High molecular weight glycosaminoglycans in AA type amyloid fibril extracts from human liver

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Abstract
Glycosaminoglycans have previously been identified in extracts of AA type hepatic amyloid fibril from a patient with amyloidosis associated with juvenile rheumatoid arthritis. The macromolecular properties of these polysaccharides are described here in more detail. By gel filtration and ion exchange chromatography glycosaminoglycans in the form of high molecular weight free chains were shown to coisolate with water extracted amyloid fibrils. About 60% of these were characterised as galactosamines (chondroitin sulphate/dermatan sulphate), whereas the remaining 40% consisted of N-sulphated glycosamines (heparin/heparan sulphate). The amyloid associated glycosaminoglycans were not part of intact proteoglycans in the fibril extracts.

Amyloidosis is a heterogeneous group of disorders caused by tissue deposition of a proteinaceous material—amyloid—characterised by a positive Congo red reaction showing yellow-green birefringence under polarised light, and a characteristic fibrillar ultrastructure.1-3 Amyloid fibrils can be extracted from affected tissue with distilled water after more soluble materials have been removed by repeated saline washes.4 This provides a relatively pure preparation of amyloid fibrils. When treated with dissociating agents like guanidine5 or urea6 followed by gel filtration the amyloid fibrils are shown to be made up of a variety of protein subunits,7 each characteristic of the different clinical categories of amyloid disease.2 So far at least 11 different amyloid fibril proteins are known by their amino acid sequence.8 Despite the heterogeneity of the fibril proteins all forms of amyloid have similar and unique ultrastructural and tinctorial properties.1-3

Several investigators have emphasised the close association between amyloid and polyanionic substances.9-11 We previously reported in this journal the presence of glycosaminoglycans in extracts of AA type hepatic amyloid fibrils associated with juvenile rheumatoid arthritis.12 The aims of the present study were, firstly, to examine in more detail the macromolecular properties of the polysaccharide moiety of this AA type amyloid and, secondly, to investigate whether these polysaccharides are present in amyloid as part of intact proteoglycans.

Materials and methods
AMYLOID FIBRIL EXTRACTION
Protein AA type amyloid fibrils were prepared from the liver of a 12 year old patient with amyloidosis associated with juvenile rheumatoid arthritis by the water extraction method of Pras et al13 with a small modification, as previously described.12 13 The aqueous amyloid fibril supernatants were either lyophilised directly or the amyloid fibrils were precipitated with 0-3 M sodium chloride.7 As the fibril proteins are kept intact during the water extraction procedure when protease inhibitors are not used, proteoglycans, if present, should also remain intact. Protease inhibitors were therefore not added until after the extraction of the amyloid fibril material from the tissue.

Normal liver tissue was subjected to the same extraction procedure.

GEL FILTRATION
The following columns, gels, and eluents were used: a 1·0×40 cm Sephadex G-50 column with 0-5 M ammonium bicarbonate buffer pH 7-5; a 1·5×100 cm Sephadex G-100 column with 0-05 M sodium acetate buffer pH 6-0, containing 6 M urea and 0·1 M sodium chloride; a 0·9×99 cm Sepharose CL-2B column with 0·05 M sodium acetate buffer pH 6-0, containing 6 M urea and 0·1 M sodium chloride; a 0·8×105 cm Sepharose CL-4B with 10 mM trometamol HCl buffer pH 6-0 containing 6 M urea; and a 0·9×96 cm Sepharose CL-6B with 10 mM trometamol HCl buffer pH 6-0, containing 6 M urea. Calibration was done with calf thymus DNA, dextran 200, thymoglobin, ferritin, catalase, aldolase, albumin, ribonuclease, insulin, and alanine. The Vt on each chromatogram was detected by the salt peak on the Sephadex columns and alanine on the Sepharose columns. The Kav values were calculated.14

Before gel filtration on the Sephadex G-100 column both the lyophilised amyloid fibrils and the corresponding normal extract were treated (15 mg/ml) with 0·05 M sodium acetate buffer pH 6·0 containing 8 M urea and 0·1 M sodium chloride and the following protease inhibitors: 10 mM benzamidine, 10 mM aminocaproic acid, and 25 mM EDTA, in addition to 10 mM dithiothreitol at 4°C over night. After fractionation the hexuronic acid content was determined, and the optical density at 260 and 280 nm read in a spectrophotometer. Selected fractions were pooled, dialysed against distilled water, and lyophilised.

PROTEOGLYCAN EXTRACTION
To search for proteoglycans the lyophilised amyloid fibril and normal liver extracts were...
treated with either 4 M guanidine HCl pH 5·8 or with 8 M urea in 0·05 M sodium acetate buffer pH 6·0 and 0·1 M sodium chloride, in the presence of the protease inhibitors 10 mM benzamidine, 10 mM aminocaproic acid, in addition to 25 mM EDTA and 10 mM dithiothreitol, at 4°C for 18 hours. Polysaccharides were also obtained from the NaCl precipitated amyloid fibrils by treatment with 4 M guanidine HCl pH 5·8 under the same conditions as above.

The materials were centrifuged at 15 000 rpm at 4°C for 15 minutes. The supernatants were collected and those treated with 4 M guanidine chloride were dialysed (mol wt cut off 6000) against a 10 mM trometamol HCl buffer pH 6·0 containing 6 M urea, and a gradient from 0·15 to 1·5 M NaCl. The ionic strength was measured by a conductivity meter, and 1·5 M NaCl was equivalent to 1900 μS. Selected fractions were pooled after determination of hexuronic acid, dialysed against distilled water, and lyophilised.

ION EXCHANGE CHROMATOGRAPHY

The extracted polysaccharides were chromatographed on a diethylaminoethyl (DEAE)-Sephacel ion exchange column. The column was calibrated and eluted with a 10 mM trometamol HCl buffer pH 6·0 containing 6 M urea, and a gradient from 0·15 to 1·5 M NaCl. The elution profile was determined by a conductivity meter, with 1·5 M NaCl being equivalent to 1900 μS. Selected fractions were pooled after determination of hexuronic acid.

CARBAZOLE REACTION

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

PROTEOGLYCAN/GLY COSAMINOGLYCAN STRUCTURE

To investigate whether the amyloid associated polysaccharides were present in the form of free (or non-covalently associated) glycosaminoglycan chains, or as part of intact proteoglycans, two different experimental procedures were used. Firstly, an aliquot of the lyophilised carbohydrate-positive material obtained after DEAE-Sephacel ion exchange chromatography was dissolved in 0·2 M trometamol HCl pH 8·0 and digested with pronase 0·2 mg/ml at 36°C for 18 hours. The digest was then gel filtered on Sepharose CL-6B. Secondly, another aliquot was dissolved in distilled water and subjected to β elimination—that is, treatment with 0·5 M NaOH for 24 hours at room temperature followed by adjustment to neutral pH with 10 M HCl and Sepharose CL-4B chromatography.

Aliquots of the lyophilised polysaccharides isolated by DEAE-Sephacel ion exchange chromatography were depolymerised as follows: galactosaminoglycans were degraded by chondroitinase ABC (EC 4.2.2.4) (Seikagaku Kogyo, Tokyo, Japan), 0·01 unit enzyme in 0·05 M trometamol HCl pH 8·0 containing 0·05 M sodium acetate and 0·05 M NaCl over night at 37°C and subsequently chromatographed on Sepharose CL-6B. Identification of polysaccharides containing N-sulphated glucosamine residues, heparin/heparan sulphate, was subsequently done by chemically specific depolymerisation using a low pH, nitrous acid treatment.

Results

AMYLOID FIBRIL PREPARATION

The amyloid laden liver was heavily infiltrated with material showing typical yellow green birefringence by polarisation microscopy after Congo red staining. The normal control organ was negative in this respect. The yield of lyophilised water extractable material from amyloid laden liver was 75 mg/g wet weight, which was eight times more than that obtained from the normal liver. The protein AA nature of the amyloidotic liver from this patient has previously been verified by amino acid sequencing of the fibril protein subunit prepared by the same procedures as described above.

GEL FILTRATION

Figure 1 shows the elution profiles of hepatic amyloid fibril and corresponding normal liver extracts on a Sephadex G-100 column. Two major protein components were obtained from the amyloid material, the Vo material and protein AA eluting in a retarded peak (fig 1a). In contrast, the normal tissue preparation eluted in only one protein peak, corresponding to the void volume (fig 1b). The most retarded peak represents salts, which give a positive optical density at 280 nm. All of the carbazole reactive polysaccharides of both the amyloid fibrils and the normal tissue extracts eluted corresponding to the Vo peak.

The top fraction of the Vo peak (fig 1) was

![Figure 1: Gel filtration on a Sephadex G-100 column of protein (A 280 nm, normal line) and carbazole positive polysaccharide material (A 530 nm, bold line) of (a) isolated hepatic amyloid fibrils and (b) the corresponding normal hepatic extract. Vi indicates the salts in both chromatograms. The column was eluted with 6 M urea/0.05 M sodium acetate/0.1 M sodium chloride pH 6·0.](http://ard.bmj.com/Downloaded from)
rechromatographed on a Sepharose CL-2B column, and fig 2 shows the elution patterns of the protein and the carbazole positive materials. All the carbazole positive material from the normal liver extract eluted in the Vo peak (fig 2), whereas that of the amyloid material was retarded with a Kav value of 0-66, corresponding to a molecular weight of about 90 kD with the protein standards used. The spectrophotometric 260/280 nm ratio in the Vo peak on the Sepharose CL-2B column showed more DNA material was present in the amyloid material than in the normal extract (data not shown).

ION EXCHANGE CHROMATOGRAPHY

The carbazole positive materials from normal liver and amyloid extracts obtained by gel filtration on Sepharose CL-2B (fig 2) were rechromatographed on DEAE-Sephacel. Figure 3 shows the carbazole reactivity. No retained carbazole positive material from the normal extract was detected with the conditions used. In contrast, the amyloid associated polysaccharides eluted in one major peak at 0-56 M NaCl.

Figure 4 shows the DEAE-Sephacel ion exchange chromatograms of the different proteoglycan extracts. All the extracts from the amyloid material showed a major carbazole positive peak at about 0-56–0-58 M NaCl. The findings indicate that the carbazole positive material from water extracted amyloid eluting in the retarded peak on fig 2 is identical to that obtained after direct extraction from the hepatic fibrils shown in fig 4. Analysis by ion exchange chromatography of the supernatant after NaCl precipitation of the amyloid fibrils (data not shown) did not disclose any carbazole positive material, showing all the polysaccharides to coprecipitate with the amyloid fibrils.

No material from the corresponding normal hepatic preparation bound the DEAE-Sephacel gel.
Molecules are made of three moieties: a protein moiety, a carbohydrate moiety, and a sulphate moiety. These moieties are connected through water and sulphate groups.

**Discussion**

The composition of amyloid has been a matter of dispute for more than a century. The term amyloid was created by Virchow in 1854 because its metachromatic property resembled that of starch, but was later for many years regarded as a misnomer because amyloid was thought to be of protein nature. The correct answer to this old question seems to be that the amyloid deposits comprise both protein and carbohydrate, and there is convincing evidence that both moieties are laid down simultaneously in the tissues.

In our previous report we showed that glycosaminoglycans in the form of dermatan sulphate, chondroitin sulphate, and heparin/heparan sulphate were present in pronase digested water extracts of hepatic AA fibrils in the order of 15 μg/mg lyophilised fibril material. We assumed that glycosaminoglycans were specifically associated with amyloid as the preparative procedure should have prevented the presence of non-associated polysaccharides in the extracts.

When amyloid fibril extracts are solubilised and subjected to gel filtration under dissociation conditions the amyloid protein is eluted as a retarded peak. In addition, a heterogeneous protein material elutes in the Vo on most columns, which includes fibronectin known to bind proteoglycans and serum amyloid protein A. A similar material is also present in corresponding preparations of normal tissues. It has been suggested that this Vo material represents contaminants in the fibril preparation, and consequently, the coisolated glycosaminoglycans might also be simple contaminants. The gel filtration experiments, however, clearly showed the amyloid associated polysaccharides are not bound to any of the Vo proteins present in the amyloid extracts.

Another possible mediator of the association between glycosaminoglycans and amyloid is the amyloid P component, a glycoprotein which is invariably present in amyloid deposits. Its
serum precursor, serum amyloid P component, a normal plasma glycoprotein of the pentraxin family, is known to bind to heparan sulphate and dermatan sulphate, and also to amyloid fibrils in a manner dependent on calcium. The addition of citrate to the saline washes during fibril preparation, which was done in this study, would remove calcium dependent bindings and thus prevent the coisolation of any glycosaminoglycans bound to amyloid fibrils or amyloid P component in this manner; indeed, the presence of amyloid P component itself in the fibril extracts is also avoided. Thus the amyloid P component does not seem to be involved in the association of the glycosaminoglycans with the fibrils, as suggested by others.

We thus assume that the polysaccharides are either integrated in or strongly associated with the fibrillar structure itself by electrostatic forces compatible with the strong negative charges characteristic of these polysaccharides. This assumption was strengthened by the observation that carboxazol reactive material with the water suspended amyloid fibrils was completely coprecipitated by 0.3 M NaCl.

Histological and immunohistochemical studies have suggested the presence of intact proteoglycan in amyloids of different protein type. The amyloid associated glycosaminoglycans studied here did not appear as parts of intact proteoglycans in the fibril preparation, however. The evidence for this is that there was no change in the elution position of the carboxazol positive material on the Sepharose columns either after β elimination or after enzymatic digestion of the amyloid extract. Such treatments should have disclosed any core protein by a change in the elution position on gel filtration, unless it were too small to be shown.

Glycosaminoglycans account for 60% and glycosamines for the remaining 40% of total carbohydrates coisolated with the hepatic AA amyloid extract studied here, confirming our previous enzymatic studies. These glycosaminoglycan chains had a remarkably large molecular size (about 90 kD), but even under the strong dissociating conditions used here we cannot rule out the possibility that they represent aggregates of smaller molecules. It should also be noted that the molecular weight estimation used here might have been inaccurate.

Amyloid fibril formation is a result of conversion of precursor protein into a characteristic fibrillar form. The role of sulphated polysaccharides in this process is not known. Glycosaminoglycans, however, occur in the tissues in close temporal and morphological relation to amyloid deposition in murine experimental amyloidosis. In vitro experiments have shown that glycosaminoglycans can initiate structural change of proteins, and thereby promote fibril formation. The role of amyloid associated glycosaminoglycans in vivo needs further elucidation.

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