Investigation into the initiation of fibril formation in dialysis related amyloidosis

Isobel Morten, Antoni Borysik, Sheena Radford and Eric Hewitt

Introduction
\(\beta_2\text{-microglobulin (}\beta_2\text{m)}\) is one of approximately twenty proteins that aggregate to form highly ordered amyloid fibrils \textit{in vivo}. \(\beta_2\text{m}\) is a small 99 residue soluble protein, which is non-covalently bound to a membrane-integrated heavy chain, forming the major histocompatibility complex (MHC) class I molecule which is expressed on the surface of all nucleated cells. \textit{In vivo}, \(\beta_2\text{m}\) is continuously shed from nucleated cells into the serum. \(\beta_2\text{m}\) is then transported to the proximal tubule of the kidney where it is degraded and excreted. As a consequence of renal failure the \(\beta_2\text{m}\) serum concentration increases by up to 60-fold. By a mechanism that is currently unresolved free \(\beta_2\text{m}\) then self-associates, forming insoluble amyloid fibrils which typically accumulate in synovial joints. As a consequence, uremic patients who have been dialysed for 10-15 years develop dialysis-related amyloidosis (DRA), a debilitating arthritic-like condition.

Hypothesis
How \(\beta_2\text{m}\) forms amyloid fibrils \textit{in vivo} is unknown. In addition, like many amyloid disorders fibril deposition occurs site-specifically, in the case of DRA the fibrils develop specifically in the synovial joints. Therefore, we hypothesised that one or more components found within the synovium may promote amyloid formation in this specialised environment. Therefore, using a combination of biochemical, cell biological and biophysical experiments we are testing if either the cells or macromolecules associated with the synovial joints of DRA patients promote fibrillogenesis.

Do macrophages promote \(\beta_2\text{m}\) amyloid fibrillogenesis?
\textit{In vitro} studies in our laboratory have shown that incubation of \(\beta_2\text{m}\) at acidic pH or the removal of the N-terminal six residues (\(\Delta\text{N6}\beta_2\text{m}\)) induces rapid fibril formation. We propose that cells within the joint capsule may internalise \(\beta_2\text{m}\), which enters the endocytic pathway and accumulates in the lysosomes. The acidic microenvironment of lysosomes (pH 4.5), coupled with its high concentration of proteases, may then stimulate fibril formation.

Macrophages are the only known cell type to be associated with \(\beta_2\text{m}\) amyloid fibrils \textit{in vivo}. To determine if \(\beta_2\text{m}\) is endocytosed into cells we N-terminally labelled the protein with fluorescein isothiocyanate (FITC) and incubated the protein at 50\(\mu\text{g/mL}\) (the concentration of \(\beta_2\text{m}\) typically found in the serum of uremic patients) with RAW 264.7 cells, a mouse macrophage cell line. Internalisation of \(\beta_2\text{m}\) was analysed using live cell confocal microscopy, and localisation of internalised \(\beta_2\text{m}\) was determined using LysoTracker, a lysosomal specific dye. Internalised \(\beta_2\text{m}\)FITC accumulates in perinuclear regions and co-localises with LysoTracker. These data are consistent with sorting of the internalised \(\beta_2\text{m}\) to lysosomes.

Lysosomal conditions were mimicked using an ammonium acetate buffer at pH 4.5. Rod-like fibrils were formed under these conditions by incubation for 24 hours at 37\(^\circ\text{C}\) whilst longer, worm-like fibrils are generated under the same conditions after incubation for 1 week. Both fibrillar forms have been shown to be amyloid-like by their ability to bind amyloid specific antibodies, the dyes thioflavin T, and Congo red as well as their appearance visualised using atomic force microscopy (AFM) (Figure 1).
Fig. 1 – Characterisation of β2m species formed at pH 4.5. A, Dot blot of β2m species incubated at pH 4.5 at time points 0, 1 and 36 days, using anti-β2m antibody (DAKO), an oligomer specific antibody (anti-oligomer) and a fibrillar specific antibody (WO1). Aβ1-40 oligomers acted as a positive control for the anti-oligomer antibody and glutathione-S-transferase (GST) acted as a negative control for all antibodies. Representative AFM images (1 µm²) of rod-like (B) and worm-like (C) β2m fibrillar species formed at pH 4.5.

To determine the effect that the lysosomal enzymes have on β2m, lysosomes were isolated from RAW 264.7 cells by subcellular fractionation and the enzymes extracted. The enzymes were incubated with monomeric β2m or preformed rod-like β2m fibrils, and the digestion products identified using N-terminal sequencing and mass spectrometry. Digestion of the monomer produces two products, an N-terminal truncation and a single cut within the region corresponding to the native E strand with the subsequent N- and C-terminal regions of the protein remaining attached via the native disulphide bond. By contrast, digestion of the fibrillar species yields only one digestion product, the same N-terminal truncation as seen when the monomer was digested. These results demonstrate that the core of the protein is inaccessible to the lysosomal enzymes when it is packed into the fibrillar structure.

Do macromolecules specific to the synovium promote fibrillogenesis from β2m?
In parallel we are testing whether macromolecules associated with the synovium can nucleate fibrillogenesis of β2m, since the monomeric protein does not nucleate fibril formation in isolation at neutral pH. We are monitoring the effect of a range of different glycosaminoglycans on fibril formation from the destabilised truncated form of β2m (ΔN6). To date we have shown that some, but not all, forms of these long-chain high molecular weight sugars can significantly enhance the nucleation of ΔN6 fibrils in vitro at physiological pH. We are now beginning to unpick the mechanisms involved in how these exciting candidate glycosaminoglycans can possibly contribute to β2m amyloidosis under physiological conditions.

Publications

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