inhibitor (lane c) was added in molar excess at 1, 2 and 6 hours (lanes d, e and f respectively) to stop the reaction. It can be observed the appearance of the fast form of α2-macroglobulin by the action of trypsin; by 2 hours (lane e) there was no significant amount of native α2-macroglobulin left in the preparation.

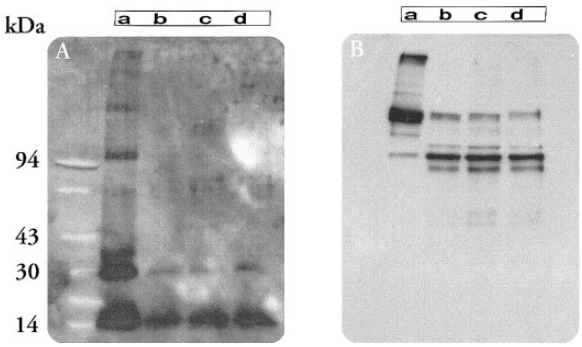

FIG. 3. α2-macroglobulin/β2-microglobulin and trypsin competitive interactions. Panel A and B represent the Western blot of the same experiment using anti β2-microglobulin and anti α2-macroglobulin antibodies respectively. α2-macroglobulin was incubated with β2-microglobulin (10/1 concentration ratio) for 24 hours (lanes a). At the end of the incubation time trypsin (200 μg/ml) was added and the reaction stopped by adding excess soybean trypsin inhibitor at 5, 10, 15 minutes (lane b, c and d). It can be observed that α2-macroglobulin bound β2-microglobulin is shifted by trypsin at the same time that α2-macroglobulin is activated (increase in the proportion of the fast form (~95 kDa)).

matched with the high mol wt ones also positive for β2-m, confirming the binding between both proteins. Further, in this experiment it was observed again that incubation with trypsin increased the amount of *activated* α2-M.

III. Degradation of Amyloid Fibrils in the Presence or Absence of α2-M

To investigate the antiproteolytic action of α2-M, we pretreated purified amyloid fibrils with α2-M before submitting them to trypsin digestion. The mixture was run in a SDS-PAGE after stopping the reaction with excess soybean trypsin inhibitor, and blotted. The antibodies used included anti-β2-m, as well as anti-κ and anti-λ light chains of immunoglobulins. Figure 4 shows that λ light chains of immunoglobulins were contained in amyloid fibrils (lane A), that trypsin reduced the amount of λ light chains of immunoglobulins contained in these fibrils (lane B) and that pre-treatment with α2-M significantly prevented this degradation (lane C). No modification of the antibody labelling signal with anti-κ light chains of immunoglobulins and anti-β2-m antibodies was observed when pre-treating amyloid fibrils with α2-M (data not shown). Therefore, α2-M does protect from degradation some but not all of the amyloid proteins in dialysis related amyloidosis.

DISCUSSION

The present study shows that the protein constituents of amyloid fibrils from dialysis related amyloidosis may be degraded *in vitro*. Amyloid fibrils in suspension are sensitive to the action of trypsin. It also shows that trypsin retains its ability to *activate* α2-M in vitro, after complexing with β2-m. Finally, it demonstrates that α2-M is able to protect λ light chains of immunoglobulins from trypsin degradation, showing that even in vitro, α2-M may protect some of the protein constituents of dialysis related amyloidosis.

Human α2-M is a 720 kDa glycoprotein consisting of four identical subunits of 180 KDa each (17). Macrophages have a high affinity receptor for α2-M, a member of the low-density lipoprotein receptor super gene family, which specifically captures the fast form of α2-M (18). By the action of proteinases, α2-M is proteolysed and following conformational changes is able to trap the proteinase; the α2-M/proteinase complex is then rapidly removed from the circulation (19,20). In vivo, α2-M is a key protein for proteinase clearance by macrophages, thereby modulating tissue protease activity. α2-M acts as a "panproteinase inhibitor" (21). However, the protease-antiprotease imbalance is not only controlled by the clearance of the proteases. In many tissues, proteases and proteinases inhibitors exist in a dynamic equilibrium. Although the gross physiological function of proteases is the prevention of undesired proteolysis, the mechanisms by which they act have been rarely elucidated.

Trypsin is a normal constituent of the serum (normal range ~300mg/l) and is mostly of intestinal origin. Like other serum proteases, serum trypsin is bound by α2-M. Our in vitro data show that trypsin is able to shift the β2-m/α2-M complex and to bind α2-M, as we observed an increase in the fast form of α2-M, even when we added trypsin to the previously formed β2-m/α2-M complexes.

The cells are important players in the protease-antiprotease dynamic equilibrium, not only because of their clearing capacities of proteinases, but also because they synthesise them (22). Although the cellular participation on the pathogenesis of amyloidosis has not been

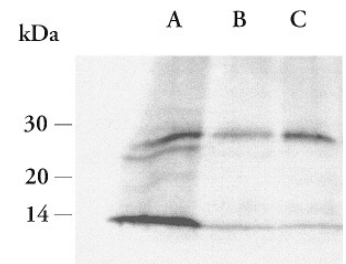

FIG. 4. Amyloid fibril degradation in vitro and protective effect of α2-macroglobulin. Purified amyloid fibrils (lane A) were incubated with trypsin (100 μg/ml) for 8 hours in the absence (lane B) or presence (lane C) of excess α2-macroglobulin. The Western blotting with anti-λ light chains of immunoglobulins is shown. It can be observed that there is a decrease in the signal in lane B (when compared with lane A) which is restored by the presence of α2-macroglobulin (lane C), showing that λ light chains of immunoglobulins may be degraded by trypsin and that this degradation is inhibited by α2-macroglobulin.

largely assessed, macrophages have been identified as the most common cell infiltrating amyloid fibrils (23). In our present in vitro study, α2-M protected λ light chains of immunoglobulins from trypsin degradation, even in the absence of macrophages. These results suggest that the conformational changes in protein/α2-M complexes may alter protein degradation.

In addition to proteases, α2-macroglobulin is able to bind a variety of other proteins (cytokines and growth factors) (24). The non-proteolytic peptides trapped by α2-M become largely protected from exogenous proteinases. Therefore, α2-M may also modulate the metabolic effects of these growth factors by modifying their catabolism (25). The protective effect of α2-M by binding the substrate of the proteases, may explain our findings on protection of λ light chains of immunoglobulins degradation by α2-M. Further, our data show that the in vitro sensitivity to trypsin degradation and its inhibition by α2-M in β2-m amyloid fibrils varies from protein to protein. Accordingly, no inhibition of degradation was observed in vitro for β2-m.

It is known that the uncleared α2-M/protease complexes may retain some proteolytic activity, particularly of small proteins and peptides (17). Contrasting with λ light chains of immunoglobulins, β2-m, with a lower mol wt, would still be sensitive to the trypsin. If this was the case, and since in our system there was no macrophages to clear the α2-M/trypsin complexes, trypsin would end by totally degrade the β2-m present in the deposits in vitro. The in vivo situation is expected to be different.

In summary, amyloid fibrils can be degraded in vitro by trypsin. The presence of α2-macroglobulin protects lambda chains of immunoglobulins from trypsin induced degradation. In a previous report, we described a binding between α2-M and β2-m. In the present study, we found that trypsin may displace β2-m from the α2-M complexes, and in the absence of cells, may completely degrade β2-m in vitro. Taken together, these data show that amyloid fibrils from β2-m amyloidosis may be processed as other amyloid forms, and most importantly, stresses the importance of the interactions between the different protein constituents of the deposits, the cellular participation still remaining unclarified.

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