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Specific glycosaminoglycans promote unseeded amyloid formation from β_2-microglobulin under physiological conditions

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Dialysis-related amyloidosis (DRA) is a complication of hemodialysis where β_2-microglobulin (β_2m) forms plaques mainly in cartilaginous tissues. The tissue-specific deposition, along with a known intransigence of pure β_2m to form fibrils *in vitro* at neutral pH in the absence of preformed fibrillar seeds, suggests a role for factors within cartilage in enhancing amyloid formation from this protein. To identify these factors, we determined the ability of a derivative lacking the N-terminal six amino acids found in *ex vivo* β_2m amyloid deposits to form amyloid fibrils at pH 7.4 in the absence of fibrillar seeds. We show that the addition of the glycosaminoglycans (GAGs) chrondroitin-4 or 6-sulfate to fibril growth assays results in the spontaneous generation of amyloid-like fibrils. By contrast, no fibrils are observed over the same time course in the presence of hyaluronic acid, a nonsulfated GAG that is abundant in cartilaginous joints. Based on the observation that hyaluronic acid has no effect on fibril stability, while chrondroitin-6-sulfate decreases the rate of fibril disassembly, we propose that the latter GAG enhances amyloid formation by stabilizing the rare fibrils that form spontaneously. This leads to the accumulation of β_2m in fibrillar deposits. Our data rationalize the joint-specific deposition of β_2m amyloid in DRA, suggesting mechanisms by which amyloid formation may be promoted.

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β_2-microglobulin (β_2m) is the nonpolymorphic subunit of the major histocompatibility complex class I molecule, which is expressed on the surface of all nucleated cells.[1] β_2m is continually shed from cell surface major histocompatibility complex molecules and is normally removed from the circulation by the kidney.[2] However, in patients with end-stage renal disease neither the kidney nor hemodialysis remove β_2m efficiently from the circulation, leading to an up to 60-fold increase in the serum concentration of β_2m. Ultimately, this leads to the deposition of β_2m amyloid fibrils, which occurs predominantly in the cartilaginous joints.[2–5] These β_2m amyloid deposits cause progressive bone and joint destruction, resulting in severe skeletal morbidity associated with dialysis-related amyloidosis (DRA).

β_2m is 99 residue protein that has a seven-stranded all β-sheet fold typical of the immunoglobulin superfamily.[6] *In vitro*, wild-type β_2m can be readily induced to form amyloid-like fibrils by partially or more completely denaturing the protein at acidic pH[7–10] and the assembly pathways and morphologies of the fibrils formed under these conditions have been studied in detail.[11,12] Recent studies have also demonstrated that a partially unfolded conformation of β_2m populated at pH 7.0 in which a non-native *trans* isomer of proline is formed at residue 32 is able to elongate fibril seeds, suggesting this species may be involved in amyloid formation *in vivo*.[13] In addition, wild-type β_2m extends preformed fibril seeds at neutral pH in the presence of sodium dodecyl sulfate,[14] mixtures of glycosaminoglycans (GAGs), trifluoroethanol[15] or GAGs alone.[16] These data indicate that although β_2m can extend preformed fibrillar seeds at neutral pH, spontaneous (unseeded) fibril formation is highly inefficient under these conditions. Identifying physiologically relevant components that promote amyloid deposition at neutral pH, therefore, remains a key issue.

Previous studies have suggested a role for collagen[17] and/or GAGs[18–21] in promoting β_2m fibrillogenesis at physiological pH, consistent with the observation that β_2m amyloid deposits are first observed in the superficial layer of cartilage.[22] One of the most extensively studied GAGs is heparin, which has been shown to significantly increase the rate of fibril formation *in vitro* of β-synuclein, gelsolin, and β-amyloid (at physiological pH).[23–25] With regard to β_2m

fibrils, heparin has been shown to increase the rate of fibril elongation at pH 7.0 in the presence of trifluoroethanol and to decrease the rate of depolymerization of fibrils formed under acidic conditions when incubated at neutral pH.[15,16,26] Whether heparin or other GAGs are also able to promote fibril deposition in the absence of added seeds, however, remains unknown.

To investigate the role of GAGs in the initiation of β_2m fibril deposition, we used ΔN6β_2m, a physiologically relevant truncated form of β_2m lacking the N-terminal six amino acids that constitutes up to 30% of the β_2m in fibrils purified from DRA patients.[27] Although retaining a native-like fold, ΔN6β_2m is destabilized compared with wild-type β_2m,[28] has an increased propensity to extend preformed fibril seeds compared with its wild-type counterpart at pH 4–8,[16,28] and can form fibrils, albeit with extremely low yields, at pH 7.0 in the absence of fibrillar seeds.[16] Fibrils formed from ΔN6 and wild-type β_2m are structurally indistinguishable;[16,28,29] indeed, the N-terminus of β_2m is solvent exposed in fibrils formed from the wild-type protein indicating that this region is not integral to fibril structure.[30–32] These properties make ΔN6β_2m an ideal model system with which to study the role of GAGs in fibrillogenesis under physiologically relevant conditions, within an experimentally tractable timeframe. All experiments were performed in the absence of seeds, at physiological temperature, pH, and ionic strength, in the absence of denaturant, detergents, or organic solvent. We show that over a 4-week period, ΔN6β_2m remains non-fibrillar at physiological pH (7.4). However, significant increases in amyloid content were observed over the same time period in reactions containing chondroitin-4-sulfate or chondroitin-6-sulfate, which are found in high concentrations in cartilage.[33,34] By contrast, hyaluronic acid, which is also abundant in cartilaginous joints,[35] had no effect. Together with analysis of the effect of these additives on the structure of native ΔN6β_2m and the rates of fibril disassembly at pH 7.4, we propose a mechanism to describe how specific GAGs promote the onset of fibril deposition within the cartilaginous joints of hemodialysis patients.

RESULTS

Heparin and chondroitin sulfate induce fibrillogenesis of ΔN6β_2m at physiological pH

A panel of GAGs was screened for their ability to promote the fibrillogenesis of ΔN6β_2m in the absence of seeds under physiological conditions (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 37°C). In contrast to previous studies of the effects of GAGs on fibril elongation,[15] no organic solvents or other non-natural additives were added. The GAGs screened were chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, heparan sulfate, and hyaluronic acid, all of which are present in the cartilaginous joints, as well as heparin, an anticoagulant used during hemodialysis.[36] Fibrillogenesis was monitored using three independent assays: immunoblotting with the amyloid-specific antibody WO1 that binds to β_2m fibrils formed both *in vitro* and *in vivo*[16,37](SE Radford, IJ Morten, and EW Hewitt, unpublished results), binding of the amyloid-specific dye thioflavin T (thioT),[38] and negative-stain electron microscopy (EM).

A standardized methodology was used in the purification and preparation of ΔN6β_2m for fibrillation assays to ensure that all experiments commenced with identical protein samples (see Materials and Methods).[8] In addition, to ensure that any differences in fibril growth rates observed could be attributed unequivocally to the GAG added, the screen was repeated three times and all reactions were set up in duplicate containing 2 mg/ml ΔN6β_2m from the same preparation with either 5.0, 0.05, or 0 mg/ml GAGs (see Materials and Methods). In all screens, none of the samples at time zero showed a significant increase in thioT fluorescence or antibody binding compared with ΔN6β_2m alone (Figure 1a and b). Likewise, no significant change was observed in reactions containing no additive or up to 5 mg/ml hyaluronic acid, even after 4 weeks of incubation (Figure 1a and 1b). Dermatan sulfate and heparan sulfate also had no significant effect on ΔN6β_2m fibrillogenesis (data not shown). Strikingly, however, in the presence of heparin, chrondroitin-4-sulfate, or chrondroitin-6-sulfate, a significant increase in thioT fluorescence was observed over time, culminating in an eight to 14-fold increase after 4 weeks (Figure 1b). Similarly, WO1 immunoreactivity was observed in the samples containing heparin, chrondroitin-4-sulfate, or chrondroitin-6-sulfate after 4 weeks incubation, but not in their absence (Figure 1a). These results were consistent in all of the screens, demonstrating that heparin, chrondroitin-4-sulfate, and chrondroitin-6-sulfate specifically promote fibril formation and that the observed effects can be attributed to the addition of these GAGs rather than differences in the ΔN6β_2m preparations. No clear concentration dependence was observed, suggesting that these GAGs are saturating at these mass ratios, mirroring the situation *in vivo* where the GAG concentration in the cartilaginous tissues is likely to exceed that of β_2m.[34] By contrast with these results, no increase in thioT fluorescence or binding by the amyloid-specific antibody WO1 was observed for wild-type β_2m even after 10 weeks incubation with 5.0 mg/ml of heparin and no fibrils were observed by negative-stain EM (data not shown), consistent with the known intransigence of wild-type β_2m to form amyloid fibrils in the absence of seeds at neutral pH.[39,40]

To further demonstrate the presence of amyloid fibrils in the reactions containing chrondroitin-4-sulfate, chrondroitin-6-sulfate, or heparin, samples were also assayed for their ability to bind the amyloid-specific dye, Congo red (Figure 2a-c). Fibrils formed in the presence of heparin (Figure 2a), chrondroitin-4-sulfate (Figure 2b), or chrondroitin-6-sulfate (Figure 2c), all bind Congo red, displaying changes in the absorbance spectrum of the dye characteristic of binding to amyloid fibrils.[41] The fibrils also show a classical amyloid-like, long-straight morphology when imaged by EM (Figure 2d–f), confirming the ability of these GAGs to promote the spontaneous formation of amyloid-like fibrils in the absence of seeds or other additives.