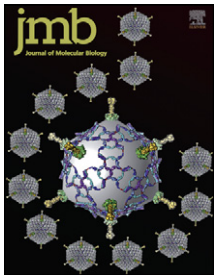

The Changing Face of Aging: Highly Sulfated Glycosaminoglycans Induce Amyloid Formation in a Lattice Corneal Dystrophy Model Protein

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Abstract

Glycosaminoglycans (GAGs) are related to multiple biological functions and diseases. There is growing evidence that GAG concentration and sulfate content increase with age. The destabilizing mutation A546T in the corneal protein TGFBIp leads to lattice-type corneal dystrophy, but symptoms only appear in the fourth decade of life. We hypothesize that this delayed phenotype can be explained by increased GAG sulfation over time. Using *in vitro* assays with the C-terminal TGFIBIp domain Fas1–4, previously shown to recapitulate many properties of full-length TGFBIp, we find that only long GAGs with multiple sulfate groups on each repeating unit increase the amount of worm-like aggregates and induce long, straight fibrils in A546T. In contrast, GAGs did not induce aggregation of wildtype Fas1–4, suggesting that the finding might be specific for lattice corneal dystrophy mutants. Our results highlight a possible role of changing GAG sulfation in the accumulation of amyloid, which also may have implications for the development of neurodegenerative diseases.

Aging involves many changes to the body. Bone and skin become more fragile, cartilage and blood vessels lose their elasticity and cellular "housekeeping" efficiency declines, leading to a large range of age-related diseases [1]. Of interest in this regard are the glycosaminoglycans (GAGs), either alone or in complex with proteins as proteoglycans (PGs), which are important for tissue structure, repair and maintenance [2] as well as signaling and infection [2–6] and embryonic development [7]. GAGs are polysaccharides built from repeating disaccharide units (disac) of glycosamines and glycoacids. After their initial synthesis *in vivo*, they undergo stereochemical modifications and sulfation, yielding a large variety of GAG structures [2]. On the basis of their sugar moieties and sulfation patterns, GAGs are categorized as heparin (Hep), heparan sulfate (HS), dermatan sulfate (DS), keratan sulfate (KS), chondroitin sulfate (CS) and hyaluronic acid (HA). GAG sulfation levels vary significantly. With three sulfates on average per disac, Hep has the highest degree of sulfation, HA does not contain any sulfates and other GAG types (HS, DS, KS and CS) have variable sulfate content dependent on their origin [2]. For the GAG types used in the current study, the average sulfation decreases from HS over DS to CS, leading to the following ranking of GAGs as regards level of sulfation: Hep > HS > DS > CS > HA. Recent reports have established a correlation between GAG concentration and sulfate content with aging (Table 1). While the total GAG load in plasma [8–10] and cartilage [11] declines over time, it increases in the brain hippocampal neurons [15], cornea [13], trabecular meshwork [14] and aorta [12], with the proportion of highly sulfated GAG types increasing dramatically. In eye-related tissues, HA concentrations decrease by 58%, while more sulfated GAGs (HS, KS and DS) increase by 40% [13,14]. GAG sulfate groups are believed to be important for maintaining the relatively

Table 1. The involvement of GAGs in the aging process

Tissue source	Decades of life	Total GAG concentration	GAG types decreasing	GAG types increasing	GAG sulfation increase	Reference
Plasma	1st to 9th	Decline	CS, DS, HS		—	[8–10]
Lumbar disk	3rd to 7th	Decline			—	[11]
Aorta	1st to 5th	Increase		CS	—	[12]
Cornea	3rd to 7th	Increase	HA	CS, DS, KS, HS	Yes	[13]
Trabecular meshwork	3rd to 7th	Increase	HA	CS, DS, KS, HS	Yes	[14]
Brain, hippocampal neurons	3rd to 7th	Increase		HS	Yes	[15]

high hydration of the stromal layer in the cornea, which contains 78% water [16]. PGs are directly involved in collagen arrangement and therefore important to maintain corneal transparency [16]. Different PG deficiencies result in blindness [17,18] or even embryonic death [19]. Corneal dystrophies (CDs) are defined as visual impairment by disruption of corneal transparency [20] and cover multiple disease mechanisms. Several CDs are caused by mutations in proteins that synthesize PGs, impairing vision through reduced ordering of collagen fibers [17,18,21]. A CD subtype with no obvious link to PGs is related to mutations in the transforming growth factor beta-induced protein (TGFBIp). Mutations in TGFBIp were first linked to visual impairment in 1997 [22]. TGFBIp is a multi-domain protein containing four consecutive fasciclin 1 (FAS1; homologous to the *Drosophila* fasciclin-1 protein Fas-1) domains (FAS1–1 to FAS1–4). TGFBIp-related CDs are divided into granular CDs (GCDs), lattice CDs (LCDs) and phenotypes with specific/mixed characteristic traits (Thiel–Behnke CD, Reis–Bücklers CD) [20,23]. The most common mutation is located at R124 in the FAS1–1 domain [20]. Outside this single location, mutations causing CDs are largely confined to the FAS1–4 domain, which harbors 49 known mutations, linked to all four different CD phenotypes [24]. LCD mutations have been suggested to be more destabilizing than GCD-causing mutations [25]. Besides different phenotypes, the mutations differ in age of onset, ranging from 35 to 40 years (A546T [26]) or even later (G594V [27]) to as early as 10 years (R555W [28,29]).

In several ways, the isolated FAS1–4 domain is a good biophysical model for full-length TGFBIp. Full-length TGFBIp and isolated FAS1–4 carrying the same mutation show the same relative stability and form similar aggregate types [25]. The mutation A546T (AT), which leads to LCD in the fourth decade of life, destabilizes both the FAS1–4 domain and full-length TGFBIp [25], leading to amyloid-like structures, that is, β-strand-rich fiber-like aggregates, which bind thioflavin T (ThT) [25,26]. Residue 546 is in the hydrophobic core of Fas1–4, and so the introduction of a polar side chain (Thr) will destabilize the protein through desolvation penalties [25]. Moreover, aggregation of AT is promoted by Hep [30]. On the basis of these observations, we hypothesize that age-related GAG sulfation might be responsible for the late onset of AT fibrillation and LCD symptoms. Here we present results in support of this hypothesis.

Monomeric FAS1–4 and Hep does not form a stable complex

Initially, we investigated whether monomeric FAS1–4 AT and wild-type (WT) directly bind Hep to form a complex. Isothermal titration calorimetry and size exclusion chromatography (SEC) experiments did not indicate complex formation. There was only a very small thermal signal ($<$0.1 kcal/mol) upon Hep titration into FAS1–4, with injections spanning a molar ratio from 0.1 to 22 disaccharides per protein monomer (Fig. S1). The thermal heats measured were more than 100-fold lower than the 10 kcal/mol measured for Hep binding to the hormone p25α [31]. Similarly, SEC did not detect any new species formed by AT or WT immediately after mixing with Hep. The protein component in FAS1–4:Hep samples (detected by absorbance at 280 nm) had the same retention time as the monomeric control for both WT (Fig. 1a) and AT (Fig. 1b). Refractive index (RI) changes, which detect both Hep and protein, contained a peak corresponding to the Hep control (Fig. 1c). Subtraction of the Hep control elution profile from the AT:Hep profile essentially eliminates the Hep peak, supporting the conclusion that the two species do not form a stable complex (Fig. 1c).

FAS1–4 AT form oligomers upon incubation

SEC showed that the WT remains monomeric after incubation with Hep for 2 days (Fig. 1a). In contrast, incubation of AT leads to larger species eluting at 14–24 min (Fig. 1b). Samples containing AT and Hep had a larger fraction of large species compared to protein-only samples. On the basis of the ultraviolet (UV) absorption signal, we estimate the large protein species to account for 46% of the total protein with AT:Hep and only 10% in the case of AT incubated alone. After incubation for 4 days, the