Presence of glycosaminoglycans in purified AA type amyloid fibrils associated with juvenile rheumatoid arthritis

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SUMMARY   Previous studies have strongly suggested an association between glycosaminoglycans and tissue deposits of amyloid. The present study was aimed at studying this association in purified preparations of hepatic amyloid fibrils obtained from human AA type secondary amyloidosis. Glycosaminoglycans were isolated by gradient ion exchange chromatography of purified amyloid fibrils treated with pronase. Degradation with specific enzymes identified the glycosaminoglycans as chondroitin sulphate, dermatan sulphate, and heparin/heparan sulphate. The total amount of glycosaminoglycans specifically coisolated with the amyloid fibrils was 15 µg/mg fibril weight. The presence of glycosaminoglycans in amyloid may play a part in the incorporation of structurally diverse protein precursors into amyloid fibrils of identical ultrastructure.

Key words: amyloidosis, protein AA.

Amyloidosis is a heterogeneous disorder both with regard to its clinical expression and the chemical composition of the amyloid deposits.[1] Despite this heterogeneity all forms of amyloid have similar or identical ultrastructural and tinctorial properties, including typical metachromasia.[2] The proteinaceous fibrils are the unique component of all amyloid deposits. It has been shown that different proteins can make up such fibrils and that there is a close relation between the chemical composition of the amyloid fibrils and the clinical category of amyloid disease.[3] The fibrils are thus made up by immunoglobulin light chain (AL) proteins or fragments in primary or myeloma associated amyloidosis,[4] whereas protein AA is the major fibril component in secondary amyloidosis.[5] The precursor for protein AA is serum amyloid A[6] as shown by recent experiments performed in our laboratory.[7] Variants of prealbumin represent the fibril protein in various familial forms of amyloidosis.[8]

The role of glycosaminoglycans or mucopolysaccharides as a possible constituent in amyloid was suggested by Oddi in 1894.[9] Investigations undertaken by Bitter and Muir in 1966 further suggested that heparan sulphate could be involved in the deposition of protein in insoluble fibrillar form in the amyloid lesions.[10]

The water extraction method of Pras and coworkers has become the standard method for isolation of amyloid fibrils from affected tissues.[11] We used this method to investigate the possible association of glycosaminoglycans with AA type human amyloid fibrils.

Materials and methods

SOURCE OF AMYLOID FIBRILS
Protein AA type amyloid fibrils were prepared from the liver of a 12 year old male patient, who had amyloidosis associated with juvenile rheumatoid arthritis. The amyloid tissue stained with Congo red and showed typical green birefringence when examined by polarisation microscopy.

Normal human liver was obtained at necropsy and used for control. The organs were kept at −20°C before use.

PURIFICATION OF AMYLOID FIBRILS

The hepatic amyloid fibrils were isolated according to the method of Pras *et al*,[11] with a small modification.[12] Briefly, the amyloid fibrils were isolated by extractions with distilled water after repeated washing of homogenised tissue with physiological saline until the optical density at 280 nm in the supernatant was negligible, usually seven to 10 times. The third and fourth 0·15 M saline washes also contained 3·68 g sodium citrate and 0·395 g trometamol (TRIS) HCl in 250 ml saline. The saline washes and the first distilled water supernatant were not studied further, while the remaining aqueous supernatants were lyophilised and kept at −20°C until used.

The normal liver was subjected to the same extraction procedure.

CONGO RED ABSORPTION

The method described by Pras *et al* was used for Congo red absorption experiments.[11] Briefly, 2·0 mg of lyophilised water supernatant from amyloid laden and normal liver tissues was suspended by sonication in 1 ml of distilled water and mixed with 5 ml of a 0·1 mg/ml solution of Congo red in physiological saline, stirred for two hours, and then centrifuged at 1000 *g* for 10 minutes. The optical densities of the supernatants were read at 490 nm in a spectrophotometer.

GEL FILTRATION

Protein AA was purified by gel filtration of lyophilised amyloid fibrils under dissociating conditions as previously described in detail.[13]

IMMUNOLOGICAL CHARACTERISATION

Double diffusion in 1% agarose gel was used for immunological characterisation of crude alkali degraded amyloid fibrils of amyloid and purified amyloid protein fractions obtained by gel filtration.[13]

Antisera to AA, AL, and prealbumin related amyloid were produced in rabbits by immunisation with amyloid fibrils degraded with 0·1 M NaOH and absorbed as previously described.[14]

ISOLATION OF GLYCOSAMINOGLYCANS

Lyophilised amyloid fibrils were dissolved (6 mg/ml) in 0·2 M trometamol HCl pH 8·0 and digested with pronase 0·2 mg/ml at 36°C for 18 hours. The digested fibrils and the digest from the corresponding extract of the normal liver were chromatographed on a diethylaminoethyl (DEAE)-Sephacel ion exchange column.[15] The column was eluted with 0·05 M sodium acetate buffer pH 4·0 with a gradient

from 0·2 M NaCl to 2·0 M NaCl. Selected fractions were pooled after determination of hexuronic acid, dialysed against distilled water, and lyophilised.

The lyophilised polysaccharide fractions were dissolved in 0·5 ml phosphate buffered saline pH 7·3.

CARBAZOLE REACTION

Hexuronic acid was determined by the carbazole method described by Bitter and Muir[16] with glucuronolactone as standard.

ENZYMATIC STUDIES

Enzymatic degradation was performed on 50 μl of the lyophilised polysaccharide material after ion exchange chromatography. Chondroitinase AC (EC 4.2.2.5) was obtained from Sigma, chondroitinase ABC (EC 4.2.2.4) and heparitinase (EC 4.2.2.8) from Seikagaku Kogyo, Tokyo, Japan. Galactosaminoglycans were degraded by sequential incubations with chondroitinase AC and chondroitinase ABC in 0·05 M trometamol HCl pH 8·0 containing 0·05 M sodium acetate, 0·05 M NaCl, and 0·1 mg/ml of bovine serum albumin.[17] To the incubation mixtures (generally 450 μl) were added 50 μl sample and 0·05 unit of the respective enzymes. Each separate incubation was performed for one hour at 37°C. The absorbance at 232 nm was read in a spectrophotometer.

Polysaccharides containing *N*-sulphated glucosamine residues—that is, heparin or heparan sulphate, were degraded by incubation with heparitinase[18] [19] in 3 mM sodium acetate buffer pH 7·0 containing 3 mM calcium chloride. Generally, 450 μl of this buffer was used with 50 μl sample and 0·002 unit of heparitinase. The samples were incubated for one hour at 43°C, and the absorbance at 232 nm was read in a spectrophotometer.

Results

CONGO RED STAINING AND ABSORPTION

The amyloid laden liver from the patient was heavily infiltrated with material showing typical yellow-green birefringence as demonstrated by polarisation microscopy of sections stained with Congo red. The normal organ was negative in this respect. The optical density at 280 nm in the second aqueous supernatant of the amyloidotic liver exceeded 1·5, whereas that of the corresponding normal organ was less than 0·5. This particular distilled water supernatant from the amyloid laden liver showed the highest degree of absorbance of Congo red (data not shown) and thus contained the largest amounts of amyloid fibrils. The second water supernatant was therefore selected for further studies.

IMMUNOLOGICAL STUDIES

Double diffusion using antisera to AA showed reactivity with crude degraded amyloid fibrils and purified protein AA from the patient. These amyloid preparations did not react with antisera to amyloid of immunoglobulin or prealbumin origin.

The normal tissue preparations did not react with the antisera to AA or any other amyloid protein. The protein AA nature of the amyloid from the patient was also confirmed by amino acid studies of the purified protein (data not shown).

COISOLATION OF POLYSACCHARIDES AND HEPATIC AMYLOID FIBRILS

Figure 1 shows the elution profile of carbazole reactive polysaccharides obtained by gradient ion exchange chromatography of amyloid fibrils from the patient after treatment with pronase. One small and three larger peaks were obtained at molarities of 0·60 (peak 1), 0·73 (peak 2), 0·80 (peak 3), and 0·93 (peak 4). This pattern was consistent throughout several runs on DEAE-Sephacel.

Table 1 shows the quantities of carbazole positive polysaccharides eluted in the various peaks. More than half of the polysaccharides was eluted at 0·73 M NaCl (peak 2), but a considerable amount (approximately 20%) was eluted in the position corresponding to the peak of a heparin standard, indicating a relatively high degree of sulphation or large molecular size of the polysaccharide associated with amyloid fibrils, or both (Fig. 1).

The total amount of such polysaccharides coisolated with amyloid fibrils extracted in the second water supernatant was approximately 15 μg/mg of lyophilised fibrils (Table 1). A corresponding extract from normal human liver yielded only trace amounts of carbazole positive polysaccharides after ion exchange chromatography and showed virtually no peaks corresponding to those of the amyloid material (Fig. 1).

ENZYMATIC CHARACTERISATION OF AMYLOID ASSOCIATED GLYCOSAMINOGLYCANS

Separate samples of the amyloid associated polysaccharide material obtained from the four ion exchange chromatography peaks (Fig. 1) were subjected to heparitinase and to sequential treatment with chondroitinase AC and chondroitinase ABC. Table 2 shows the relative amounts of degradable material using the respective enzymes.

The glycosaminoglycans which are degraded by chondroitinase AC and chondroitinase ABC represent chondroitin sulphate and dermatan sulphate, and more than 60% of the total polysaccharides is accounted for by these glycosaminoglycans. In peaks 3 and 4 the large amount of material degraded by chondroitinase ABC representing dermatan sulphate was particularly noted. The amount of heparin/heparan sulphate as shown by heparitinase degradation varied from 20% to approximately 40% in the four peaks. The highest proportion of
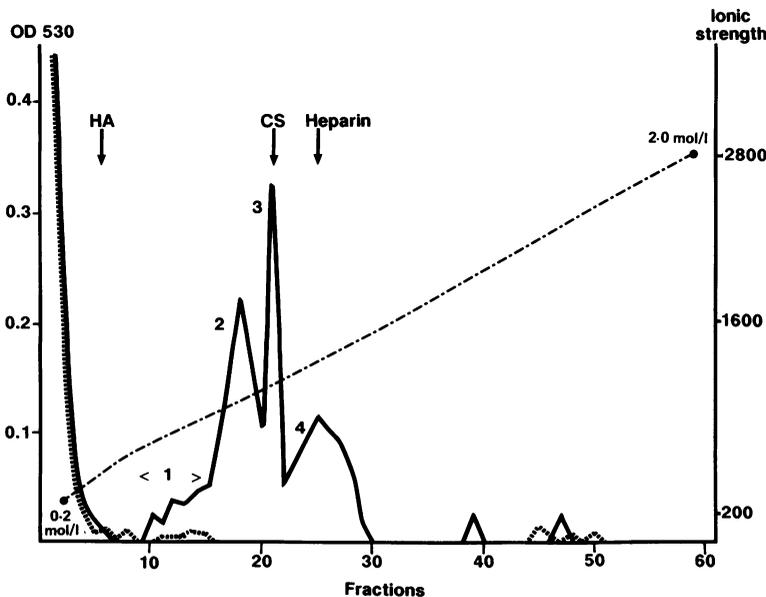
Fig. 1 *Carbazole positive material (glycosaminoglycans) eluted by gradient DEAE-Sephacel ion exchange chromatography of AA type amyloid fibrils (———) and corresponding normal tissue extract (- - - -) treated with pronase. The molarity range of the linear NaCl gradient was 0·2–2·0 mol/l (—·—·). Fractions were pooled according to the four peaks indicated on the figure. HA=hyaluronic acid; CS=chondroitin sulphate.*

Table 1   *Amount of carbazole positive amyloid associated polysaccharide eluted by DEAE-Sephacel ion exchange chromatography*

Peak No*	Molarity of peak fraction	Absorbance at 530 nm in 50 μl sample	Polysaccharides† μg (%)	Polysaccharides expressed as μg/mg of lyophilised amyloid fibrils
1	0·60	0·024	53·4 (11·4)	1·7
2	0·73	0·155	258·0 (55·1)	8·6
3	0·80	0·026	57·6 (12·3)	1·9
4	0·93	0·045	99·6 (21·3)	3·3
Total			468·6	15·5

*See Fig. 1.
†Glucuronolactone was used as standard.

Table 2   *Relative amounts* of degradation products after enzymatic treatment of polysaccharide fractions obtained by ion exchange chromatography, determined by absorbance at 232 nm*

Enzyme used	Peak 1†	Peak 2	Peak 3	Peak 4
Heparitinase	37·2	38·6	24·8	21·3
Chondroitinase AC	57·8	61·4	55·2	41·3
Chondroitinase ABC	5·0		20·0	35·6

*Percentage of total absorbance at 232 nm after heparitinase and chondroitinase ABC treatment.
†See Fig. 1.

heparin/heparan sulphate was found in the ion exchange chromatography peak 2 (Fig. 1), containing 55% of the total content of carbazole positive material (Table 1) of this AA type hepatic amyloid fibril preparation.

Discussion

More than a century ago it was suggested that amyloid was mainly a proteinaceous material.[20] It should be remembered, however, that Virchow who created the term amyloid in 1854 based this on its starch-like staining with iodine.[21] Later on, several investigators emphasised the specific association between amyloid and polysaccharides.[10 22 23] Pras and coworkers suggested the presence of non-protein material (heparitin sulphate and chondroitin sulphate B) in water extracted amyloid.[24] Furthermore, studies on experimental amyloidosis have shown a clear temporal relation between the in situ occurrence of glycosaminoglycans and the deposition of amyloid.[25] Indeed the accumulation of glycosaminoglycans even appears to precede the formation of detectable amyloid fibrils. Snow and Kisilevsky also demonstrated a close ultrastructural

relation between amyloid fibrils and glycosaminoglycans in tissue sections, and enzymatic treatment by the sections indicated the presence of chondroitin sulphate and heparin/heparan sulphate.[26]

In the present work we confirmed the specific association of polysaccharides with AA type, human hepatic amyloid. Using the standard method for purification of amyloid fibrils[11] we have shown here that significant amounts of polysaccharide are co-isolated with such fibrils. The evidence for this is as follows: (*a*) The purification procedure for amyloid fibrils[11] involves several washings of homogenised tissue with physiological saline before the extraction of amyloid fibrils with water. The polysaccharides were specifically coisolated with the water extracted hepatic amyloid fibrils and not with the corresponding extract from normal liver. (*b*) Carbazole positive polysaccharide material was obtained by ion exchange chromatography at elution positions characteristic of glycosaminoglycans.[15] (*c*) The treatment of the polysaccharides derived from amyloid fibril with specific enzymes provided direct biochemical proof of their identity as glycosaminoglycans.

It appears, therefore, that glycosaminoglycans are integrated in or intimately associated with human AA type amyloid fibrils. This is compatible with the histochemical and structural observations by Snow and coworkers, who showed a close association between glycosaminoglycans and splenic AA type amyloid fibrils in mice.[26] The composition of glycosaminoglycans in amyloid deposits may vary with different clinical and chemical types of amyloidosis, age of the affected individual, disease duration, and organs or tissues involved. This possibility is being studied further.

The role of glycosaminoglycans in the formation of amyloid fibrils has not been established. The highly negatively charged glycosaminoglycan chains may be involved in protein folding and thus in the

formation of fibrils.[27] It has also been suggested that glycosaminoglycans may modulate the fibril formation of other proteins, like collagen.[28] Furthermore, it has recently been suggested that glycosaminoglycans may be part of the so called amyloid enhancing factor.[29 30] This factor is poorly defined but appears to consist of both polysaccharide and protein.[29] Amyloid enhancing factor activity is present in highly purified amyloid fibrils[30] and has been shown to accelerate considerably the formation of AA experimental amyloid.[31] Amyloid enhancing factor activity has been observed in tissue extracts from AA, AL, and prealbumin related amyloidosis.[32]

If it is assumed that glycosaminoglycans are essential for amyloid enhancing factor activity then polyanionic sugars may be a significant constituent common to amyloids of different protein composition. The striking ultrastructural homology of amyloid fibrils having such diverse protein structure may thus be caused by the presence of glycosaminoglycans as their 'common denominator'.

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