Isolation and partial characterization of heparan sulphate proteoglycans from human hepatic amyloid

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Proteoglycans were isolated from human amyloidotic liver by extraction with guanidine, followed by trichloroacetic acid precipitation, DEAE-Sephacel ion-exchange chromatography, and Sepharose CL-6B gel chromatography. A significant portion of the material was found to be free chondroitin/dermatan sulphate chains (30 %), whereas the predominant part was heparan sulphate proteoglycan (HSPG) (70 %). The approx. molecular mass of the HSPG was 200 kDa, as measured by gel electrophoresis and gel chromatography. The molecular mass of the core protein was shown to be 60 kDa by SDS/PAGE following de-aminative cleavage of the heparan sulphate chains. The heparan sulphate chains were liberated from the core protein by alkali treatment and found to have a molecular mass of approx. 35 kDa by Sepharose CL-6B gel chromatography. The core protein was shown, by immunoblotting, to react with a monoclonal antibody against bovine basement membrane HSPG. The presence of HSPG in amyloid deposits was further confirmed by immunohistochemistry on tissue sections from amyloidotic liver using the same antibody.

INTRODUCTION

The term amyloidosis refers to a heterogeneous group of diseases (rather than a single disease entity) defined by the deposition of proteinaceous material, amyloid, in various tissues or organs (Husby & Sletten, 1986). The clinical syndromes are characterized by the tissues and organs involved. In Alzheimer's disease, the most common form of human amyloidosis and the major cause of dementia (Davies, 1991), the amyloidotic lesions are located in senile plaques in the brain and do not appear to be systemic. In contrast, reactive amyloidosis, a rare complication of common inflammatory conditions such as rheumatoid arthritis, tuberculosis or osteomyelitis, is a systemic disorder in which several organs are affected (Husby, 1985).

Electron microscopy studies have revealed that amyloid has a fibrillar ultrastructure (Glennner, 1980). These fibrils, formed from different precursor proteins in the various clinical forms of amyloidosis, are easily extractable in water after the removal of more soluble proteins by saline washings (Pras *et al.*, 1968), and so far at least 15 different amyloid fibril proteins are known by their amino acid sequences (Husby *et al.*, 1991). Despite the heterogeneity of the fibril proteins, all forms of amyloid share similar ultrastructural and tinctorial properties. Some of the characteristic properties of amyloid, such as metachromasia after Crystal Violet staining, do not depend on the fibrillar structure or on the fibril protein, but on the presence of glycosaminoglycans (GAGs) in amyloid deposits (Puchtler & Sweat, 1966). In fact, the recognition and characterization of heparan sulphate (HS), as distinct from heparin, were carried out in material obtained from the livers of horses with amyloidosis (Linker *et al.*, 1958).

Heparan sulphate proteoglycans (HSPGs) are most commonly found at cell surfaces or in extracellular matrices (ECMs) such as basement membranes (BMs) in virtually all mammalian tissues (Gallagher *et al.*, 1986). Interaction of these macromolecules with other ECM components, such as laminin, collagen and fibronectin, contributes to the general structure of the BM (Yurchenco & Schittny, 1990). Furthermore HSPGs have a

major functional role in a variety of dynamic processes such as cell adhesion and migration (Ruoslahti, 1989). Proteoglycans (PGs) and GAGs also play a critical role in the pathophysiology of BM-related diseases, including diabetes, atherosclerosis, and cancer metastases (Gallagher *et al.*, 1986).

The pathophysiological mechanisms in amyloid deposition are unknown, but the increased amount of GAGs in the amyloid-laden organs has initiated an interest in the possible role of these polysaccharides in amyloidogenesis. Biochemical studies of water-extracted fibrils have identified the amyloid-associated GAGs as predominantly being of the HS and chondroitin/dermatan sulphate (CS/DS) type (Magnus *et al.*, 1989). Most reports have been performed on enzyme-digested fibril material (Brandt *et al.*, 1974; Ohishi *et al.*, 1990), but a few studies have used protease inhibitors during the extraction procedures of the GAGs (Magnus *et al.*, 1991a,b; Nelson *et al.*, 1991; Stenstad *et al.*, 1991a). We have reported the predominance of free GAG chains in the isolated fibril material from amyloid (Magnus *et al.*, 1991c); more recently, however, we were able to obtain an intact HSPG from water-extracted amyloid fibrils from human spleens (Stenstad *et al.*, 1991b).

These findings prompted us to investigate whether HSPG could be extracted from an amyloidotic tissue. The results presented here show the isolation and partial characterization of HSPG from human hepatic amyloid.

MATERIALS AND METHODS

Materials

The amyloid-laden liver (3.6 kg) of a 12-year-old male patient with amyloidosis associated with juvenile rheumatoid arthritis was obtained at autopsy and kept frozen at -20°C . The liver (420 g) of a 3-year-old child who died in an accident was obtained and used as a normal control.

Guanidinium chloride from Fluka (Buchs, Switzerland) was further purified by filtration through activated charcoal. Urea (Fluka) was de-ionized by treatment with mixed-bed resin from

Abbreviations used: AA, amyloid A; GAG, glycosaminoglycan; PG, proteoglycan; HS, heparan sulphate; CS, chondroitin sulphate; DS, dermatan sulphate; ECM, extracellular matrix; BM, basement membrane; HS PG, heparan sulphate proteoglycan; PMSF, phenylmethanesulphonyl fluoride; APAAP, alkaline phosphatase-anti-alkaline phosphatase; TBS, Tris-buffered saline; EHS, Engelbreth-Holm-Swarm.

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Bio-Rad (Richmond, CA, U.S.A.) before use. DEAE-Sephacel, Sepharose CL-6B, Sepharose CL-4B, Sephadex G-50 Superfine, SDS/PAGE 8–25 % and 4–15 % Phast gels were from Pharmacia LKB Biotechnology (Uppsala, Sweden). 1,9-Dimethyl-Methylene Blue was obtained from Aldrich Chemicals (Milwaukee, WI, U.S.A.) and Toluidine Blue from Difco Laboratories (West Molesey, U.K.). Heparinase (EC 4.2.2.7), heparitinase (EC 4.2.2.) and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Kogyo, Tokyo, Japan. Phast System (Pharmacia) was used for SDS/PAGE and Western immunoblotting.

PG extraction

All extraction and purification procedures were carried out at 4 °C. Portions (5 g) of frozen liver tissue were powderized in liquid nitrogen with a mortar. PGs were extracted with (10 × volume/weight in g) 4 M-guanidinium chloride/50 mM-sodium acetate, 10 mM-aminohexanoic acid, 10 mM-benzamidine hydrochloride, 10 mM-EDTA, 5 mM-N-ethylmaleimide and 0.5 mM-phenylmethanesulphonyl fluoride (PMSF), pH 5.0. The homogenate was gently mixed for 24 h and then centrifuged at 2000 g for 10 min in a Sorvall centrifuge at 4 °C. The supernatant was thereafter filtered through a layer of nylon mesh.

Precipitation by trichloroacetic acid

Trichloroacetic acid precipitation was performed as described by Lyon & Gallagher (1991). Briefly, ice-cold 100 % (w/v) trichloroacetic acid was added to the filtered extract to give a final concentration of 10 % (v/v). The precipitate was centrifuged and the supernatant neutralized to pH 7.

Enzymic treatment with benzon nuclease

The neutralized supernatant after trichloroacetic acid precipitation was extensively dialysed against 6 M-urea/10 mM-Tris/HCl/0.15 M-NaCl/10 mM-EDTA/10 mM-aminohexanoic acid, pH 8.0. Thereafter 250 units of enzyme was added and the mixture was incubated for 2 h at 37 °C. The absorbance at 260 nm was measured before and after enzyme digestion, and the material subjected to ion-exchange chromatography as described below.

Ion-exchange chromatography

Batches of the extract, the neutralized supernatant and the precipitate after trichloroacetic acid precipitation, with or without benzon nuclease digestion, were extensively dialysed against 6 M-urea/10 mM-Tris/HCl/0.15 M-NaCl/10 mM-EDTA/10 mM-aminohexanoic acid, pH 8.0. The material was then batch-adsorbed on to 5 ml of DEAE-Sephacel gel, equilibrated in the same buffer, by gentle mixing overnight at 4 °C. The gel was poured into a column and washed with the same urea buffer. Thereafter the washing was continued with the same buffer, except that the pH was changed to 5.0. The column was then washed with 6 M-urea/10 mM-Tris/HCl/10 mM-EDTA/10 mM-aminohexanoic acid/0.3 M-NaCl, pH 5.0, before the PGs were eluted using a gradient of 0.3–1.0 M-NaCl (200 ml total volume) in the 6 M-urea solution (pH 5.0). Fractions (5 ml) were collected at a flow rate of 15 ml/h. After fractionation the hexuronic acid content was determined by the carbazole method described by Bitter & Muir (1962), and the absorbance at 280 nm was read in a Hitachi spectrophotometer. The ionic strength was measured by a conductometer, where 1.0 M-NaCl was equivalent to 1200 μ S. Selected fractions were pooled.

Chondroitinase ABC digestion

Pooled fractions from the DEAE-Sephacel column were extensively dialysed against distilled water and lyophilized before chondroitinase ABC digestion. Alternatively, the pooled frac-

tions were concentrated to approx. 5 ml by reverse osmosis against solid poly(ethylene glycol) and was then dialysed against 50 mM-NaCl/50 mM-Tris/HCl/10 mM-aminohexanoic acid/10 mM-EDTA/5 mM-N-ethylmaleimide, pH 8.0. Chondroitinase ABC (0.1 unit) and PMSF (final concentration of 0.25 mM) were then added. After an overnight incubation at 37 °C, a further addition of PMSF and 0.05 unit of enzyme was made, followed by a further 5 h incubation. The digest was either run on a DEAE-Sephacel ion-exchange column under the conditions already described, or on a Sepharose CL-6B column (see below). Selected fractions, after determination of hexuronic acid content, were precipitated with 9 vol. of 95 % (v/v) ethanol at –20 °C. After 2 h the precipitate was recovered by centrifugation at 16000 g for 10 min. The pellet was washed with a small volume of 75 % (v/v) ethanol, air-dried and then redissolved in 6 M-urea/10 mM-Tris/HCl/10 mM-EDTA/10 mM-aminohexanoic acid/0.15 M-NaCl, pH 6.0.

Gel filtration

The following columns, gels and eluants were used: a 1.0 cm × 40 cm Sephadex G-50 Superfine column was eluted with 0.2 M-ammonium bicarbonate buffer, pH 7.5; a 0.8 cm × 105 cm Sepharose CL-4B column and a 0.9 cm × 96 cm Sepharose CL-6B column were both eluted with 6 M-urea/10 mM-Tris/HCl/0.5 M-NaCl, pH 6.0. Urea was used because of its compatibility with the carbazole method. The columns were calibrated with dextran 200, thyroglobulin, ferritin, immunoglobulin, albumin, ribonuclease, insulin and 1,6-dinitropyridylalanine. The V_i on each chromatogram was detected by 1,6-dinitropyridylalanine. The K_{av} values were calculated as described (Laurent & Killander, 1964). The Sepharose CL-6B column was additionally calibrated using ³⁵S-labelled Na₂SO₄ and radiolabelled CS of known molecular mass, and the K_{av} value calculated and used for reference. [³H]Chondroitin 4-sulphate was a gift from Dr. U. Lindahl (Swedish University of Agricultural Sciences, Uppsala, Sweden).

Aliquots of the polysaccharide material isolated by DEAE-Sephacel ion-exchange material before and after depolymerization with chondroitinase ABC were subsequently chromatographed on Sepharose CL-6B or Sephadex G-50 Superfine. After fractionation, the hexuronic acid content was determined, and the absorbance at 280 nm measured in a spectrophotometer. Selected fractions were pooled, either dialysed against distilled water and lyophilized, or precipitated with ethanol as described above.

Antibodies

HSPG. A commercially available monoclonal HSPG antibody, IgG₁ subclass (Chemicon International, Temcula, CA, U.S.A.), was used for Western immunoblotting and immunohistochemistry. This antibody against purified bovine glomerular HSPG was raised and characterized by Kemeny *et al.* (1988) and recognizes the core protein of this HSPG. The monoclonal antibody showed no cross-reactivity against laminin, type IV collagen or fibronectin. Furthermore, immunohistochemistry has demonstrated its reactivity with HSPG moieties in the BM in a variety of human tissues (Kemeny *et al.*, 1988).

Amyloid A (AA). AA protein is the fibril protein of amyloid deposits in reactive amyloidosis (Husby & Sletten, 1986). A commercially available monoclonal antibody against AA protein, IgG_{2a} subclass (Dako, Glostrup, Denmark), was used for immunohistochemical staining. This antibody reacts with amyloid deposits of AA type in all organs and tissues.

Immunohistochemistry

Alkaline phosphatase–anti-alkaline phosphatase (APAAP)

was used for immunohistochemical investigations (Cordell *et al.*, 1984). Tissue blocks were immersed in 4% (w/v) formaldehyde before embedding in paraffin. After sectioning, the paraffin sections were de-waxed with xylene and some specimens were treated with pronase before addition of primary antibody. An APAAP Kit system (Dako) was used with anti-(mouse immunoglobulins) antibody as the second antibody.

Electrophoresis

Phast System (Pharmacia) was used for performing SDS/PAGE. Gradient gels [8–25% and 4–15% (w/v) polyacrylamide] was stained with Coomassie Blue for detecting proteins and Toluidine Blue [1% (w/v) in 20% (v/v) methanol] for polysaccharides. Semi-dry electrophoretic transfer to nitrocellulose after SDS/PAGE of the intact HSPG and the HSPG core protein was performed (Jägersten *et al.*, 1988), before Western immunoblotting. Briefly, after incubation in blocking solution [3% (w/v) gelatin in Tris-buffered saline (TBS) 20 mM-Tris/HCl, 0.5 M-NaCl; pH 7.5], the nitrocellulose was incubated with anti-(HSPG) antibody as the primary antibody (1:25 or 1:50 dilution), in a solution of 0.1% gelatin, 0.05% Tween 20 in TBS overnight at room temperature. After washing with 0.05% Tween in TBS, an alkaline phosphatase-conjugated rabbit (anti-mouse IgG) antibody at a 1:2000 dilution (Dako) was added for 1 h. The blot was developed by addition of enzyme substrate solution prepared as previously described (Blake *et al.*, 1984).

Other analytical procedures

The recovery of PGs/GAGs was determined by use of the 1,9-dimethyl-Methylene Blue colorimetric assay with CS (Sigma) as the standard, because the presence of DNA and guanidinium chloride interferes with the carbazole reaction. The increased absorbance at 525 nm was analysed manually in a Hitachi spectrophotometer as described by Farndale *et al.* (1986). The protein content was monitored by use of the Bio-Rad Protein Assay, using the Bio-Rad Protein Standard 1 as standard.

Polysaccharides containing *N*-sulphated glucosamine residues (heparin/HS) were depolymerized by nitrous acid, pH 1.5 (Shively & Conrad, 1976), before SDS/PAGE. Parallel samples were also chromatographed on a Sephadex G-50 column and the elution profile determined by the carbazole reaction, and compared with that of untreated material. These polysaccharides were also digested with heparinase and heparitinase (0.002 unit of enzyme/mg of polysaccharide material) in a 0.05 M-Tris/HCl buffer, pH 7.4, with 1.0 mM- CaCl_2 , at 40 °C overnight before gel filtration (Linker & Hovingh, 1972).

Alkali borohydride treatment, known to disrupt the serine-xyloside linkage which is the attachment site for GAG chains to the protein backbone, was employed to elucidate whether intact PGs were present or not. Briefly, aliquots of lyophilized polysaccharide material were treated with 0.05 M-NaOH with 1.0 M- NaBH_4 for 48 h at 40 °C, and thereafter neutralized with HCl and subsequently gel-filtered on a Sepharose CL-6B column. The elution profiles based on hexuronic acid determination were compared with those of untreated aliquots (Heinegård & Sommarin, 1987).

RESULTS

The amyloidotic liver used in this study was shown to be heavily infiltrated with material displaying typical yellow/green birefringence when analysed by polarization microscopy after Congo Red staining. Furthermore, APAAP immunohistochemistry revealed positive staining of the amyloid deposits with the monoclonal anti-AA antibody as shown in Fig. 6(a) (see also separate section about immunohistochemistry). Isolated fibril

proteins from liver deposits of this particular patient have previously been shown to be of the AA type (Magnus *et al.*, 1989).

Isolation of polysaccharides from human liver

Polysaccharides were isolated from sections of an amyloidotic and a normal liver by 4 M-guanidine extraction and DEAE-Sephacel ion-exchange chromatography. As shown in Fig. 1(a) the carbazole-positive material eluted as a broad peak ranging from 0.5–0.7 M-NaCl. The polysaccharide peak was heavily contaminated with protein and DNA, and subsequent chromatography of the pooled peak fractions on a column of Sepharose

Fig. 1. DEAE-Sephacel ion-exchange chromatography of guanidine-extracted material from human amyloidotic and normal liver

PGs were extracted from human amyloidotic liver (a and c) and normal liver (b and d) using 4 M-guanidine and protease inhibitors before trichloroacetic acid precipitation as described in the Materials and methods section. The crude extract (a and b) and the supernatant after trichloroacetic acid precipitation (c and d) were thereafter dialysed into Tris/HCl buffer containing 6 M-urea and protease inhibitors, before being subjected to DEAE-Sephacel ion-exchange chromatography. The column was washed with 6 M-urea/10 mM-Tris/HCl/10 mM-EDTA/10 mM-aminohexanoic acid/0.3 M-NaCl, pH 5.0, followed by a gradient of 0.3–1.0 M-NaCl (200 ml total volume) in the same buffer. Fractions (5 ml, at a flow rate of 15 ml/h) were analysed for protein (A_{280}) (—) and GAGs by the carbazole reaction (A_{530}) (---). Selected fractions were pooled as indicated, and subjected to further analyses.

Table 1. Purification of polysaccharides from human normal liver and amyloidotic liver

Experimental details are given in the Materials and methods section.

Sample	Proteins				Polysaccharides			
	Normal		Amyloidotic		Normal		Amyloidotic	
	Content (mg/g of liver)	Recovery (%)	Content (mg/g of liver)	Recovery (%)	Content (μ g/g of liver)	Recovery (%)	Content (μ g/g of liver)	Recovery (%)
Extract	241.5	100	175.1	100	880.6	100	3665.2	100
Trichloroacetic acid supernatant	155.6	64.4	59.4	33.9	324.0	36.8	2236.0	61.0
DEAE-Sephacel	16.5	6.8	1.7	1.0	75.0	19.9	1485.0	49.4

Fig. 2. Sepharose CL-6B gel chromatography of human amyloid-associated PGs obtained after DEAE-Sephacel ion-exchange chromatography

Pooled carbazole-positive material (peak 1, Fig. 1c) was subjected to Sepharose CL-6B gel chromatography, and the elution patterns of untreated material (a), chondroitinase ABC-digested material (b) and chondroitinase ABC and alkaline borohydride-treated material (c) are shown. The column was eluted with 6 M-urea/10 mM-Tris/HCl, pH 6.0, containing 0.5 M-NaCl. Fractions (1 ml) were collected and analysed for GAG content (A_{530}).

CL-4B did not improve the degree of purification of the polysaccharide (result not shown). Digestion of samples with benzonase before the ion-exchange chromatography step (results not shown) reduced the amount of DNA binding to the column, but large amounts of protein still contaminated the carbazole-positive material in a similar way to that shown in Figs. 1(a) and 1(b). However, trichloroacetic acid precipitation of the guanidine extract significantly reduced the protein content (Figs. 1c and 1d), and resulted in a highly improved separation of the protein and the polysaccharide material after ion-exchange chromatography. Comparing the elution profiles of the polysaccharides obtained in the supernatant and the corresponding precipitate after trichloroacetic acid precipitation it was evident that only

insignificant amounts of carbazole-positive material were obtained in the precipitates. Furthermore, the amyloid-associated polysaccharides retained in the supernatant required a higher NaCl concentration in order to be discharged from the DEAE-Sephacel ion-exchange column (Fig. 1c), than did the material obtained from the corresponding precipitate (results not shown). From Fig. 1(c) it is also evident that the polysaccharide from the amyloidotic material is resolved into two peaks when a trichloroacetic acid-precipitation step is added before the DEAE-Sephacel filtration. The material eluting after ion-exchange chromatography was therefore pooled as shown in Fig. 1(c), and the two separate fractions were subjected to further analyses. Table 1 lists the amounts of protein and polysaccharide found in the crude extract, the supernatant after trichloroacetic acid precipitation and in the pooled fractions (peaks 1 and 2, total) after ion-exchange chromatography from both the normal and the amyloidotic liver. The small amounts of polysaccharides obtained per gram of fresh liver tissue (75 μ g/g) after DEAE ion-exchange chromatography and the limited tissue supply prevented further detailed studies on PGs from normal human liver tissue. This was at variance to the situation in amyloidotic liver tissue where the recovery of polysaccharide was 1485 μ g/g of tissue, approx. 20 times more than was recovered from the normal liver tissue employing identical methods. The pooled peak 1 (Fig. 1c) contained approx. 56% and peak 2 approx. 44% of the total polysaccharide recovered from the amyloidotic liver. The chromatogram shown in Fig. 1(c) was consistent throughout several extractions of sections from the same liver. SDS/PAGE revealed the presence of three proteins with approximate molecular masses of 30, 55 and 67 kDa both in the peak 1 and peak 2 material.

Characterization of the PGs from human amyloidotic liver

The molecular mass of the isolated material was analysed by subjecting the two fractions separately to Sepharose CL-6B gel chromatography in 6 M-urea. From Fig. 2 it is seen that the peak 1 material (see Fig. 1b) eluted with a peak K_{av} of 0.36 (Fig. 2a), whereas the peak 2 material eluted with a K_{av} of 0.32 (Fig. 3a).

The peak 1 material was treated with chondroitinase ABC followed by gel chromatography. From Fig. 2(b) it is evident that only 10% of the material is susceptible to the enzyme treatment and is hence of the CS/DS type. The material resistant to chondroitinase ABC treatment was subjected to de-aminative cleavage, known to depolymerize heparin and HS-related GAGs. When analysed by Sephadex G-50 gel chromatography the remaining 90% of the polysaccharide in this fraction was depolymerized to oligosaccharides (Fig. 4). The peak 1 fraction from Fig. 1(c) therefore contains predominantly HS. The PG nature of this HS was further demonstrated by gel chromatography of this material after alkaline borohydride treatment,

Fig. 3. Sepharose CL-6B gel chromatography of human amyloid-associated PGs obtained after DEAE-Sephacel ion-exchange chromatography

Peak 2 material (see Fig. 1c) was pooled and subjected to Sepharose CL-6B gel chromatography. Untreated material is shown in (a), chondroitinase ABC-treated material in (b). The material from peak 2 (Fig. 1c) was subjected to nitrous acid treatment. The material resistant to chemical depolymerization was isolated on Sephadex G-50, and rechromatographed on Sepharose CL-6B before and after alkali borohydride treatment (●; c). Elution conditions were as described in the legend to Fig. 2.

Fig. 4. Sephadex G-50 gel chromatography of human amyloid-associated HSPG before and after nitrous acid depolymerization

The purified HSPG (Fig. 2b) was subjected to Sephadex G-50 gel chromatography before and after nitrous acid, pH 1.5, depolymerization (●). The column was eluted with 0.2 M-NH₄CO₃, pH 7.5, and fractions were analysed for GAG content (A_{530}).

which is known to result in the liberation of free GAG chains from PG molecules. From Fig. 2(c) it is evident that the peak is eluted at a K_{av} of 0.39, as compared to 0.31 in untreated chondroitinase ABC-resistant material (Fig. 2b). Based on the results published by Wasteson (1971), such a K_{av} value for the free HS chains should indicate an approximate molecular mass of 35 kDa. The major portion of the material recovered from peak (Fig. 1c) is, based on these results, HSPG.

Peak 2 material (Fig. 1c) was subjected to the same treatment as peak 1, and from Fig. 3(b) it is evident that approx. 65 % of

Fig. 5. Western immunoblotting of native and HNO₂-treated HSPG with monoclonal anti-(BM HSPG) antibody

The purified HSPG (Fig. 2b) was subjected to SDS/PAGE under reducing conditions before and after HNO₂ depolymerization of the HS chains. One gel was stained with Coomassie Blue (a); lane 1, standards; lane 2, untreated material; lane 3, HNO₂-treated material. (b) Western immunoblotting with the monoclonal anti-(BM HSPG) antibody; lane 1, standard; lane 2, untreated material; lane 3, HNO₂-treated material.

the material is depolymerized and eluting in the V_t position after chondroitinase ABC treatment. The material resistant to enzymic treatment, and eluting as several minor peaks up to K_{av} of 0.5, was completely depolymerized to oligosaccharides after HNO₂ treatment (results not shown). Peak 2 material therefore contains a mixture of CS/DS (65 %) and HS (35 %). To investigate whether these GAGs were of PG nature, peak 2 material was subjected to preparative chondroitinase ABC and HNO₂ treatments. The HNO₂-resistant material was subjected to alkali treatment, Sephadex G-50 and Sepharose CL-6B gel chromatography. No shift in elution position (K_{av} 0.30) could be observed when compared with untreated material (Fig. 3c). The CS/DS material therefore appeared to contain free GAG chains only, in accordance with previous reports (Magnus *et al.*, 1991a,b,c; Stenstad *et al.*, 1991a; Nelson *et al.*, 1991). Due to the limited amounts of chondroitinase ABC-resistant material available only the two major peaks (see Fig. 3b), eluting with K_{av} values of 0.19 and 0.33 respectively, could be subjected to alkali treatment and gel chromatography. The 0.19 peak material was shifted to a K_{av} of 0.30 and the 0.33 peak was shifted to a K_{av} of 0.38 (results not shown). Pronase treatment of these two HS fractions yielded almost identical elution profiles to those obtained after alkali treatment of the same materials (results not shown). The major portion of all the HS isolated from the amyloidotic liver is therefore HSPG. HS accounted for 70 % of the GAGs isolated from this amyloidotic liver.

The purified HSPG (Fig. 2b) was subjected to SDS/PAGE. Toluidine Blue staining of the gel indicated a molecular mass of approx. 200 kDa for the intact HSPG (result not shown). When aliquots of the same material were run on a Sepharose CL 6B column, the peak eluted with a K_{av} of 0.31, corresponding to a molecular mass of approx. 230 kDa compared with the protein standard used. The HNO₂ depolymerization of the HS chains resulted in the appearance of a 60 kDa protein, which could not be seen in the lane of the untreated material (Fig. 5). To characterize further this protein the monoclonal anti-(basement membrane HSPG) antibody was used in immunoblotting. This antibody recognized the 60 kDa protein released from the isolated HSPG by HNO₂ treatment (Fig. 5), suggesting that the amyloidotic liver does contain HSPG related to the HSPGs of the BM.

Immunohistochemistry

To obtain further evidence for the presence of a BM-type HSPG in amyloid deposits in human liver, formalin-fixed liver

Fig. 6. Immunohistochemical staining of amyloidotic liver sections

Tissue sections from human amyloidotic liver were studied with monoclonal anti-AA antibody (a) and a monoclonal anti-(BM HSPG) antibody. A distinct immunoreactivity of amyloid with the anti-AA antibody is demonstrated exclusively within the amyloid deposits (a). In (b) it is evident that the monoclonal anti-HSPG antibody also stains the amyloid deposits and the BMs of small blood vessels (V) and bile canaliculi (B).

specimens were subjected to immunohistochemical studies. A distinct immunoreactivity, using an antibody raised against the AA protein, could be detected exclusively within the amyloid deposits (Fig. 6a). From Fig. 6(b) it is evident that the monoclonal anti-HSPG antibody also stained the amyloid deposits. In addition, staining of the BMs of small blood vessels and bile canaliculi could also be observed. No staining was detected in liver parenchymal cells outside the amyloid deposits.

DISCUSSION

HSPGs, a complex and heterogeneous family of macromolecules, are ubiquitous components of BM, ECM and cell surfaces (Gallagher *et al.*, 1986). Most of the HSPGs characterized have been isolated from cell cultures or animal tissues. Only a limited number of studies have been performed on the isolation and characterization of PG from human tissues, with the exception of cartilage (Isemura *et al.*, 1987; van den Heuvel *et al.*, 1989, 1990; Stöcker *et al.*, 1991). Furthermore, few reports deal with the PG structure of tissues afflicted with pathological conditions (Iozzo & Wight, 1982; Dalferes *et al.*, 1987; Alini & Losa, 1991).

Using histochemical methods the temporal relationship between amyloid deposition and GAG accumulation in the tissues in experimental amyloidosis has been demonstrated (Snow & Kisilevsky, 1985). Immunohistochemical investigations using antibodies to the Engelbreth-Holm-Swarm (EHS) tumour HSPG (Hassell *et al.*, 1980), have demonstrated the presence of this protein moiety in mouse spleen and liver during experimental amyloidosis (Snow *et al.*, 1988). Furthermore, immunoreactivity

using antibodies against another distinct HSPG has been demonstrated in human renal amyloid deposits (Norling *et al.*, 1988).

In the present study we have isolated and partially purified a HSPG from human amyloidotic liver by guanidine extraction. As demonstrated in the Results section, a trichloroacetic acid precipitation step, previously reported by Lyon & Gallagher (1991), was important for the separation of a large portion of the protein contaminants from the polysaccharides. The approximate size of the HSPG (K_{av} 0.31, Fig. 2b) of 200 kDa, and the core protein of 60 kDa was demonstrated on SDS/PAGE. The size of the HS chains was shown to be approx. 35 kDa. Western immunoblotting experiments demonstrated the immunoreactivity to an anti-(HSPG) monoclonal antibody. The presence of this HSPG in amyloid deposits was also confirmed by immunohistochemical investigations (Fig. 5b).

The core protein of this bovine BM HSPG has a relatively large molecular mass, approx. 130 kDa, but different molecular masses of HSPG core proteins from different sources ranging from 10 to 400 kDa have been reported (Hassell *et al.*, 1986). In a recent publication Soroka & Farquhar (1991) characterized a novel HSPG (150–300 kDa) with a core protein size of approx. 40 kDa found in ECM of liver sinusoids and BMs. One of the earliest reported antibodies to BM HSPG was raised against HSPG isolated from the EHS sarcoma (Hassell *et al.*, 1980). Studies employing this antibody have revealed cross-reactivity, and therefore some sequence identity, between the HSPG core protein derived from EHS tumour and other BM HSPGs *in vivo*. However, isolation of these cross-reactive HSPGs has demonstrated tissue-specific variations in the molecular masses of the core proteins (Kato *et al.*, 1988). Furthermore, post-mortem or storage proteolytic changes of the HSPG core protein isolated in this study cannot be excluded. The other high-molecular-mass HSPGs isolated (Fig. 3b) are presently the subject of further studies.

The presence of a high-molecular mass CS/DS moiety is in concert with previous results reported by us (Magnus *et al.*, 1991a,b,c; Stenstad *et al.*, 1991a). The demonstration of two proteins, of 15 and 17 kDa on SDS/PAGE after chondroitinase ABC digestion may represent the core proteins, fragments thereof, or merely contaminating proteins, and this needs to be studied in further detail.

The increase of total polysaccharide content in amyloidotic organs compared with normal organs has been well documented (Bitter & Muir, 1966; Dalferes *et al.*, 1967). However, further work is needed to compare the structure and type of the amyloid-associated PGs and GAGs with the scarce amounts obtained from the normal organs utilizing the same procedures.

The presence of HSPG, in addition to other BM components such as laminin, fibronectin and collagen type IV in amyloid deposits (Lyon *et al.*, 1991) invites the speculation as to whether there is a disturbance in the synthesis, or organization of the components in the BM in the pathogenesis of amyloid deposition (Kisilevsky, 1990). The presence of high-molecular-mass, possibly free CS/DS chains, in amyloid deposits may result from enhanced production by polymorphonuclear cells invading the tissues during amyloidogenesis. Although these products are usually generated and retained in intracellular compartments, synthesis of free GAG chains from cells in culture has been reported (Wilson & Rider, 1991; Piepkorn *et al.*, 1989). Alternatively, these GAGs could be the result of a selective increase in the catabolism of CS PG in the liver, or PGs sequestered from circulating blood. The increased amount of GAGs in acute-phase serum has been demonstrated during amyloidogenesis in experimental animals also (Snow *et al.*, 1987).

In conclusion, this biochemical study has demonstrated the presence of one major HSPG moiety and high-molecular-mass

CS/DS in human hepatic amyloid. The isolation and characterization of PGs from human amyloid reported in this study constitute a basis for further investigations of the significance of these moieties in the pathogenesis of amyloid.

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