Investigation into the Role of Macrophages in the Formation and Degradation of β₂-Microglobulin Amyloid Fibrils*

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Isobel J. Morten¹, Walraj S. Gosal², Sheena E. Radford, and Eric W. Hewitt³

From the Institute of Molecular and Cellular Biology and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

Dialysis related amyloidosis is a serious complication of long-term hemodialysis in which β₂-microglobulin (β₂m) forms amyloid fibrils that deposit predominantly in cartilaginous tissues. How these fibrils form in vivo, however, is poorly understood. Here we perform a systematic investigation into the role of macrophages in the formation and degradation of β₂m amyloid fibrils, building on observations that macrophages are found in association with β₂m amyloid deposits in vivo and that these cells contain intra-lysosomal β₂m amyloid. In live cell imaging experiments we demonstrate that macrophages internalize monomeric β₂m, whereupon it is sorted to lysosomes. At lysosomal pH β₂m self-associates in vitro to form amyloid-like fibrils with an array of morphologies as visualized by atomic force microscopy. Cleavage of the monomeric protein by both macrophages and lysosomal proteases isolated from these cells results in the rapid degradation of the monomeric protein, preventing amyloid formation. Incubation of macrophages with preformed fibrils revealed that macrophages internalize amyloid-like fibrils formed extracellularly, but in marked contrast with the monomeric protein, the fibrils were not degraded within macrophage lysosomes. Correspondingly β₂m fibrils were highly resistant to degradation by high concentrations of lysosomal proteases isolated from macrophages. Despite their enormous degradative capacity, therefore, macrophage lysosomes cannot ameliorate dialysis-related amyloidosis by degrading pre-existing amyloid fibrils, but lysosomal proteases may play a protective role by eliminating amyloid precursors before β₂m fibrils can accumulate in what may represent an otherwise fibrillogenic environment.

The amyloidoses are a class of conformational diseases associated with the aggregation of proteins or peptides into insoluble amyloid fibrils (1). More than 20 proteins and peptides are currently known to form amyloid deposits that are associated with human disease (1). One such protein is β₂-microglobulin (β₂m),⁴ the precursor of amyloid fibrils formed in dialysis-related amyloidosis (DRA), a debilitating complication of long-term hemodialysis (2–4). β₂m forms the non-covalently bound light chain of the major histocompatibility class I complex, which is expressed on the plasma membrane of all nucleated cells (5). Cells continually shed β₂m from their surface, whereupon the protein is normally removed from the serum by degradation in the proximal tubule of the kidney (3). In patients with end stage renal disease, however, neither the kidney nor the dialysis membrane can remove β₂m from the circulation, resulting in a 10–50-fold increase in the concentration of β₂m in the serum (2–4). The sustained high concentration of β₂m therein appears to be one key initiating factor in the aggregation of this protein into insoluble amyloid fibrils, which typically accumulate in the musculoskeletal system resulting in bone and joint destruction (2–4).

How amyloid fibrils form from β₂m in vivo is poorly understood. Because intact wild-type β₂m is unable to form amyloid-like fibrils at neutral pH in vitro in the absence of pre-formed seeds, a role for amyloid-associated factors (6, 7), limited proteolysis (8, 9), or interaction with collagen (10) or glycosaminoglycans (11, 12) have all been implicated in the initiation of amyloid deposition in vivo. Interestingly, although macrophages are found in association with amyloid deposits in patients suffering from DRA, and macrophages and macrophage-like cells cultured in vitro have been shown to promote the aggregation of a variety of amyloidogenic precursors into amyloid-like fibrils (13–16), the role of these cell types in DRA remains poorly understood (17, 18). Importantly in this regard, β₂m fibrils have been found within the lysosomes of macrophages associated with amyloid in patients suffering from DRA (19), raising the intriguing possibility that these organelles could play a role in the amyloidogenesis of β₂m either by the acidic pH, therein promoting the self-assembly of β₂m into amyloid fibrils (20–24), or by proteolytic cleavage, producing fragments with increased amyloid propensity (8, 9, 25). Alternatively, the macrophages associated with β₂m amyloid deposits may play a protective role in DRA by capturing extracellular amyloid by phagocytosis for degradation (26).

To determine the role of macrophages in DRA, we performed a systematic examination of whether macrophage lysosomes act as a site to enhance the initiation of β₂m amyloid

⁴The abbreviations used are: β₂m, β₂-microglobulin; AGE, advanced glycan end product; AFM, atomic force microscopy; DRA, dialysis-related amyloidosis; EM, electron microscopy; FITC, fluorescein-5-isothiocyanate; ThT, thioflavin T; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
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formation or if these organelles instead can capture and degrade β₂m amyloid. In a combination of experiments using purified lysosomal extracts, live cell imaging, and flow cytometry, we demonstrate that whereas fibril formation from β₂m occurs at lysosomal pH, monomeric β₂m that has been internalized into macrophage lysosomes is rapidly and completely degraded by lysosomal proteases. By contrast, β₂m fibrils were found to be highly resistant to lysosomal proteolysis and were not degraded after their uptake by macrophages. Together these findings demonstrate that whereas macrophages remove potential amyloidogenic precursors of β₂m by their degradation, the fibrils themselves, once formed, are highly resistant to lysosomal degradation. Thus, the infiltration of macrophages into the joint areas in the late stages of DRA (27) exacerbates disease by secreting inflammatory cytokines and chemokines (18, 28, 29), but cannot remove the amyloid deposits that develop therein.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise indicated all reagents were obtained from Sigma-Aldrich.

Expression and Labeling of Recombinant β₂m—Recombinant β₂m was expressed and purified as described previously (21). The N terminus of β₂m was conjugated with fluorescein-5-isothiocyanate (FITC; “isomer I,” Molecular Probes) by incubating the wild-type protein with FITC at pH 7.4 for 2 h at room temperature with stirring to generate the singly labeled conjugate β₂mN-FITC. The protein-dye conjugate was separated from free dye by gel filtration on a PD10 column (Amersham Biosciences). β₂mN-FITC was then purified by anion exchange chromatography using a 6-ml Resource Q column (Amersham Biosciences). The resultant pure protein was dialyzed, and labeling at a single site was confirmed by mass spectrometry. β₂m was also labeled at multiple sites with FITC by incubation of the dye and protein at pH 9.4 for 2 h at room temperature with stirring. The resultant protein conjugate, β₂mFITC-multi, was separated from free dye by gel filtration and subsequent dialysis. Analysis by mass spectrometry demonstrated that β₂mFITC-multi was a mixture of β₂m species labeled with 1, 2, 3 or 4 FITC molecules at a ratio of 1:2:2:1, respectively.

Formation of Fibrils from β₂mFITC-multi—β₂mFITC-multi (0.5 mg/ml) was incubated with 1 mg/ml unlabeled β₂m in phosphate-buffered saline (136 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄), pH 7.4, in the presence of 10% (v/v) β₂m amyloid seeds formed from recombinant β₂m at pH 2.5 that were subsequently stabilized by the addition of heparin (6). The samples were incubated for 6 weeks at 37 °C with agitation at 200 rpm. The fibril preparation was centrifuged at 14,000 × g in a microcentrifuge for 10 min to pellet insoluble material. The insoluble material was then analyzed by probing dot blots with a polyclonal anti-β₂m antibody (DAKO) and the generic anti-amyloid antibody, WO1 (30), as described previously (24, 30). Negative-stain electron microscopy (EM) was performed on the insoluble pellet fraction as described previously (6). The presence of fluorescent β₂m fibrils in the pellet was confirmed by SDS-PAGE. The fibril pellet was resuspended into SDS-PAGE sample buffer, which did not contain reducing agent, heated at 95 °C for 5 min, and resolved on a 15% Tris-glycine SDS-PAGE gel. Multimers of the β₂mFITC-multi were detected by illumination with UV light. Quantification of the fibril yield was performed as previously described (12).

Cell Culture—RAW 264.7 mouse macrophage cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine at 37 °C in 5% CO₂.

Live Cell Imaging of the Uptake of Monomeric and Fibrillar β₂m by RAW 264.7 Macrophages—10⁶ RAW 264.7 cells were plated in 34-mm glass-bottom culture dishes (Iwaki) and grown overnight. Cells were then incubated in culture medium containing either 50 μg/ml monomeric β₂mN-FITC or 50 μg/ml monomeric β₂mFITC-multi or 10 μg/ml of fibrils incorporating β₂mFITC-multi in the presence of LysoTracker (Molecular Probes) for 2 h at 37 °C in 5% CO₂. Cells were washed to remove the non-cell associated FITC-conjugated β₂m and either imaged immediately or incubated for up to 24 h in culture medium at 37 °C in 5% CO₂. Cells were imaged using a Zeiss Axiosvert 200M inverted confocal microscope with a plan-apochromat ×63 oil immersion lens. Fluorescence excitation was by argon ion and He:Ne1 lasers.

Flow Cytometric Analysis of the Uptake of Monomeric and Fibrillar β₂mFITC by RAW 264.7 Macrophages—RAW 264.7 cells were plated in 6-well dishes (Costar) and grown overnight at 37 °C in 5% CO₂. Cells were then incubated in culture medium with either 50 μg/ml monomeric β₂mFITC-multi or 10 μg/ml fibrils incorporating β₂mFITC-multi for 2 h and washed to remove non-cell-associated β₂mFITC. Alternatively, the cells were grown in the presence of 50 μg/ml unlabeled β₂m for 10 days after which monomeric β₂mFITC-multi (50 μg/ml) was added. The cells were either analyzed immediately or incubated for a further 2–24 h at 37 °C in 5% CO₂. Before flow cytometric analysis the cells were washed 3 times with phosphate-buffered saline 0.1% (w/v) bovine serum albumin and detached from the culture dish by scraping, and cell-associated fluorescence was analyzed with a BD Biosciences FACSARia and Cell Quest software. The cell population was gated to exclude debris, and 5000 gated events were recorded.

Fibril Formation at pH 4.5—Recombinant β₂m (1 mg/ml) was incubated in 100 mM ammonium acetate, pH 4.5, at 37 °C with or without agitation at 200 rpm. Samples were dot-blotted and probed with anti-β₂m (DAKO), anti-oligomer (A11), or anti-fibrillar (WO1) antibodies as described previously (24, 30, 31). Fibrils were visualized by atomic force microscopy (AFM) as described previously (24). Worm-like and rod-like fibrils formed at pH 4.5 were diluted 1:100, and long straight fibrils formed at the same pH were diluted 1:20 with deionized water before analysis by AFM. Images (2.5 μm²) were acquired using a scan rate of ~2 Hz, and images are shown as artificial zoom (2.5 times original) for clarity. Congo red binding was performed as described (32) using 5 μM Congo red and 5 μM β₂m. Thioflavin T (ThT) fluorescence was performed using a final concentration of 20 μM ThT and 10 μM β₂m samples. ThT fluorescence was quantified using a PFI Quantamaster C-61 spectrofluorimeter with excitation at 440 nm and emission scan between 450 and 600 nm as described previously (12).
Congo Red Birefringence—Congo red birefringence was measured using a protocol adapted from Tennent (33). Briefly, a fibril suspension in phosphate-buffered saline was applied to glass slides coated with bovine gelatin and dried overnight at 37 °C. The slides were then fixed with a solution of 96% (v/v) ethanol and 4% (v/v) formaldehyde for 5 min, rinsed in water for 5 min, and air-dried. Slides were immersed in a filtered saturated NaCl solution containing 80% (v/v) ethanol and 0.1% (v/v) sodium hydroxide for 20 min, drained, and immersed into a filtered saturated NaCl and Congo red solution containing 80% (v/v) ethanol and 0.1% (v/v) sodium hydroxide for 20 min. The slides were rinsed for 10 s in 3 sequential baths of 100% ethanol and then rinsed in water for 5 min, and air-dried. Slides were immersed in a filtered saturated NaCl solution containing 80% (v/v) ethanol and 0.1% (v/v) sodium hydroxide for 20 min. The slides were then heated at 95 °C for 5 min and resolved on 15% Tris-Tricine gels. The protein bands were visualized by staining with Coomassie Blue. In parallel, samples were analyzed by dot-blotting with anti-βm antibodies as well as antibodies specifically labeled with FITC (βmN-FITC) (see “Experimental Procedures”).

RESULTS

Monomeric β2m Is Internalized and Sorted to Lysosomes in RAW 264.7 Macrophages—How the formation of β2m amyloid is initiated in vivo remains an open question, the intransigence of the protein to form amyloid fibrils at pH 7.0 in vitro suggesting a key role of one or more factors within the biological environment in stimulating this process (35). Previous studies have shown that monomeric β2m is endocytosed by macrophages (36), raising the possibility that transit to the low pH and protease-rich environment of lysosomes could play a role in DRA. To investigate this possibility we first examined the fate of monomeric β2m following its internalization in the model macrophage cell line, RAW 264.7. To enable detection of the protein in live cell imaging experiments, the N terminus of β2m was specifically labeled with FITC (β2mN-FITC) (see “Experimental Procedures”). RAW 264.7 macrophages were incubated with monomeric β2mN-FITC at 50 μg/ml, a concentration equivalent to that of β2m in the serum and synovial fluid of patients undergoing long-term hemodialysis (2–4). Lysosomes were then visualized with LysoTracker dye, a fluorescent probe that accumulates in cellular compartments of acidic pH (Fig. 1). After 2 h of incubation at 37 °C, β2mN-FITC was clearly localized to intracellular punctate structures that were also labeled by the LysoTracker dye, consistent with internalization of β2m and its subsequent sorting to lysosomes in this cell type.

β2m Forms Amyloid-like Fibrils at Lysosomal pH—Previous studies have shown that β2m forms amyloid-like fibrils with different morphologies when incubated under acidic conditions in vitro (20, 21, 24). To determine whether amyloid-like fibrils can also be generated at the pH of lysosomes, pH 4.5 (37, 38), monomeric β2m was incubated at pH 4.5 either with or without agitation, and fibril formation was monitored by immunoblotting using anti-β2m antibodies as well as antibodies able to recognize protofibrillar species (A11) (31) or amyloid fibrils (W01) irrespective of the protein sequence (30). In addition, the presence of fibrils was monitored using ThT fluorescence and Congo red absorbance, whereas the morphology of aggregated species was examined using tapping mode AFM (see “Experimental Procedures”) (Fig. 2). The data revealed that incubation of β2m at pH 4.5 in the absence of agitation resulted in...
in the formation of short (<50 nm) rod-like fibrils after 24 h, whereas longer fibrils with a worm-like morphology were formed after 36 days under these conditions (Fig. 2. A and B, i and ii). These species are reminiscent of the fibrils formed previously at lower pH values at high ionic strengths (20, 21, 24). By contrast, fibrils with a long, straight morphology that more closely resemble amyloid fibrils purified from DRA patients (10) were generated when the samples were incubated at pH 4.5 with agitation (Fig. 2Bi).i). These observations are consistent with previous findings that the generation of fibrils with a long straight morphology involves nucleated assembly that is enhanced in vitro by agitation (24). The amyloid-specific antibody WO1 (30) bound to all of these samples (Fig. 2A), whereas only samples containing the rod-like and worm-like fibrils were recognized by the anti-oligomer antibody, A11 (31), consistent with previous results on βm fibrils with these morphologies formed under more acidic conditions (24).

To confirm that the fibrils formed at pH 4.5 are amyloid-like, their ability to bind the amyloid-specific dyes Congo red and ThT was also analyzed (Fig. 2C). In the presence of each of the fibrillar species formed at pH 4.5 the absorbance spectrum of Congo red showed a characteristic increase at 540–550 nm compared with that of the dye in the presence of monomeric βm, consistent with these fibrils having amyloid-like structures (Fig. 2C) (32). For the long straight fibrils, binding of Congo red also gave rise to red-green birefringence when the stained fibrils were viewed with cross-polarized light (see “Experimental Procedures”), although this was not so apparent for the rod-like and worm-like fibrils (data not shown). In addition, each of the fibril types formed at pH 4.5 displayed an increase in fluorescence at 459–600 nm upon incubation with ThT (Fig. 2D) and an ~120-nm red shift in the excitation spectrum of ThT from 327 to 441 nm (data not shown), characteristic of binding of ThT to amyloid-like material (39, 40). Together the data demonstrate that the acidic pH associated with lysosomes promotes the spontaneous self-assembly of βm into amyloid-like material, resulting in fibrils with a variety of morphological types, reminiscent of those formed under more acidic conditions in vitro (24, 41).

Lysosomal Proteases Cleave Monomeric βm into Discrete Fragments—Removal of the N-terminal six residues of βm destabilizes the native structure of the protein and enhances its ability to self-associate into fibrils both at acidic and neutral pH (6, 8, 25). To determine whether lysosomal proteases are also able to form N-terminal-cleaved fragments of βm, lysosomes were isolated from RAW 264.7 cells by subcellular fractionation. The lysosomes were then lysed by freeze-thaw, and the preparation was centrifuged to remove residual membranes (see “Experimental Procedures”). To examine the ability of the lysosomal proteases to cleave βm at pH 4.5, monomeric βm was incubated at this pH with different concentrations of lyso-
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In vivo rapid and quantitative degradation of β2m into small fragments unable to self-assemble into amyloid fibrils.

Next we sought to identify the three major cleavage products generated by limited proteolysis of β2m with lysosomal enzymes. Thus, a sample of monomeric β2m was incubated with lysosomal supernatant containing the equivalent of 0.05 units cathepsin B for 4 h at 37 °C, and the resulting fragments were identified by N-terminal sequencing and mass spectrometry. As anticipated, the upper band (band A in Fig. 3B) corresponds to full-length β2m. Band B, by contrast, contained a combination of two cleavage products that had either 10 or 11 amino acids removed from the N terminus of the protein, whereas the C terminus remained intact. The protein in band C had an intact N terminus but lacked the C-terminal 38 residues, whereas band D contained β2m missing the 65 N-terminal amino acids but with an intact C terminus (Fig. 3B). Analysis of the fragments by mass spectrometry in the absence of a reducing agent demonstrated that the β2m cleavage products in bands C and D were in fact derived from a single species in which residues 62–65 had been removed, with the two resultant fragments linked by the natural disulfide bond between residues 25 and 80 (Fig. 3, B and C). This was confirmed by non-reducing SDS-PAGE, in which bands C and D ran as a single species (data not shown). Interestingly, this species is reminiscent of a natural cleavage product of β2m, ΔK58, which is known to have increased amyloid potential compared with the wild-type protein (9, 42). A fragment lacking the N-terminal 10 amino acids is also found in amyloid deposits in patients with DRA (43).

Monomeric β2m Is Rapidly Degraded by Macrophages—The data presented above demonstrate the potential of the lysosomal environment to stimulate the assembly of β2m into amyloid fibrils in vivo by its low pH, by limited proteolytic cleavage, or both. Therefore, to determine the fate of monomeric β2m internalized by macrophages in living cells, β2m was labeled with FITC at multiple sites (β2mFITC-multi), and the resulting protein (50 μg/ml) was incubated with RAW 264.7 macrophages for 2 h. Uptake of the protein into lysosomes was then visualized using LysoTracker dye (Fig. 4A). As expected, β2mFITC-multi co-localized with the LysoTracker dye in punctate structures, consistent with endocytosis and subsequent sorting of β2mFITC-multi to lysosomes (Fig. 4A).
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However, after incubation for a further 2 h in the absence of extracellular \( \beta_m \)-FITC-multi, there was a marked reduction in the intensity of cell-associated FITC fluorescence (Fig. 4A), consistent with degradation of \( \beta_m \) by lysosomal proteases and loss of the fluorophore. This was corroborated by flow cytometry, in which a substantial reduction in the level of cell-associated \( \beta_m \)-FITC-multi fluorescence was observed after a 2-h chase in the absence of \( \beta_m \)-FITC-multi and a complete loss of FITC fluorescence after 24 h (Fig. 4B). The loss of the \( \beta_m \)-FITC-multi fluorescence signal could not be attributed to photobleaching, as control experiments in which FITC-dextran was internalized into lysosomes showed no significant reduction in the fluorescence signal over 24 h when analyzed by both flow cytometry and fluorescence microscopy (data not shown).

Furthermore, when preincubated with \( \beta_m \), the RAW 264.7 macrophages were still able to degrade \( \beta_m \)-FITC-multi rapidly, indicating that prolonged incubation with this concentration of \( \beta_m \) does not overload the proteolytic capacity of the lysosomes (Fig. 4C).

The data demonstrate, therefore, that although at a pH corresponding to that of the lysosomes \( \beta_m \) can assemble into amyloid fibrils, this process is relatively slow (although it could be accelerated by other factors within lysosomes). By contrast, the proteolytic capacity of the lysosomal proteases results in the rapid degradation of the amyloid precursors, resulting in their removal before fibril formation can commence. Rather than exacerbating amyloid deposition, therefore, the results suggest an important role for lysosomal proteases in the degradation of potentially dangerous amyloid precursors after their endocytic uptake.

\( \beta_m \) Fibrils Are Internalized and Persist in Macrophages—As well as degrading monomeric or small molecular weight precursors of amyloid, we next asked whether macrophages also have the ability to degrade \( \beta_m \) amyloid fibrils formed extracellularly. Amyloid-like fibrils were formed from a mixture of \( \beta_m \)-FITC-multi and unlabeled \( \beta_m \) (1:2 ratio) at pH 7.4 by seeding elongation of amyloid-like fibrils formed from the wild-type protein at pH 7.25 in the presence of heparin (see “Experimental Procedures”). Under these conditions amyloid-like fibrils that are stable at pH 7.4 are generated. Previous studies have shown that fibrils generated in this manner are long and straight in morphology, bind Congo red, resulting in red-green birefringence, and give rise to a fiber diffraction image consistent with the cross-\( \beta \) architecture of amyloid (6, 44). The fibrils incorporating \( \beta_m \)-FITC-multi were also long and straight in morphology and bound by the amyloid-specific antibody, W01 (Fig. 5, A and B). To confirm that these fibrils had incorporated \( \beta_m \)-FITC-multi, the insoluble material was also shown to be fluorescent. To further demonstrate that \( \beta_m \)-FITC-multi was incorporated into fibrils, we took advantage of our observation that \( \beta_m \) in long straight fibrils, but not monomeric \( \beta_m \), forms a ladder of higher molecular weight species when resolved by SDS-PAGE under non-reducing conditions (45). This is presumably because the disulfide bond required for fibril assembly (46, 47) is retained under these conditions, whereas when long straight fibrils are resolved by SDS-PAGE under reducing conditions, this bond is broken, and \( \beta_m \) is observed as a single species. Indeed, when analyzed by SDS-PAGE under non-reducing conditions, \( \beta_m \)-FITC-multi fibrils formed a ladder of higher molecular weight species, whereas only a single species was observed for the monomeric \( \beta_m \)-FITC-multi (Fig. 5C).

Together these data are consistent with the assembly of \( \beta_m \)-FITC-multi into long straight fibrils that are amyloid-like.

To determine whether fibrils are taken up into macrophage lysosomes, RAW 264.7 macrophages were incubated with \( \beta_m \)-FITC-multi fibrils for 2 h at 37°C, washed to remove any non-cell-associated amyloid fibrils, and then analyzed by confocal microscopy (Fig. 5D). In these cells the \( \beta_m \)-FITC-multi fibrils co-localized with the LysoTracker dye and were present within vacuolar structures that could be visualized by phase contrast microscopy. Because of their length (>0.75 μm, Fig. 5B), the most plausible route by which the \( \beta_m \)-FITC-multi fibrils were internalized by the RAW 264.7 macrophages was via phagocytosis (48, 49). Indeed, this would be consistent with EM studies of \( \beta \) amyloid fibrils were imaged apparently phagocytosing \( \beta_m \) amyloid (26) and in which \( \beta_m \) amyloid was localized within the lysosomes of these cells (19). Remarkably, and by contrast with the results obtained using monomeric \( \beta_m \)-FITC-multi, the cells incubated...
with β2mFITC-multi fibrils retained FITC fluorescence after the 2-h chase (Fig. 5D). This finding was confirmed by flow cytometric analysis (Fig. 5E). In these experiments, most but not all cells had captured β2mFITC-multi fibrils after the 2-h incubation, although there was a wide variation in the level of FITC fluorescence, suggesting that individual cells captured differing quantities of the fibrils. The level of cell-associated fluorescence did not decrease after 2 or even 24 h of chase, suggesting that the β2mFITC-multi fibrils are highly resistant to degradation in these cells (Fig. 5E). The failure to degrade the β2mFITC-multi fibrils was not due to a block in phagosome maturation, as the internalized fibrils co-localized with the LysoTracker dye (Fig. 5D). Furthermore, there is no evidence that the inability of these cells to degrade the fibrils was due to cell death. The cells remained attached to the culture dishes, and no gross morphological changes in either cell or nuclear structure were observed that would be consistent with either necrotic or apoptotic cell death (data not shown).

In Vitro Analysis of the Proteolysis of β2m Fibrils by Lysosomal Enzymes—The data presented above demonstrate that β2m fibrils are internalized but not degraded by RAW 264.7 macrophages. One potential explanation for their persistence in these cells is that the fibrils are resistant to degradation by lysosomal proteases. To test if this was the case, monomeric β2mFITC-multi and the β2mFITC-multi fibrils were incubated with lysosomal supernatant containing the equivalent of 0.6 units of cathepsin B or 0, 4, or 24 h at 37 °C. Digests were either analyzed by 15% Tris-Tricine SDS-PAGE or dot-blotted. Dot blots were probed with anti-β2m and WO1 anti-amyloid antibodies (24, 30, 31). Identification of cleavage products of lysosomal digest of rod-like β2m fibrils by N-terminal sequencing.
bation (Fig. 6A). Similar results were observed when rod-like, worm-like, and long straight fibrils formed from β2m at pH 4.5 in vitro were incubated with lysosomal supernatant (Fig. 6B). Each fibril type displayed an increased resistance to proteolysis over that of the monomer, with only partial digestion of the intact protein (worm-like and long straight fibrils) or complete digestion into a single band slightly smaller than the intact wild-type protein (rod-like fibrils) occurring within this time (Fig. 6B). Interestingly, proteolysis of all three fibril types generated a fragment of the same size, consistent with the lysosomal proteases accessing the same site in β2m irrespective of the fibril type. Furthermore, no loss in WO1 immunoreactivity was observed for any fibrillar sample even after 24 h of incubation (Fig. 6B), whereas AFM imaging revealed that fibrils were still present in the samples after digestion for this time (data not shown). N-terminal sequencing indicated that the common cleavage site product formed contains β2m truncated by either 10 or 11 residues from the N terminus (Fig. 6, B and C). Remarkably, these cleavage sites mirror those found in amyloid from DRA patients (43, 50) and fibrils formed at acidic pH in vitro cleaved by chymotrypsin (51) as well as some of the sites observed during limited proteolysis of monomeric β2m with lysosomal enzymes (Fig. 3B). Thus, although lysosomal proteases may protect cells by the degradation of the monomeric protein, they appear unable to degrade preformed fibrils, generating fibrils that remain assembled but are truncated at their N terminus akin to amyloid material formed in hemodialysis patients.

DISCUSSION

Despite their acidic pH and ability to proteolytically generate fragments from monomeric β2m with potentially increased amyloid propensity (6, 8, 12), our data do not support a role for macrophage lysosomes in enhancing the initiation of fibril formation from β2m in vivo. Thus, although monomeric β2m is sorted to lysosomes in RAW 264.7 macrophages, the immense proteolytic capacity of this organelle results in the rapid degradation of the monomeric precursor, essentially precluding amyloid formation by rapid and complete degradation of the amyloid precursor (Fig. 7). Although limited proteolysis of monomeric β2m resulted in the generation of characteristic fragments lacking 10 or 11 residues from the N terminus (ΔN10β2m and ΔN11β2m), the enhanced fibrillogenic potential of these fragments is unfulfilled in lysosomes due to their rapid degradation. Rather than enhancing β2m amyloid deposition, lysosomal proteases appear to play a protective role, degrading the monomeric protein before it can self-assemble into protease-resistant fibrillar forms. The ability to rapidly and completely degrade monomeric β2m is not unique to macrophages; lysosomal proteases isolated from HeLa cells also rapidly digest monomeric β2m, cleave at the same sites as lysosomal proteases from RAW 264.7 macrophages, and correspondingly inhibit fibril formation (data not shown). Lysosomal proteolysis, thus, appears to be a generic protective mechanism, preventing the self-assembly of amyloidogenic β2m by rapid and exhaustive proteolysis after endocytic uptake and lysosomal sorting.

In a previous study mononuclear cells isolated from the blood of dialysis patients using a procedure that utilized hepa-
Amyloid fibrils in microglia (54, 55), suggesting that resistance to lysosomal degradation may be a property shared by other extracellularly generated amyloid fibrils.

Irrespective of their morphology, βm fibrils were resistant to lysosomal proteolysis. Whereas monomeric βm was cleaved by lysosomal proteases between residues 61/62 and 65/66, these sites were not cleaved in long straight, rod-like and worm-like fibrils, indicating that these residues are masked in all of the fibrillar species. Despite their differing morphologies, long straight, rod-like and worm-like fibrils, therefore, must possess elements of common structure. Furthermore, the N-terminal 10–11 residues were accessible to cleavage by lysosomal proteases between residues 61/62 and 65/66, these residues of βm have been identified in ex vivo amyloid (43, 50).

No cleavage at this site was observed when βm was incubated with either synovial fluid or human serum (data not shown). Hence, these data suggest, therefore, that lysosomal proteolysis of the βm fibrils by macrophages associated with the ADR amyloid plaques may be responsible for the generation of these two cleavage products in vivo.

The inability of macrophages to degrade βm fibrils may have profound consequences for the development of the pathology associated with DRA. First, because macrophages (and presumably other cell types) are unable to degrade existing βm amyloid deposits, the amyloid load will increase as a function of the time of hemodialysis, fibril deposition occurring more rapidly once the first fibrils are formed and seeds are generated. Second, the βm amyloid deposits in vivo are unlikely to be biologically inert. Indeed, βm fibrils are modified by advanced glycan end products (AGEs) in vivo (56, 57), and this material is known to activate macrophages to release cytokines that increase collagenase expression in synovial cells (58, 59). The failure to remove βm amyloid, therefore, would be predicted to lead to the chronic activation of macrophages by AGE-βm, resulting in the continual secretion of cytokines that promote bone and joint destruction, leading to the pathological consequences of DRA. Consistent with this notion, macrophage recruitment is accompanied by the symptomatic phase of DRA in which bone erosions are first observed (27).

Overall, therefore, lysosomal uptake by macrophages and other cell types may reduce amyloidogenicity by decreasing the concentration of amyloid precursor, but due to their resistance to lysosomal proteolysis, the amyloid fibrils once formed cannot be removed resulting in the chronic inflammatory response associated with DRA.

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