Biochem. J. (1991) 275, 67–73 (Printed in Great Britain)

Isolation and characterization of the integral glycosaminoglycan constituents of human amyloid A and monoclonal light-chain amyloid fibrils

Stephen R. NELSON,* Malcolm LYON,† John T. GALLAGHER,† Edward A. JOHNSON‡ and Mark B. PEPYS*§

*Immunological Medicine Unit, Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, †Cancer Research Campaign Department of Medical Oncology, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, and ‡National Institute for Biological Standards and Control, Potters Bar, Herts., U.K.

Amyloid fibrils were isolated by extraction in water from the livers and spleens of four patients who had died of monoclonal, light-chain (AL)-type, systemic amyloidosis and one with reactive systemic, amyloid A protein (AA)-type amyloidosis. Each fibril preparation contained 1–2% by weight of glycosaminoglycan (GAG) which was tightly associated with the fibrils and not just co-isolated from the tissues with them. After exhaustive digestion of the fibrils with papain and Pronase, the GAGs were specifically precipitated with cetylpyridinium chloride and were identified by cellulose acetate electrophoresis and selective susceptibility to specific glycosidases. All the preparations contained approximately equal amounts of heparan sulphate and dermatan sulphate. There was no evidence for the presence of chondroitin sulphate or other GAGs. Fine structural analysis by oligosaccharide mapping in gradient polyacrylamide gels, following partial digestion with specific glycosidases, showed very similar structures among the heparan sulphates and the dermatan sulphates, respectively. GAGs were also extracted by solubilizing amyloid fibrils in 4 M-guanidinium chloride followed by CsCl density-gradient ultracentrifugation. Although a minor proportion of the GAG material obtained in this way was apparently in the form of proteoglycan molecules, most of it was free GAG chains. The presence in amyloid fibrils of different types, in different organs and from different patients of particular GAG classes with similar structures supports the view that these molecules may be of pathogenic significance.

INTRODUCTION

Amyloidosis is a disorder of protein metabolism in which autologous proteins are deposited in the tissues in a characteristic fibrillar form, leading to disruption of normal structure and function [1–2]. If major viscera are involved, as in systemic amyloidosis of AL (immunoglobulin light chain) or AA (reactive systemic) type, the condition is usually fatal. There is no specific effective means for promoting regression and resolution of the deposits.

Amyloid fibrils are formed from different precursor proteins in different forms of amyloidosis, but it has long been recognized that amyloid-laden organs and the amyloid deposits themselves contain sulphated glycosaminoglycans (GAGs) [3–12]. Indeed, these materials are responsible for the uptake of iodine by the deposits, which led Virchow to name the condition 'amyloid', meaning starch-like. The GAGs are ubiquitous components of mammalian organs and tissues, located predominantly in the extracellular matrix or on cell surfaces and have been reviewed by Gallagher [13]. They are most commonly present as proteoglycans in which the GAGs are covalently linked to a variety of core proteins [13,14].

There has lately been a resurgence of interest in GAGs in amyloid. The demonstration that they are universally present in all forms of amyloid which have been tested [15–21], and that in experimental systems their deposition occurs at the same time as the appearance of amyloid fibrils [22,23], suggest that GAGs may participate in the pathogenesis of amyloid fibril formation and/or persistence. These results also raise the question of whether GAGs may be related in some way to the presence of amyloid P component (AP) [24,25], a non-fibrillar serum protein which is also always found in all types of amyloid regardless of the nature of the fibril protein [2].

Hitherto the association of GAGs with amyloid fibrils *per se*, rather than their mere co-deposition in affected tissues, has been surmised from ultrastructural histochemical staining [17,19] and from analysis of fibril preparations [9], in which co-isolation of GAGs was not [26] or could not be [27] excluded. We report here that GAGs are present in purified preparations of both AA and AL fibrils and that they separate with the fibrils under salt conditions which specifically precipitate amyloid fibrils but not proteoglycans or free GAG chains. Furthermore, isolation of the GAGs themselves, their identification and structural characterization have revealed a notable consistency of GAG constituents in both AA and AL amyloid, in different patients and in different organs from the same patient.

MATERIALS AND METHODS

Isolation of amyloid fibrils

Amyloid-containing organs were removed at autopsy within 8 h of death and were frozen at −20 °C in portions of 20–30 g. The patients (identified by their initials), their organs and the

Abbreviations used: GAG, glycosaminoglycan; AA, amyloid A protein; AL, amyloid monoclonal light-chain protein; CPC, cetylpyridinium chloride; BCA, bicinchoninic acid: T, acrylamide; C, bisacrylamide (cross-linker); AP, amyloid P component.

§ To whom correspondence should be addressed.

Table 1. Relative amounts of different GAGs associated with amyloid fibrils

All GAG preparations in solution at 1 mg/ml were run in a single cellulose acetate electrophoresis, stained with Alcian Blue and densitometrically scanned. The proportions of each sample are shown which remained at the origin (zero mobility) and which migrated in the positions of heparan sulphate and dermatan sulphate respectively.

Patient	Type of amyloid	Organ	GAG (% of total)		
			Zero mobility	Heparan sulphate	Dermatan sulphate
M.C.	AL	Spleen	9	59	32
M.S.	AL	Liver	18	50	32
J.P.	AL	Spleen	8	34	58
H.H.	AL	Spleen	7	48	45
W.C.	AA	Spleen	12	44	44
		Liver	10	54	36

types of amyloid which were studied are listed in Table 1. Amyloid fibrils were extracted from the tissue by the method of Pras *et al.* [28], except that the initial homogenizations were in 140 mM-NaCl/10 mM-Tris/HCl containing 10 mM-EDTA and 0.1% (w/v) NaN_3 at pH 8.0. Homogenization in this buffer was repeated up to 12 times until no further soluble material absorbing at 280 nm was obtained. Homogenization in water then yielded soluble amyloid fibrils, predominantly in the second and third water washes, which typically had A_{280} values of 1–3. The whole procedure from thawing the frozen tissue to completion of the water extraction took 2 days, with homogenization being conducted at 21 °C and centrifugation and overnight storage at 4 °C.

Portions of the fibril preparations were adjusted to 10 mM-NaCl by addition of 4 M-NaCl, and then centrifuged at 15000 *g* for 15 min to sediment the fibrils [26]. Other portions were adjusted to 2 M-NaCl by addition of 4 M-NaCl, mixed together by rotation at 21 °C overnight and then centrifuged under the same conditions. The supernatant was separated and assayed for GAGs. The remainder of each fibril preparation in water was freeze-dried and stored desiccated at room temperature.

Extraction of GAGs by proteinase digestion

Freeze-dried fibrils (100 mg) were suspended in 20 ml of 0.1 M-sodium phosphate buffer, pH 6.5, containing 5 mM-EDTA and 0.79 mg of cysteine hydrochloride/ml, and 0.25 mg of papain (EC 3.4.22.2; Sigma, Poole, Dorset, U.K.)/ml was added. The mixture was then incubated for 16 h at 37 °C with stirring, after which it was heated to 100 °C for 30 s, to inactivate the papain, and then cooled. Digestion was continued by addition of 80 ml of 10 mM-Tris/HCl, pH 7.8, containing 10 mM-EDTA and 100 mg of Pronase (from *Streptomyces griseus*; Boehringer-Mannheim, Lewes, East Sussex, U.K.) and incubated with stirring at 37 °C. After 24 h, a further 100 mg of Pronase was added and incubation continued for another 24 h. The mixture was then centrifuged at 15000 *g* for 30 min to remove the traces of insoluble material which remained. This precipitate was tested in each case and contained insignificant amounts of GAG. Cetylpyridinium chloride (CPC) (Sigma) was added to the supernatant at 21 °C to a final concentration of 0.06% (w/v) to precipitate the GAGs [29], and the mixture was centrifuged at 1000 *g* for 30 min. The supernatant was removed and the precipitate re-dissolved in 1 or 2 M-NaCl before re-precipitation of the GAGs alone with ethanol at 95% (v/v) (final concn.) for 16 h at 4 °C. The precipitate was washed three times with 100% ethanol and finally dissolved in water. This solution was extensively dialysed against water and then freeze-dried.

Extraction of GAGs by density-gradient centrifugation

Freeze-dried amyloid fibrils were solubilized by incubation at 4 °C for 16 h with 4 M-guanidinium chloride (Aldrich, Gillingham, Dorset, U.K.), further purified by treatment with activated charcoal, 1% (w/v) Triton X-100 (Sigma), 20 mM-phosphate, pH 7.4, containing proteinase inhibitors [100 mM-6-aminohexanoic acid, 20 mM-benzamidine hydrochloride, 10 mM-EDTA, 5 mM-*N*-ethylmaleimide and 0.5 mM-phenylmethanesulphonyl fluoride (all from Sigma)]. A small amount of residual insoluble material was removed by centrifugation at 1000 *g* for 10 min. The supernatant was adjusted to a volume of 12 ml and final density 1.45 g/ml by addition of 4 M-guanidinium chloride and solid CsCl (molecular-biology grade; Boehringer), before centrifugation in a Beckman L5 ultracentrifuge at 130000 $g_{av.}$ for 66 h at 15 °C. The bottom of the tube was then pierced and the contents collected as 1 ml fractions, the density of which was determined by weighing 0.1 ml aliquots and the uronic acid content of which was monitored by the orcinol assay.

Analysis of GAGs

Glycosidase digestion. The GAG material from proteinase digestion or density gradient centrifugation was dissolved in water at 1 mg/ml and then adjusted to 100 mM-Tris/1 mM-$CaCl_2$/0.5% (w/v) BSA, pH 7.0. Chondroitinase ABC (EC 4.2.2.4; from *Proteus vulgaris*; Seikagaku Kogyo Co., Tokyo, Japan via ICN Biomedical, High Wycombe, Bucks., U.K.) and chondroitinase AC II (EC 4.2.2.5; from *Arthrobacter aurescens*; Seikagaku Kogyo Co. via ICN) were both used at 1.5 units/mg of GAG at 37 °C. Heparinase I (EC 4.2.2.7 from *Flavobacterium heparium*; ICN) was used at 60 munits/mg of GAG at 30 °C and heparinase III (EC 4.2.2.8; from *Flavobacterium heparium*; ICN) was used at 60 munits/mg of GAG at 43 °C. In each case the GAG was incubated with enzyme for 2 h and digestion was assessed by monitoring absorbance at 232 nm, by Azure A-staining a spot of the reaction mixture placed on paper and by cellulose acetate electrophoresis. Control incubations with standard preparations of pure GAGs, chondroitin 4-sulphate, dermatan sulphate, heparan sulphate and mucosal heparin were performed on each occasion to confirm activity of the enzymes.

For oligosaccharide mapping experiments, glycosidase digestions of 15 μg amounts of GAG at 1 mg/ml in water were performed as follows: 1 unit of chondroitinase ABC/ml in 50 mM-Tris/50 mM-NaCl, pH 8.0, at 37 °C for 16 h; 1 unit of chondroitinase AC/ml in 50 mM-Tris/50 mM-NaCl, pH 7.3, for 16 h at 37 °C; 20 munits of heparinase I/ml in 100 mM-sodium acetate/0.1 mM-calcium acetate, pH 7.3, at 30 °C for 16 h (all these enzymes were from Seikagaku Kogyo Co.); 40 munits of heparinase III (Grampian Enzymes, Aberdeen, Scotland, U.K.)/ml in 100 mM-sodium acetate/0.1 mM-calcium acetate, pH 7.3, at 43 °C for 16 h; combined 20 munits each of heparinases I, II (Grampian Enzymes) and III/ml in 100 mM-sodium acetate/0.1 mM-calcium acetate, pH 7.3, and 37 °C for 16 h.

Electrophoretic analyses. Cellulose acetate electrophoresis was run in 0.1 M-barium acetate, pH 5.0 [30], stained with 1.0% (w/v) Alcian Blue (BDH, Poole, Dorset, U.K.) in 1% (v/v) acetic acid, destained in 1% acetic acid and rendered transparent [31] for photography. PAGE of intact GAG chains was run on 20%-acrylamide (T)//2%-bisacrylamide (C)–30%-T/5%-C gradient gels with a 4%-T/5%-C stacking gel and the discontinuous buffer system of Laemmli [32], but without SDS. Electrophoresis

was at 250 V through the stacking gel and 500 V through the resolving gel until the Phenol Red marker reached the bottom of the gel. GAGs were then stained with 0.5% (w/v) Alcian Blue in 2% (v/v) acetic acid, and de-stained with 2% acetic acid. Oligosaccharide maps of GAG digests were obtained in 24%-T/2%-C–30%-T/5%-C gradient PAGE with a 4%-T/5%-C stacking gel run under the same conditions as above. After electrophoresis the oligosaccharides were fixed with aq. 0.08% (w/v) aqueous Azure A, de-stained with water and then revealed with ammoniacal silver [33].

GluN:GalN ratios. GAG material from proteinase digestions was dissolved at 0.5 mg/ml in 4 M-HCl in a screw-capped vial, the air displaced by nitrogen, and heated for 16 h at 110 °C. The micro-filtered solution was taken to dryness by rotary evaporation, and the residue dried twice more after additions of water. It was analysed for glucosamine (GluN) (derived from the heparan sulphate) and galactosamine (GalN) (from dermatan sulphate) by the ligand exchange chromatographic procedure of Navratil *et al.* ([34]; see also [35]). A column (30 cm × 0.5 cm) of copper-loaded Bio-Rex 70 (Bio-Rad) ion-exchange resin (−400 mesh, fines removed) was used, equilibrated with aq. 1 M-NH_3 containing 0.1 mM-$CuSO_4$. Detection was at 250 nm; both hexosamines gave identical peak areas for equal mass.

Detection of GAGs

Qualitative detection of GAGs in fibril preparation, fractions and digests was performed by staining, with 0.08% (w/v) Azure A (Sigma), a dried 2 μl spot of the material on Whatman no. 1 filter paper. Excess stain was removed by rinsing in tap water, and the method could detect approx. 2 μg of GAG. Uronic acid in GAGs was measured quantitatively in the range 4–60 μg/ml by the orcinol reaction using standards of glucuronolactone [36].

Detection of protein

The presence of protein in extracted GAG fractions was sought by monitoring absorbance at 280 nm, by the bicinchoninic acid (BCA; Pierce Chemical Co., Chester, U.K.) variation of the Lowry method [37], and by SDS/PAGE analysis [32] with Coomassie Blue staining. Protein radio-iodination was attempted with ^{125}I-Bolton–Hunter reagent (du Pont/New England Nuclear Products, Dreieich, Germany; sp. radioactivity 2200 Ci/mmol) at 0 °C for 30 min in 100 mM-sodium phosphate buffer, pH 8.0. Known control intact proteoglycans, run in parallel, were labelled very efficiently under these conditions. GAG chains were released from proteoglycan by treatment with 50 mM-NaOH containing 1 M-sodium borohydride at 45 °C for 24 h, followed by neutralization with 1 M-acetic acid.

RESULTS

Association of GAGs with amyloid fibrils

Amyloid fibrils were isolated by water extraction from the liver and spleen of one patient who died with AA amyloidosis and from the spleens of three patients and the liver of one other patient who died with AL amyloidosis. Despite very extensive homogenization and washing of the tissues with Tris-buffered saline containing EDTA, procedures likely to extract any soluble proteoglycan and/or GAG chains, before water extraction was performed, all the fibril preparations contained GAGs. When the fibril solutions were made to a final concentration of 10 mM-NaCl and then centrifuged at 15000 *g* for 15 min all the GAGs were recovered in the precipitate. Incubation of fibril preparations with 2 M-NaCl eluted only up to 18% of the total GAG in a soluble form.

Isolation of GAGs from amyloid fibrils by proteinase digestion

The amyloid fibril preparations were sequentially digested with papain and Pronase, leaving very little insoluble residue, and GAGs within the soluble digest were then specifically precipitated with CPC. In nine separate procedures performed on material from five different patients, the total recovery of GAG from the initial fibril preparation was between 0.9 and 2.7% (w/w). The absorbance spectra of these GAG preparations showed a minor peak at 260 nm, suggesting the presence of traces of DNA, but there was no significant absorbance at 280 nm. The virtual absence of protein was confirmed by the absence of any protein staining band when samples were subjected to SDS/PAGE, and by values in the BCA assay of only 20–25 μg of protein/mg dry weight of GAG preparation.

All the preparations of isolated GAGs gave two major bands in cellulose acetate electrophoresis, with mobilities corresponding to dermatan sulphate and heparan sulphate markers (Fig. 1). In addition, some preparations also contained a trace of material which remained at the origin; this behaviour is typical of commercial heparin preparations, but we have not characterized this material in detail (Fig. 1; Table 1). The identity of the two main bands was confirmed by their selective susceptibility to the specific glycosidases chondroitinase ABC and heparinase III respectively (Fig. 2). Chondroitinase AC, which cleaves chondroitin sulphate but not dermatan sulphate, had no effect on the cellulose acetate electrophoretograms. In one case (W.C.; AA amyloid, spleen), heparinase I had a more marked effect than heparinase III, indicating the presence of a more highly sulphated heparan sulphate species.

Three samples of GAG (M.S.; AL liver) were isolated by variations in proteolytic procedures and analysed for GluN/GalN ratios by ligand-exchange chromatography. All gave identical ratios: 75% GluN/25% GalN (coefficient of variation of four replicate assays < 5%). Another sample (W.C.; AA liver) again gave the same ratio; after incubation with chondroitinase AC there was no change, but after chondroitinase ABC treatment the percentage of GalN decreased to 7.5%. These results would suggest a heparan sulphate/dermatan sulphate ratio of 3:1, which is higher than the ratios deduced from

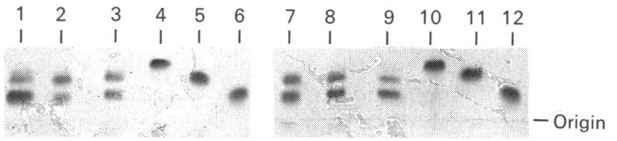

Fig. 1. Cellulose acetate electrophoresis of GAGs isolated from amyloid fibrils

Lanes: 1, M.C. AL liver; 2, J.P. AL spleen; 3, H.H. AL liver; 4, chondroitin sulphate; 5, dermatan sulphate; 6, heparan sulphate; 7, W.C. AA spleen; 8, W.C. AA liver; 9, M.S. AL liver; 10, chondroitin sulphate; 11, dermatan sulphate; 12, heparan sulphate.

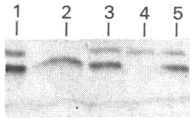

Fig. 2. Cellulose acetate electrophoresis of GAGs from isolated hepatic AL amyloid fibrils (patient MS)

Lanes: 1, starting material; 2, after chondroitinase ABC digestion; 3, chondroitinase ABC digestion buffer control; 4, after heparinase III digestion; 5, heparinase III digestion buffer control.

scanning densitometry of Alcian Blue-stained GAGs (approx. 1.5:1; Table 1). Nevertheless, the data are clearly consistent in demonstrating that heparan sulphate is the major GAG in these samples. The discrepancies might be accounted for by the zero mobility material observed on electrophoresis which could be heparin-like (i.e. GluN-containing), or the more highly sulphated dermatan sulphate may bind more Alcian Blue than heparan sulphate.

Isolation of GAGs from hepatic AA amyloid fibrils by solubilization in guanidinium chloride

Freeze-dried amyloid fibrils (W.C.; AA liver) were almost completely solubilized in 4 M-guanidinium hydrochloride. Density-gradient centrifugation of the soluble material in CsCl/4 M-guanidinium chloride at a starting density of 1.45 g/ml clearly separated the major protein components, accumulating at the top of the tube, from the orcinol-positive material, most of which ($\sim 70\%$) sedimented to the bottom of the tube with a density $\geqslant 1.60$ g/ml (Fig. 3). A minor component ($\sim 30\%$) banded near the centre of the tube with a density of 1.45–1.50 g/ml (Fig. 3). The orcinol positivity at the top of the tube appeared to be interference from the high concentration of protein present. In the experiment shown (Fig. 3) the combined uronic acid recovery from the starting material of freeze-dried fibrils was approx. 0.65% (w/w). A similar distribution of uronic acid was also obtained from an extract of liver AL amyloid fibrils (result not shown).

The high-density material, without any further treatment, resolved into two components on cellulose acetate electrophoresis, a predominant band corresponding to a heparan sulphate marker and a second band corresponding to a dermatan sulphate marker, a pattern very similar to that obtained with the GAGs isolated by proteinase digestion. In 20–30%-acrylamide-gradient PAGE, the material entered the gel and migrated a short distance; this mobility was unaffected by digesting it beforehand with papain (results not shown). Furthermore, it did not become labelled at all with ^{125}I-Bolton–Hunter reagent under conditions in which known proteoglycans were efficiently iodinated. These observations, taken together, demonstrated that a major portion of the total GAG present in the amyloid fibrils, both heparan sulphate and dermatan sulphate, was in the form of large, free GAG chains rather than intact proteoglycan molecules.

In contrast, the less abundant medium-buoyant-density material did not enter the 20–30%-gradient gel until it had been treated with alkaline borohydride (result not shown). However, after this treatment it also resolved into the same two components on cellulose acetate electrophoresis as did the high-buoyant-density material, corresponding to dermatan sulphate and heparan sulphate. There is thus clear evidence for the presence of some GAG, of both the classes represented in amyloid fibrils, in a form covalently linked to protein(s), presumably proteoglycan core protein(s).

Structural characterization of amyloid fibril GAGs

The GAGs isolated after proteinase digestion of amyloid fibrils from each individual source were subjected to exhaustive selective digestion with chondroitinase ABC to destroy the dermatan sulphate, and, in separate experiments, with combined heparinases I, II and III to destroy the heparan sulphate. In each case the size distribution of the remaining intact class of GAG was analysed by gradient PAGE and stained with Alcian Blue (Fig. 4). In general, the heparan sulphate molecules were of larger average size than the dermatan sulphates and they also were considerably more polydisperse. Within each class of GAG, however, the size distribution was broadly similar between

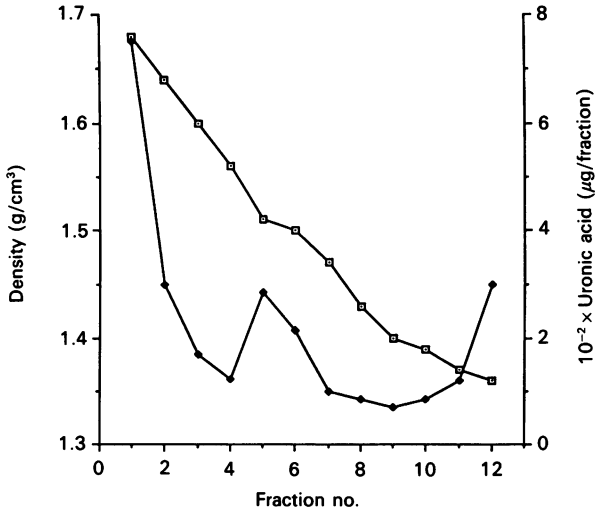

Fig. 3. CsCl density gradient ultracentrifugation of guanidinium solubilized hepatic AA amyloid fibrils

Distribution of uronic acid-containing material across the density gradient is shown.

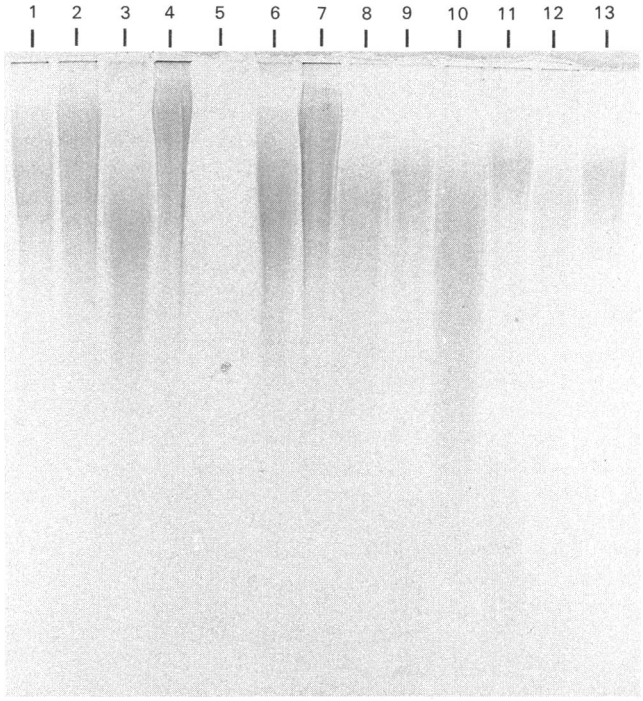

Fig. 4. Gradient PAGE analysis of amyloid fibril GAGs after exhaustive selective glycosidase digestion

Alcian Blue stain. Lanes: 1–7, chondroitinase ABC digestion; 8–13, combined heparinase I, II and III digestion. Lanes 1 and 8, H.H. AL spleen; lanes 2 and 9, W.L. AA liver; lanes 3 and 10, W.C. AA spleen; lane 4, M.S. AL liver; lane 5, blank; lanes 6 and 12, M.C. AL spleen; lanes 7 and 13 W.C. AA liver.

different patients, different organs and different types of amyloid, with the exception of the material from the spleen of patient W.C. with AA amyloid in which both the heparan and dermatan sulphates were somewhat smaller.

The structures of the heparan and dermatan sulphates in all GAG isolates were probed by selective digestion with glycosidases of known limited substrate specificity, followed by mapping of the complex mixtures of resistant oligosaccharides by PAGE and

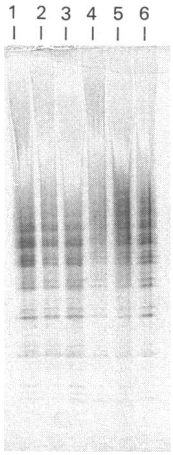

Fig. 5. Gradient PAGE analysis of amyloid fibril GAGs after exhaustive digestion with chondroitinase AC

Silver stain. Lanes: 1, H.H. AL spleen; 2, W.C. AA liver; 3, W.C. AA spleen; 4, M.S. AL liver; 5, M.C. AL spleen, 6, W.C. AA liver.

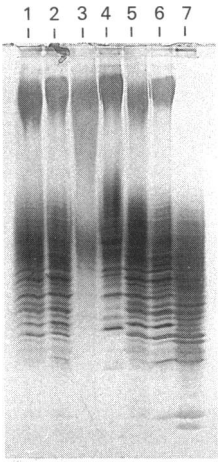

Fig. 6. Gradient PAGE analysis of amyloid fibril GAGs after exhaustive digestion with heparinase III

Lanes: 1, H.H. AL spleen; 2, W.C. AA liver; 3, W.C. AA spleen; 4, M.S. AL liver; 5, M.C. AL spleen; 6, W.C. AA liver; 7, heparan sulphate standard.

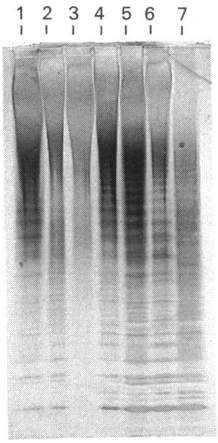

Fig. 7. Gradient PAGE analysis of amyloid fibril GAGs after exhaustive digestion with heparinase I

Lanes: 1, H.H. AL liver; 2, W.C. AA liver; 3, W.C. AA spleen; 4, M.S. AL liver; 5, M.C. AL spleen; 6, W.C. AA liver; 7, heparan sulphate standard.

staining with Azure A and ammoniacal silver [33]. Exhaustive digestion with chondroitinase AC, which cleaves GlcA (glucuronic acid)-containing sequences, produced a strikingly similar ladder of resistant oligosaccharides in all samples (Fig. 5). These oligosaccharides, comprising the IdoA-rich sequences, will have the general structure ΔGlcAβ1-[3GalNAc(S)β1 $\rightarrow$ 4IdoAα1]$_n$-3GalNAc(S), with some scope for heterogeneity in the levels of sulphation. Their presence confirmed the presence of dermatan sulphate, as opposed to chondroitin sulphate, in the fibril GAGs. The close correspondence between samples indicated that the distribution of iduronate residues, that is the range in value of n, and probably the overall content of iduronate was very similar in all the samples.

Exhaustive digestion with heparinase III, which is specific for GlcNAc/GlcNSα1 $\rightarrow$ 4GlcA linkages, regardless of sulphation of C-6 of the hexosamine [38], produced considerable depolymerization (Fig. 6). In contrast, heparinase I, which is specific for GlcNS($\pm$6S)α1 $\rightarrow$ 4IdoA(2S) linkages, had a more limited effect (Fig. 7). Both these patterns of breakdown are consistent with the presence of heparan sulphate rather than heparin and therefore confirm the cellulose acetate electrophoresis results. Although there were subtle differences in the amounts of certain oligosaccharides, there was generally more correspondence than difference, indicating a considerable overall similarity in the heparan sulphate structures in the GAGs from different sources. The only exception was, once again, the material from the spleen of patient W.C. with AA amyloid, the heparin sulphate of which was poorly degraded by heparinases I and III though the pattern of digestion of its dermatan sulphate by chondroitinase AC was typical.

DISCUSSION

There is extensive evidence that amyloid-laden organs, in both human and experimental animal amyloidosis, contain increased amounts of GAGs [3–8,10–12,15,16,18,20–23]. Crude preparations of amyloid fibrils have also been shown in the past to contain GAGs [9], and ultrastructural histochemical staining for highly sulphated GAGs [17,19] has confirmed their apparently intimate association with amyloid fibrils both in tissue sections and after extraction. More recently, amyloid fibrils isolated by the water extraction method have been studied. Magnus *et al.* [26] isolated AA fibrils from the liver of a single patient and, after Pronase digestion, extracted and identified chondroitin sulphate, dermatan sulphate and heparin/heparan sulphate. Ohishi *et al.* [27] isolated fibrils of AA (two patients), AL (three patients) and β_2-microglobulin type (two patients) by water extraction, but also included, with the water-soluble fibrils, the top layer of the sediment, i.e. material not displaying the characteristic solubility in water of pure amyloid fibrils. After papain and Pronase digestion, heparan sulphate and dermatan sulphate were identified in all except the β_2-microglobulin type. In addition, chondroitin sulphate, heparin, keratan sulphate and hyaluronic acid were detected in some of the preparations.

The aims of the present study were: (1) to establish the presence of GAGs as integral constituents of isolated amyloid fibrils rather than merely materials co-isolated from amyloidotic organs in the fibril extraction procedures; (2) to identify and characterize structurally, in more detail than hitherto, these integral GAG constituents of amyloid fibrils. A notable feature of amyloid fibrils of both AA and AL type is that although they are insoluble and therefore sedimentable in saline buffers at physiological ionic strength, they are soluble in pure water and can thereby be extracted and separated from tissue debris [28]. Furthermore, once 'dissolved in water', amyloid fibrils are readily precipitated even by low concentrations of added salt [28]. In

contrast, intact proteoglycans and free GAG chains, provided they are not tightly bound to other insoluble structures, are highly soluble both in physiological saline and in water. Amyloidotic tissue was therefore very extensively and repeatedly homogenized and washed with buffered saline containing EDTA, continuing until no further material absorbing at 280 nm was eluted. Amyloid fibrils were then extracted by further homogenization in water and separated carefully and completely from all the remaining insoluble, sedimented material. It is well recognized that not all the amyloid fibrils are obtained in this way [28] and that a portion remain in the tissue debris, but at least the fibrils which are released should not be contaminated with other GAG-containing tissue constituents. As a further and critical check that the GAGs detected in the fibril preparations were tightly associated with the fibrils themselves, we demonstrated that they were co-precipitated with the fibrils on addition of just 10 mM-NaCl, conditions which clearly would not affect free proteoglycans or GAG chains themselves. Furthermore, even washing of the fibrils with 2 M-NaCl released only a minor proportion of the GAGs, indicating that the GAGs identified and characterized here were indeed integral components of, or at last very firmly bound to, amyloid fibrils.

These fibril-associated GAGs were present at an abundance of about 1–2% (w/w) of the freeze-dried fibrils, in agreement with previous reports [9,26,27]. They consisted of heparan sulphate, dermatan sulphate and traces of material which migrated like heparin in cellulose acetate electrophoresis. The identity of the major GAG classes was established unequivocally by specific digestion with particular glycosidases and by detailed oligosaccharide mapping. There was no evidence for the presence of chondroitin sulphate or other GAGs reported by others [26,27].

A notable finding was the striking similarity both in GAG composition and in GAG structure between preparations from spleens and livers of patients with both AA and AL amyloidosis. Only the fibrils from the spleen of the one case of AA amyloid contained GAGs which were more than subtly different from the others, including the fibrils from that patient's own liver. This remarkable restriction to just two major classes of GAG, and the overall general consistency of their structures, suggest that the presence of these GAGs is not just an epiphenomenon but, as proposed by others [3–12,15–23], may contribute to pathogenesis of amyloid-fibril deposition and/or persistence.

Our finding of some apparently intact proteoglycans in the amyloid fibrils is in agreement with previous reports of immunohistochemical staining of amyloid deposits with antibodies to heparan sulphate core protein [20–39]. However, the bulk of the GAG present in the fibril preparations was in the form of free chains. Since there is generally an unavoidable delay in obtaining and freezing amyloid tissue after death, and since the buffers used for fibril extraction did not contain proteinase inhibitors, it is possible that some degradation of core proteins *post mortem* and *in vitro* may have occurred. However, intact proteoglycans can be obtained from normal or non-amyloid tissues under similar conditions, so it is possible that the finding of abundant free GAG chains reflects, at least in part, the true situation *in vivo*. Free polysaccharides or oligosaccharides derived from the degradation of proteoglycans are found in a variety of haemopoietic cells [40,41] and epithelial cells [13], but under normal conditions of culture they are generated and retained in intracellular compartments rather than being secreted into the growth medium. In amyloidosis, such polysaccharides could be diverted to a secretory pathway as a reaction to the build-up of insoluble fibres in the cellular environment. Alternatively, excessive breakdown of extracellular proteoglycans could occur in this disease. A further possibility is that some of the amyloid-associated GAGs are sequestered from circulating blood. Small quantities of GAGs, including heparan sulphate (though not dermatan sulphate) are found in human plasma samples [42], although their origins and detailed molecular structure are unknown.

In summary, the present study clearly establishes that GAGs are tightly associated with amyloid fibrils of different structure. A degree of specificity in the association is evident because only polysaccharide containing iduronate and complex sulphation patterns, heparan sulphate and dermatan sulphate, are present in the fibril extracts. The sources of these polysaccharide chains, the process involved in their cleavage from the core proteins and their incorporation into amyloid fibrils are matters of considerable interest.

This work was supported in part by the Medical Research Council, by the Cancer Research Campaign and by the Trustees of the Hammersmith and Queen Charlotte's Special Health Authority. We thank Beth Sontrop for expert preparation of the manuscript.

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Received 10 July 1990/2 October 1990; accepted 11 October 1990