Characterization of proteoglycans associated with mouse splenic AA amyloidosis

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We here report for the first time on the chemical characteristics of proteoglycans associated with mouse splenic reactive AA amyloid. Amyloid was induced in CBA/J mice by two different procedures; conventional casein treatment and by employing Freund's complete adjuvant, accelerated by Trypan Blue. Pulse-labelling was employed at distinct stages during amyloid development, followed by [³⁵S]proteoglycan characterization of organ extracts. Repetitive ³⁵S injections were also administered during the phase where amyloid deposition occurred most rapidly. Proteoglycans were extracted with guanidine in the presence of protease inhibitors and purified. The results showed that the production of proteoglycans is dramatically enhanced during amyloidogenesis, the glycosaminoglycan and proteoglycan accumulation being not only dependent on alterations in

proteoglycan catabolism, but rather on increased synthesis. The increment could be demonstrated even at the stage before microscopic detection of amyloid deposits, clearly suggesting that the upregulation of proteoglycan expression precedes amyloid fibril formation. Two major proteoglycans were found to accumulate in advanced splenic amyloid; one a heparan sulphate proteoglycan of approx. 200 kDa with a core protein of 70 kDa, the other a chondroitin sulphate proteoglycan of smaller size. Moreover, free dermatan sulphate chains seemed to specifically accumulate in the organs during amyloid fibrillogenesis. We suggest that free glycosaminoglycans may be a specific feature of amyloidosis, and that different proteoglycans and glycosaminoglycans play a role in formation and stabilization of amyloid fibrils *in vivo*.

INTRODUCTION

Amyloidosis is a group of disorders characterized by the deposition of amyloid in extracellular matrices. The unique component of amyloid is known to be the amyloid fibril; a proteinaceous rod of indefinite length with prominent β -pleated sheet secondary peptide structure [1,1a]. During the last decade, evidence has been mounting that the association between sulphated polysaccharides and amyloid is universal, i.e. glycosaminoglycans (GAGs) and proteoglycans (PGs) are co-deposited with amyloid fibrils in all kinds of amyloid studied so far [2]. Moreover, it seems clear that these events also occur coincidentally with an accumulation of other extracellular matrix- and basement-membrane (BM)-derived elements, such as collagen IV and laminin [3], fibronectin [4] and vitronectin [5]. Employing immunohistochemical methodology, the appearance of the core protein of the large BM heparan sulphate (HS) PG (perlecan) has been reported for several amyloids [6] as well as a smaller vascular BM HSPG [7,8]. In previous studies we have characterized biochemically GAGs and PGs derived from various human tissues afflicted with different amyloids [8–10]. A specific association between certain GAGs/PGs and purified amyloid fibrils has also been disclosed [10,11].

The aim of the present work was to isolate and characterize biochemically PGs/GAGs found in experimentally induced amyloid A (AA). Mouse experimental amyloidosis was chosen (serving as a suitable model for the investigation and understanding of the pathogenesis of amyloidosis), representing an equivalent to human secondary AA amyloidosis. The model offers the opportunity to avoid any post-mortem proteolysis, which might possibly explain why significant quantities of free

GAG chains appear in a diversity of human amyloid preparations as previously described [11]. Thus, mouse splenic amyloid was employed as this particular organ is most severely afflicted with amyloid deposits, resembling the stage of disease found in affected human tissues at the time of autopsy. Moreover, an experimental animal model is far less material-consuming due to the possibility of *in vivo* GAG radiolabelling. Consequently, *de novo* synthesis of PGs could be analysed at different stages of amyloidogenesis. Additionally, repetitive radiolabelling during the phase of massive amyloid deposition would allow identification of which PGs and GAGs accumulate in the deposits. The mouse model has previously been employed in some studies addressing the association between GAGs/PGs and amyloid; however, most employed immunohistochemical and histochemical methods [6] which do not necessarily clarify the types and quantities of PGs actually present.

MATERIALS AND METHODS

Materials

Na₂³⁵SO₄ (National Institute of Energy Research, Kjeller, Norway) was diluted with 0.9% sterile saline. Liquid-scintillation cocktail (Ultima Gold XR) was purchased from Packard (Canberra, Australia). Casein (technical grade), aminohexanoic acid, *N*-ethylmaleimide, phenylmethanesulphonyl fluoride, Trypan Blue, thioflavine-T, calf thymus DNA, dinitropyridylalanine and chondroitin A were obtained from Sigma (St. Louis, MO, U.S.A.). Guanidinium chloride (Fluka, Buchs, Switzerland) was further refined by means of filtration through activated charcoal. Urea (Fluka) was de-ionized by treatment with mixed-bed resin (Bio-Rad, Richmond, CA, U.S.A.) before use. DEAE-Sephacrose

Abbreviations used: GAG, glycosaminoglycan; PG, proteoglycan; BM, basement membrane; HS, heparan sulphate; CS, chondroitin sulphate; DS, dermatan sulphate; AA, amyloid A; CFA, Freund's complete adjuvant; aCFA, accelerated CFA; DMB, Dimethylmethylene Blue; APAAP, alkaline phosphatase-anti-alkaline phosphatase.

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fast flow, Sepharose CL-6B, Sephadex G-50 fine, prepacked PD10 Sephadex G-25 columns, gradient (4–15 % and 8–25 %) SDS/polyacrylamide Phastgels with bufferstrips, PAG GelBond films, ExcelGel bufferstrips were products of Pharmacia LKB Biotechnology (Uppsala, Sweden). Chondroitinase ABC (EC 4.2.2.4), chondroitinase ACII (EC 4.2.2.5), chondroitinase B (EC 4.2.2.) and heparitinase (EC 4.2.2.8.) were purchased from Seikagaku Kogyo (Tokyo, Japan), benzamidine from Fluka and 1,9-Dimethylmethylene Blue (DMB) from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Freund's complete adjuvant (CFA) was obtained from Behring (Germany). Phast System (Pharmacia) was employed for small-size SDS/PAGE and Multi-phor II (Pharmacia) for large-size gels.

Antibodies

Affinity-purified rabbit anti-(mouse IgG) was obtained from Dako (Glostrup, Denmark). Monoclonal antibodies against purified BM HSPG were purchased from Chemicon (Temecula, CA, U.S.A.). This antibody was raised against bovine glomerular HSPG [12], but cross-reacts with human BM- and human amyloid-derived HSPG core proteins [8,9]. Monoclonal antibodies against human syndecan, N-syndecan and two distinct monoclonal antibodies against human fibroglycan, expected to cross-react with mouse variants, were provided by Dr. Guido David, University of Leuven, Belgium. Polyclonal antibodies against mouse AA were provided by Dr. Reinhold P. Linke, Max Planck Institute, München, Germany.

Laboratory animals

Amyloid-susceptible CBA/J mice were chosen because attempts to induce amyloidosis in Balb/C mice did not succeed. 10-week-old inbred female mice (Charles Rivers, Wiga, Germany) were raised on pelletized feed (RM1-E, Special Diets Service, U.K.) and water *ad libitum*. The average weight of the animals and their spleens were 25 g and 90 mg respectively.

Amyloid induction

Two different protocols were employed to induce amyloidosis in the animals. Saving time and effort, accelerated amyloidosis was induced in groups of mice by weekly subcutaneous injections of 0.3 ml of CFA emulsified in PBS in a 1:1 (v/v) ratio. An aliquot (1.0 ml) of 1 mg/ml Trypan Blue in PBS was administered intraperitoneally one day before administering the first dose of adjuvant, serving as the enhancer in this protocol [13].

Another group of 12 animals were subjected to a conventional amyloid induction protocol [14] receiving subcutaneous injections of 0.5 ml of 10 % (w/v) casein in aq. solution, pH 7, for 5 days a week for a period of 14 weeks. The animals were all subjected to partial splenectomy after the tenth week to assess the degree of amyloid deposition in the tissue. No animals were lost during this procedure.

Biolabelling of PGs/GAGs

To analyse the *de novo* synthesis of sulphated polysaccharides, groups of 15 animals each were killed at different stages of accelerated CFA (aCFA)-induced amyloid deposition. Three animals in each group were given intraperitoneal injections of 75 MBq (2 mCi) of ^{35}S 2 h before harvesting the spleens.

During the final 2 weeks of induction half of the animals subjected to both casein and aCFA treatments were given intraperitoneal injections of ^{35}S ($\text{Na}_2^{35}\text{SO}_4$) to enable character-

Figure 1 Algorithm visualizing the induction of mouse splenic amyloidosis and radiolabelling of proteoglycans

ization of the PGs/GAGs accumulating during amyloid deposition; 6.2 MBq (167 μCi) were administered for 5 days a week. Normal control animals were subjected to the same ^{35}S labelling.

The animals were killed by cervical dislocation and the blood evacuated from the circulation by cardiectomy to prevent contamination of tissue PG extracts by polysaccharide components derived from serum. After removal from the animals, the organs were all immediately immersed in liquid nitrogen to prevent any *post mortem* proteolysis and kept frozen until subjected to further treatment. The procedures are summarized in Figure 1.

Histological examination

Tissue specimens were immersed in 4 % buffered formaldehyde before paraffin embedding, sectioning and dewaxing and were subsequently subjected to standard haematoxylin–eosin, Congo Red [15] and thioflavine-T staining [16].

Electron microscopy

Following cervical dislocation, *in situ* fixation was performed on some animals. Briefly, infusion of 4 % formaldehyde in PBS was carried out through the left ventricle after opening the right atrium. Tissue specimens were further fixed in the same buffer, embedded in Epon, sectioned, stained and examined.

Immunohistochemistry

Alkaline phosphatase-anti-(alkaline phosphatase) (APAAP) was employed for immunohistochemical investigations. After sectioning and dewaxing, the tissue specimens were subjected to Pronase digestion for 8 min before addition of primary antibody. An APAAP-kit (Dako) was subsequently utilized for staining [17].

PG extraction

Portions of mouse splenic tissue were pulverized in a mortar mill (Retsch, Germany) following thorough cooling in liquid nitrogen. PGs were extracted with 4 M guanidinium chloride/50 mM sodium acetate containing 10 mM aminohexanoic acid, 10 mM benzamidine hydrochloride, 10 mM EDTA, 5 mM *N*-ethylmaleimide and 0.5 mM phenylmethanesulphonyl fluoride, pH 5.8. The homogenate was mixed vigorously for 24 h at 4 °C. The extract was subsequently subjected to trichloroacetic acid precipitation as previously described [18], giving a significant decrease in the amount of contaminating proteins and nucleic acids [9]. Briefly, ice-cold 100% (w/v) trichloroacetic acid was added to the extract to give a final concentration of 10% (v/v). Centrifugation was then performed and the supernatant neutralized dropwise with NaOH to pH 7.

Ion-exchange chromatography

The neutralized supernatant from trichloroacetic acid precipitation was extensively dialysed against 6 M urea/10 mM Tris/HCl/10 mM EDTA/10 mM aminohexanoic acid with 0.15 M NaCl, pH 5.8, at 4 °C. The material was then subjected to DEAE-Sepharose ion-exchange chromatography, essentially as described elsewhere [9]. The runs were carried out at 4 °C. Samples (100–250 µl) from each fraction were mixed with 2–3 ml of liquid-scintillation cocktail before counting. Measurements of absorbance at 280 nm, as well as conductivity in each fraction, were also performed.

Detection of GAGs

The DMB colorimetric assay for sulphated GAG quantification according to Farndale et al. [19] was performed in order to determine the elution profile of the total [PG/GAG] in extracts eluting from ion-exchange chromatography gels.

PG/GAG characterization

Pooled fractions from the DEAE ion-exchange chromatography were extensively dialysed against cold distilled water and lyophilized. Alternatively, peak fractions were distributed as 500 µl aliquots, desalted on a prepacked Sephadex G-25 column (PD10) and subjected to vacuum-concentration before further characterization. Various enzymic and chemical treatments were carried out as follows: chondroitinase ABC, chondroitinase ACII and chondroitinase B (dermatanase) digestions were performed at 37 °C for 15 h [20] and heparitinase digestion at 40 °C. Depolymerization of heparan sulphate was also done by low-pH nitrous acid treatment as previously described [21]. β -elimination was performed by 24 h incubation with 0.05 M NaOH in 1.0 M NaBH₄ at 45 °C [22].

Gel filtration

Gel filtration was performed on a 0.8 cm × 95 cm Sepharose CL-6B column eluted with 6 M urea/10 mM Tris/HCl/0.5 M NaCl,

pH 5.8; a 1.0 cm × 50 cm Sepharose CL-2B column eluted with the same buffer; a prepacked PD-10 Sephadex G-25 column and 4 cm³ Sephadex G-50 fine columns, eluted with cold distilled water. Calibration of the CL-6B column was performed as described elsewhere [10]. All runs were performed at 4 °C.

Electrophoresis

Phase System (Pharmacia) was employed for small-size SDS/PAGE. Materials obtained from ion-exchange chromatography were subjected to Sepharose CL-6B gel filtration followed by desalting on PD-10 columns and lyophilization. The samples were subsequently prepared and run as previously described [8]. Long (25 cm) ultrathin (0.5 mm) 3–12% (w/v) polyacrylamide gels with 3% stacking gels were cast on GelBond PAG films using the Laemmli buffer system [23] and run horizontally with ExcelGel bufferstrips on constant current. The gels were subsequently fixed and stained with 0.2% Coomassie Blue/0.1% Alcian Blue followed by silver staining with preceding oxidation [24,25].

Western blotting

Semi-dry electrophoretic transfer to nitrocellulose membranes was performed following SDS/PAGE [26]. After 24 h incubation in blocking solution [3% (w/v) gelatin in Tris-buffered saline, pH 7.5] and incubation with primary anti-HSPG antibodies for 16 h, alkaline phosphatase-conjugated rabbit anti-(mouse IgG) at 1:1000 dilution was added and incubated for 1 h. Development was carried out with *NN*-dimethylformamide containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitrobluetetrazolium chloride (NBT) [27].

RESULTS

Animals and organs

The total weight of the amyloidotic animals varied between 30 and 36 g. The average masses of the organs derived from the aCFA-induction protocol, at different stages, are shown in Table 1.

Histological examination

Significant hyperplasia and cellular infiltration was apparent in splenic sections taken from mice 2 days after aCFA-induced acute-phase reaction. No amyloid deposits could be detected by

Table 1 Increment of splenic weight during the course of accelerated adjuvant-induced amyloidosis along with the relative quantities of ³⁵S-labelled proteoglycans/glycosaminoglycans synthesized during *in vivo* radiolabelling for a 2 h interval

For further details, see Materials and methods section.

Time	Average splenic mass (mg)	Relative amount of [³⁵ S]glycosaminoglycans	[³⁵ S]glycosaminoglycans/splenic mass ratio
Normal control	85	1.0	1.0
2nd day	150	9.3	5.3
10th day	190	11.7	5.2
8th week	380	54.2	12.1
10th week	500	73.7	12.5

Figure 2 Light microscopy of splenic amyloid deposits in early (a) and advanced (b) stages

(a) The micrograph shows splenic amyloid deposits in the early phase, localized in the perifollicular zone. The specimen was stained with fluorescent thioflavine-T ($\times 120$ magnification). (b) The micrograph shows splenic casein-induced amyloid deposits in an advanced stage ($\times 120$).

immunohistochemical staining employing anti-AA antibodies, Congo Red or thioflavine-T staining (pre-amyloid phase). After 10 days of stimulation, however, moderate perifollicular amyloid had developed, representing early-phase amyloidosis (Figure 2a). Abundant amyloid deposits had developed after 8 weeks of adjuvant stimulation with deposits infiltrating most follicles as

well as the red pulp parenchyma, demonstrating its progressive increment. Finally, after 10 and 13 weeks of treatment, most of the original tissue architecture was deranged due to massive amyloid infiltration, representing advanced amyloidosis (Figure 2b).

Microscopy of stained tissue sections obtained from partial splenectomy after 10 weeks of casein challenge revealed amyloidosis in three-quarters of the animals, localized exclusively in the perifollicular zone (cf. Figure 2a). The degree of amyloid deposition was sparse, comprising no more than 5–10% of the tissue. After 14 weeks of treatment, all animals had developed amyloidosis corresponding to advanced disease with abundant deposits throughout the spleens. The amyloid deposits were estimated to comprise more than 70% of the specimens.

Electron microscopy

Amyloid fibrils, randomly arranged, could be demonstrated in the deposits. Abundant monocytoïd cells appeared in the periphery of the deposits, with extensive cytoplasmic processes and invaginations wherein fibrils occurred in close proximity to the plasma membrane (not shown). In some cells condensed vacuolized amyloid fibrils could be demonstrated intracellularly, as described previously [28].

PG/GAG synthesis

In Table 1, the synthesis of GAGs/PGs after 2 h of radiolabelling at the different stages of amyloidogenesis is demonstrated relative to the incorporation in normal control animals. The incorporation of ^{35}S in GAGs was increased approx. 6-fold even before microscopic amyloid deposition occurred (pre-amyloid phase). The same level of synthesis was detected after 10 days (early amyloid phase). The production of sulphated polysaccharides was increased even more, approx. 12-fold (^{35}S -labelled material relative to splenic mass), in the advanced stages of amyloidosis (8, 10 and 13 weeks respectively).

Ion-exchange chromatography

Ion-exchange chromatograms of splenic extracts obtained from *in vivo* ^{35}S incorporation for 2 h at distinct phases of amyloidogenesis (Figure 3a) and normal spleens (Figure 3c), exhibited a similar elution pattern. However, the total amounts differed (Table 1). The amount of material eluting in peak 2 comprised 10–15% of the total material. DMB analysis of the amyloid-derived material eluting from the gel (Figure 3a) revealed a difference between the total accumulated material and that

Figure 3 DEAE-Sepharose ion-exchange chromatography profiles derived from extracts of mouse spleens, ^{35}S -labelled *in vivo*

For further details, see the Materials and methods section. (a) Amyloidotic extracts ^{35}S -labelled for 2 h ($\diamond$) with DMB analysis for total GAG contents ($\blacklozenge$). (b) Amyloidotic extracts repetitively ^{35}S -labelled ($\diamond$). (c) Normal splenic extracts repetitively ^{35}S -labelled ($\diamond$). (1.0 M NaCl corresponds to 1200 μS .)

Figure 4 Sepharose CL-6B elution profiles of peak 1-derived [^{35}S]polysaccharides

(a) Material from amyloidotic animals (2 h labelling): untreated ($\diamond$), alkaline-borohydride-treated ($\blacklozenge$), chondroitinase ABC-digested ($\triangle$); (b) HSPG obtained from chondroitinase ABC digestion (bracket) with ($\blacklozenge$) and without ($\diamond$) alkaline-borohydride treatment; (c) HNO_2 -treated amyloidotic material; (d) CSPG obtained from HNO_2 treatment (bracket) with ($\blacklozenge$) and without ($\diamond$) β -elimination; (e) material from pre-amyloidotic animals (2 h labelling) untreated ($\diamond$) and HNO_2 -treated material with ($\blacktriangle$) and without ($\triangle$) alkaline-borohydride treatment; (f) chondroitinase ABC-digested material from normal ($\triangle$) and pre-amyloidotic animals with ($\blacklozenge$) and without ($\diamond$) β -elimination; (g) HNO_2 -treatment of accumulated ^{35}S -labelled polysaccharides from severely amyloidotic organs with ($\blacklozenge$) and without ($\diamond$) β -elimination; (h) CS obtained from HNO_2 treatment (bracket) re-chromatographed with ($\blacklozenge$) and without ($\diamond$) β -elimination; (i) chondroitinase ABC digestion with ($\blacklozenge$) and without ($\diamond$) alkaline-borohydride treatment; (j) chondroitinase ABC-digested accumulated ^{35}S -labelled polysaccharides from normal preparations (Figure 3c, peak 1) with ($\blacklozenge$) and without ($\diamond$) β -elimination and HNO_2 -treated material with ($\bullet$) and with ($\circ$) β -elimination. Eluent: 6M urea/10 mM Tris/HCl/0.5 M NaCl, pH 5.8; V_0 19 ml, V_i 53 ml. (For further details, see the Materials and methods section.)

synthesized *de novo*. Notably, the peak 2:peak 1 ratio increased significantly when GAGs were detected by DMB analysis versus ^{35}S measurements.

Materials derived from casein-treated mice eluted as two peaks; the first (peak 1) appearing polydispersely at 0.67 M NaCl, and the second (peak 2) eluting at 0.75 M NaCl (Figure 3b), an elution pattern resembling that previously found for a variety of human amyloids [8]. The corresponding normal control extracts also eluted as two peaks, peak 1 at a slightly lower ionic strength (0.63 M NaCl) (Figure 3c). However, the relative amount of ^{35}S -labelled material eluting in the respective peaks varied when comparing the amyloidotic and normal tissue extracts. Peak 2 approximated to 42% of the total amyloid-derived material, as contrasted to 25% of the total material obtained from the normal control extract (Figure 3c). Notably, peak 2 from aCFA-treated mice, subjected to repetitive radio-labelling, contained significantly less material (approx. 20%) than the corresponding peak from casein treatment.

DEAE-Sepharose peak 1-derived polysaccharides

^{35}S pulse-labelling

On Sepharose CL-6B it was demonstrated that about half of the material (Figure 3a, peak 1) obtained from heavily amyloidotic spleens (10 weeks of aCFA treatment), depolymerized on β -elimination, represent PGs (Figure 4a). The presence of an HSPG and free HS chains (Figure 4a) was disclosed by chondroitinase ABC digestion. Chondroitinase AC treatment revealed a similar profile (not shown). The PG was re-chromatographed, with and without prior β -elimination (Figure 4b) visualizing the purified PG with a K_{av} of 0.23–0.25 and HS

chains with a K_{av} between 0.45 and 0.49. According to protein molecular-mass standards the HSPG had a molecular mass of approx. 250 kDa. Complete depolymerization of the HS chains was accomplished by HNO_2 treatment (results not shown).

Following HNO_2 treatment of materials in peak 1 (Figure 3a), two closely eluting peaks emerged; a chondroitin sulphate (CS) PG and free CS chains (Figure 4c), the latter quantitatively in excess. The CSPG fraction obtained was also re-chromatographed on Sepharose CL-6B following dialysis and lyophilization. Untreated aliquots eluted at K_{av} 0.31 and alkaline-borohydride-treated aliquots released free GAG chains with K_{av} 0.41 (Figure 4d).

Gel filtration of the materials from pre-amyloidotic spleens (2 days aCFA treatment) is shown in Figure 4(e). A rather polydisperse peak (K_{av} 0.53) appeared on CL-6B. The same results were obtained with materials derived from early-phase amyloid extracts as well as normal controls. Subjected to HNO_2 treatment 15–20% of the polysaccharides depolymerized and alkaline borohydride treatment did not change the elution profile, indicating the presence of free CS chains (Figure 4e) (confirmed by chondroitinase ABC digestion).

The pre-amyloidotic HS fractions eluted at K_{av} 0.32 and 0.62 respectively (Figure 4f), representing an HSPG of lower molecular mass compared with that found in extracts of heavily amyloidotic spleens (Figure 4b). Additionally, minute amounts appeared in V_0 , but were not studied further. Following β -elimination, the HSPG was replaced by a peak at K_{av} 0.62 (Figure 4f).

A smaller HSPG, obtained from chondroitinase ABC-digested normal controls, occurred at K_{av} 0.40 before β -elimination (Figure 4f), accounting for approx. 30% of the HS fraction. By re-extracting the trichloroacetic acid-precipitable pellets from

Table 2 Distribution of ^{35}S -labelled macromolecules in extracts of normal and amyloidotic mouse spleens

Table 2 summarizes the distribution between accumulated ^{35}S -labelled glucosaminoglycans (HS) and ^{35}S -labelled galactosaminoglycans (CS/DS) obtained from advanced amyloidotic mouse spleens. The organs were extracted with 4 M guanidine in the presence of protease inhibitors, purified by trichloroacetic acid precipitation and subjected to ion-exchange chromatography. Characterization was performed utilizing chemical and enzymic treatments as described in the Materials and methods section.

Origin of tissue extracts	Glucosaminoglycans		Galactosaminoglycans	
	PG (%)	GAG (%)	PG (%)	GAG (%)
Amyloidotic mouse spleen	20	5	15	60
Normal mouse spleen	—	23	—	77

Figure 5 Sepharose CL-6B elution profiles eluting in peak 2 on ion-exchange chromatography

(a) HNO_2 -treated materials from amyloidotic organs (Figure 3a) with ($\blacklozenge$) and without ($\diamond$) β -elimination. (b) Digestion with chondroitinase ABC ($\diamond$), chondroitinase ACII ($\blacklozenge$) and chondroitinase B digestion ($\triangle$). (c) Pre-amyloidotic preparation untreated ($\diamond$) and chondroitinase AC II-digested ($\blacklozenge$). (d) HNO_2 -treated materials from normal organs radiolabelled for 2 h ($\triangle$) and repetitively with ($\blacklozenge$) and without ($\diamond$) β -elimination. (Eluent: 6 M urea/10 mM Tris/HCl/0.5 M NaCl, pH 5.8; V_0 19 ml; V_i 53 ml.)

normal controls, a relatively higher amount of the HS fraction occurred as PGs, a feature not exhibited with the amyloid-derived extracts.

Accumulated ^{35}S -labelling

The galactosaminoglycans in peak-1 aliquots from severely amyloidotic spleens (Figure 3b) eluted as two major peaks on gel

filtration (Figure 4g). The first eluted as the CSPG described above (Figure 4d) (K_{av} 0.31), with CS chains eluting at K_{av} 0.41, whereas the larger peak (K_{av} 0.62) appeared as free GAG chains (Figure 4h), depolymerizing in the presence of both chondroitinases ABC and ACII, thus representing CS of lower molecular mass.

Approx. 25% of the total material eluting from DEAE-Sephacrose chromatography was glucosaminoglycans (HS), as demonstrated by HNO_2 -susceptibility (Table 2). When treated with chondroitinase ABC, most of the ^{35}S -labelled HS accumulating in amyloid occurred as PGs (K_{av} 0.23–0.25) (Figure 4i). Thus, the HSPGs synthesized in severely amyloidotic organs do not tend to decrease in size during the course of amyloidogenesis.

Approx. 20% of the accumulated [^{35}S]polysaccharides from normal controls (Figure 3c) appeared to be HS in the form of free GAG chains (K_{av} 0.55–0.65) (Figure 4j). No HSPGs could be traced upon repetitive radiolabelling using this protocol. The galactosaminoglycans, susceptible to chondroitinase ACII digestion (not shown), also occurred in the form of free GAG chains (K_{av} 0.62) (Figure 4j).

DEAE-Sephacrose peak 2-derived polysaccharides

Polysaccharides from amyloidotic animals, radiolabelled for 2 h or repetitively for 2 weeks (Figures 3a and 3b), eluted as one main peak at K_{av} 0.41 on Sepharose CL-6B (Figure 5a). Following β -elimination the K_{av} did not change. When subjected to chondroitinase ABC, all of the material depolymerized whereas chondroitinase ACII treatment revealed a relatively low enzyme susceptibility (Figure 5b). Chondroitinase B treatment of this material is displayed in the same panel, showing the appearance of relatively long digestion fragments in addition to oligo- and disaccharides, indicating the presence of a dermatan sulphate (DS)/CS co-polymeric structure.

The corresponding material obtained from normal controls, pre-amyloidotic tissue and early-phase amyloid had the same size as that from massive amyloid deposits (K_{av} 0.41) and depolymerized upon chondroitinase ABC treatment (results not shown). However, the free GAGs were less resistant to chondroitinase ACII treatment (Figure 5c). Also, elution patterns of chondroitinase-digested normal control preparations disclosed the similarity with the DS/CS chains found in the pre-amyloidotic and early amyloid deposition phase (not shown). However, the GAG chains obtained from repetitive radiolabelling of the normal controls appeared to have a significantly reduced molecular mass (K_{av} 0.55) (Figure 5d) when compared with those obtained from amyloidotic extracts, as well as normal preparations obtained after 2 h of ^{35}S pulse-labelling (K_{av} 0.41).

In Table 2, the relative distribution between accumulated [^{35}S]PGs and [^{35}S]GAGs (from peaks 1 and 2 respectively) found in extracts of amyloidotic and normal spleens is summarized.

SDS/PAGE and Western blotting

Materials eluting in peak 1 after DEAE-Sephacrose isolation of casein-induced amyloidotic spleens (Figure 3b) were run preparatively on a Sepharose CL-6B column, and the PG fractions pooled, exhaustively dialysed, lyophilized and subjected to SDS/PAGE. Electrophoresis of materials recovered after Sepharose CL-6B gel filtration revealed one major PG with molecular mass 180–190 kDa, as well as a smear with extremely disperse migration characteristics (Figure 6a, lane 1). Following chondroitinase ABC digestion all of the latter disappeared, leaving the HSPG (lane 2). The DS preparation obtained from HNO_2

Figure 6 SDS/PAGE analysis of (a) peak 1-derived materials (Figure 3b) and (b) isolated amyloidotic HSPG, recovered after chondroitinase ABC treatment and subsequent Sepharose CL-6B gel filtration

(a) Fractions containing proteoglycans were pooled after preparative Sepharose CL-6B gel filtration, exhaustively dialysed and lyophilized. Lane 1, untreated sample; lane 2, chondroitinase ABC-digested material. Materials obtained from peak 2 (Figure 3b) following HNO_2 -treatment are displayed in lane 3. Electrophoresis was carried out horizontally, employing long 3–12% gradient gels. (b) Lane 1, HNO_2 -depolymerized sample; lane 2, untreated sample. Mini-electrophoresis was performed employing precast 4–15% linear gradient gels.

depolymerization of peak 2 (Figure 3b) migrated as a smear corresponding to a molecular mass between 50 and 70 kDa (lane 3).

Figure 6(b) shows the SDS/PAGE analysis of isolated amyloidotic HSPG preparation (Figure 4b) recovered after chondroitinase ABC treatment and subsequent Sepharose CL-6B gel filtration. HNO_2 -depolymerized and untreated samples are shown (lanes 1 and 2 respectively). Following depolymerization of the HS side chains, a core protein with a molecular mass of 70 kDa occurred (lane 1). A control experiment employing heparitinase as a substitute for HNO_2 revealed the same remarkably clear 70 kDa band (results not shown). This band was subjected to Western blotting with the anti-(human fibroglycan), anti-(human syndecan) and anti-(BM HSPG) antibodies. However, all failed to react with the isolated core protein (results not shown).

Summary

Using *in vivo* radiolabelling, amyloidogenic stimulation clearly increased the synthesis of splenic PGs. This increment appeared before fibril deposition. Galactosaminoglycans accounted for 70–80 % of splenic PG/GAG synthesis at the different stages of amyloid formation. In normal, pre-amyloidotic and early-phase amyloidotic organs, the labelled polysaccharides appeared mainly as free GAG chains, indicating a very rapid turnover of PGs in splenic tissue. In severely amyloidotic tissue, however, the presence of significant quantities of intact PGs was demonstrated. The PGs were a 200 kDa HSPG with a 70 kDa core protein and a smaller CSPG respectively. Using multiple ^{35}S injections, it appeared that free, relatively high-molecular-mass DS/CS chains accumulated in the tissue during amyloid deposition, and that

their co-polymeric structure changed in the course of amyloidogenesis, favouring DS structure.

DISCUSSION

In the present investigation, [^{35}S]polysaccharides, pulse-labelled *in vivo*, were extracted from mouse spleens at different stages during the course of reactive amyloidogenesis. In addition, repetitive ^{35}S injections were administered during the period when most of the amyloid was deposited, providing the opportunity to characterize the PGs and GAGs accumulating in amyloid. Exhaustive induction protocols were employed with intent to obtain seriously afflicted tissues, mimicking the pathology of those observed in humans dying from advanced AA amyloidosis [8,9].

It seems that normal splenic tissue exhibits a very rapid turnover of PGs as only very limited amounts of intact PGs could be traced. Consequently, it is possible that PGs are synthesized and almost immediately internalized or shedded and decomposed, thus releasing free GAG chains. The distribution between galactosaminoglycans and glucosamines was approx. 3:1 for both amyloidotic and normal control organs (Table 2), a proportion which has been suggested previously [29]. Moreover, splenic parenchyma do not contain much extracellular matrix/connective tissue, although haematopoietic and immunological cells, which are known to synthesize and contain CS/DS, are abundant [30].

In extracts of heavily amyloidotic spleens two principal PG populations appeared to be produced and accumulated. First, a small CSPG was found in advanced amyloid. Secondly, a 200 kDa HSPG occurred, possessing a core protein of 70 kDa and HS chains of about 30 kDa. The molecular mass is approximately the same as the (bovine vascular) BM HSPG [31] and those previously isolated from various human amyloids, which have core proteins displaying immunological identity [8,9]. Moreover, neuroblastoma-derived DSPGs and HSPGs of identical size also seem to cross-react immunologically [32], suggesting the presence of closely related core protein which may be substituted with either HS or DS/CS residues. Definite conclusions should not be drawn as to the nature of this HSPG as none of the antisera in this study reacted with the core protein on Western blots. However, to our knowledge no reports have previously been carried out aimed at a biochemical characterization of PGs derived from splenic tissue, regardless of species, so the PG might even represent a hitherto undescribed molecule.

Free DS chains obviously accumulate in amyloid deposits, comprising about 40 % of the accumulated ^{35}S -labelled macromolecules, and hence exceeding the amount synthesized at different stages during amyloid development, as well as that accumulating in normal spleens. However, the DS/CS:HS ratio did not differ significantly in normal and amyloidotic spleens (Table 2), indicating that increased epimerase activity might also be involved during development of reactive amyloidosis. Some variation in DS quantity, comparing preparations from adjuvant- and casein-treated animals, could depend on real differences in GAG distribution due to different stimuli. However, most of the amyloid deposited in casein-challenged mice occurred after 10 weeks, as verified by partial splenectomy. Thus it seems plausible that the observed variations might also reflect different phases of amyloid deposition.

Noteworthy, the results are in agreement with those obtained with various human amyloids [8–11], indicating that free DS/CS chains are major polysaccharide components of amyloid deposits. Moreover, the occurrence of highly sulphated DS during amyloidogenesis has previously been described for Balb/C mice [33].

Also, the presence of free GAG chains, especially in cell cultures, has been reported [34].

Several investigators have failed to detect CSPG/DSPGs in amyloid deposits by immunohistochemistry [8,35,36]. Intriguingly, the sole exception is the presence of anti-decorin immunostaining in the peripheries of Alzheimer's β -amyloid [37], which might support the hypothesis that DSPGs are co-deposited with amyloid fibrils initially, subsequently releasing free DS chains which strongly associate with the fibrils.

In the present investigation, only a minute fraction of the total material had a molecular mass comparable with that of intact perlecan [38]. Although this low-density BM HSPG has previously been reported to be converted into a high-density variant [39], the present results, as well as preliminary results utilizing shorter labelling intervals, do not suggest that the present HSPG is derived from a larger precursor.

The fact that smaller HSPG variants appeared in the normal, pre-amyloidotic and early-phase amyloidotic tissue extracts might be explained by partial degradation of HSPGs, a process which seems to be more rapid in the normal state. It might also be a reflection of altered synthesis during disease resembling the effects of transforming growth factor- β stimulation on cultured cells, hence producing larger PGs [40].

The significance of PGs in amyloid fibrillogenesis is still poorly understood. Such molecules definitely accumulate during disease development, but the present work shows for the first time evidence that the *in vivo* production of sulphated polysaccharides is actually enhanced. Thus, it seems that increased GAG quantities in amyloids do not merely depend on a passive accumulation depending on an altered catabolism of PGs. The results thus support the work of Linker and Carney [29] who reported an increased ^{35}S incorporation in GAGs obtained from cultures of tissue slices of amyloidotic versus normal organs. The fact that the increased PG synthesis actually precedes the deposition of amyloid fibrils in the extracellular matrix is also suggested from previous histochemical investigations [41] in addition to the recent demonstration that perlecan gene expression is enhanced before amyloid formation [42]. The potential significance of such carbohydrates for amyloid formation is suggested by the conformational effect of GAGs on the mouse amyloid fibril precursor protein, SAA 2 [43]. Furthermore, HSPGs and laminin strongly interact with the β -protein precursor [44,45], and sulphate ions are shown to induce conformational changes for both the β -protein [46] and SAA-derived fragments [47].

To conclude, it thus seems that extracellular matrix elements, and especially PGs/GAGs synthesized *in situ*, may impart fibrillogenetic and/or fibril-stabilizing properties during fibril deposition. We believe that the polysaccharides may take an active part in the anchoring of fibril protein precursors to the site of deposition and their conversion into an insoluble fibrillar form.

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