Amyloid-specific Heparan Sulfate from Human Liver and Spleen*

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Heparan sulfate proteoglycans are consistently accumulated in tissues affected by amyloidosis and have been implicated in the mechanism of amyloid deposition. To study this relationship, heparan sulfate was isolated from liver and spleen of patients with AA amyloidosis and from normal organs and subjected to structural analysis. The polysaccharides were deaminated with nitrous acid, and the products were reduced with NaB\(_3\)H\(_4\) to yield labeled oligosaccharides. Disaccharides obtained by selective deamination of intact or N-deacetylated polysaccharides were separated and quantified by anion-exchange high performance liquid chromatography and thus defined the composition of N-sulfated block regions or the entire heparan sulfate chains, respectively. The heparan sulfate samples derived from liver or spleen with AA-type amyloidosis were all similar in composition, regardless of tissue source, but differed from either control material. These findings suggest that secondary amyloidosis is associated with the deposition in the affected tissues of a heparan sulfate with a specifically modified structure.

The deposition of proteins as insoluble amyloid fibrils in various organs is characteristic of a diverse set of medical disorders. In Alzheimer’s disease, the polypeptide of 40–42 amino acid residues, the A\(_\beta\) protein, is the main constituent of amyloid in the cerebral parenchyma and vasculature (1, 2). AA amyloidosis, related to chronic infections and some malignant tumors, is characterized by the accumulation of the AA polypeptide, which is derived from SAA, a normal acute phase protein (3–6). The occurrence of heparan sulfate (HS)\(^3\) glycosaminoglycan is a consistent and early finding in amyloid lesions, and the polysaccharide is therefore believed to participate in the process of amyloid deposition (7–11). HS chains that usually occur covalently bound to proteins in proteoglycan structures are composed of alternating units of hexuronic acids, N-glucosamine (GlcN). The GlcN residues are either N-acetylated or N-sulfated, and in addition O-sulfate groups occur at various positions. The substitution pattern of the polysaccharide chain is highly variable and thus accounts for the expression of HSs of different structure in different tissues (12–14) and, indeed, in different cells from the same tissue (15, 16). It is generally assumed that HSs exert their biological effects largely through interactions with proteins and that these interactions are strongly dependent on the structural properties of the HS ligand (for review see Ref. 17).

HS was previously implicated with the pathogenesis of Alzheimer’s disease through the demonstration of the polysaccharide in the characteristic amyloid plaques of cerebral cortex (18). However, the amounts of HS in diseased cortex were found to be only marginally increased over those in control tissue. Furthermore, analysis of HS isolated from normal and diseased cerebral cortex failed to demonstrate any significant structural difference (13).

Contrary to Alzheimer’s disease, secondary amyloidosis shows dramatically increased amounts of HS in afflicted organs, such as kidney, liver, and spleen (9, 19). In the present study we compared the O-sulfation patterns of HS from liver and spleen of patients with AA amyloidosis with those of HS from the corresponding healthy organs. Unexpectedly, all amyloid HS samples, regardless of tissue source, showed highly similar composition, which differed from that of any of the control polysaccharides. These findings point to the generation of a novel HS species that is associated with the amyloidotic disease.

EXPERIMENTAL PROCEDURES

Isolation and Purification of HS—Tissue samples from amyloidotic liver and spleen were obtained at autopsy from two patients with secondary amyloidosis. The first patient (designated A1) died at the age of 12 from AA amyloidosis associated with juvenile rheumatoid arthritis. The second patient (designated A2) died at the age of 13 from AA amyloidosis secondary to mesothelioma. Control samples of normal liver and spleen were derived from a 3-year-old (C1) and a 35-year-old (C2; spleen only), both dead by accident. HS was isolated from the tissues essentially as reported (13). Tissue samples of ~5 g (wet weight) were homogenized in 5 volumes of ice-cold acetone with a Turrax homogenizer and were then subjected to lipid extraction and proteolytic digestion with Pronase and papain. The sulfated polysaccharides were recovered by anion-exchange chromatography as described (13), except that the column was eluted in one step, with 2 M LiCl in 0.05 M sodium acetate, pH 4.0, after having been washed with 0.2 M LiCl in the same buffer. The material recovered in the high-salt fraction was digested with chondroitinase ABC and with nuclease, and enzyme-resistant polysaccharide (HS) was isolated by gel chromatography (5). The recovered HS was quantified by carbazole analysis for hexuronic acid (20).

Structural Analysis of HS—The isolated HS samples were subjected to compositional analysis, following degradation by either one of two different procedures (see also Ref. 14). In one approach, the polysaccharide was cleaved at N-sulfated GlcN units by treatment with HNO\(_3\) at pH 1.5, and the resultant deamination products were reduced with NaB\(_3\)H\(_4\). The recovered \(^3\)H-labeled disaccharides represent N-sulfated block structures in the intact polymer. A polysaccharide sample of ~20 \(\mu\)g was initially treated with 5 \(\mu\)mol (unlabeled) of NaB\(_3\)H\(_4\) for 3 h to block any reducible group associated with the intact polymer. The product was recovered by gel chromatography (Sephadex G-25 (PD-10 column; Pharmacia Biotech) in 0.2 M NH\(_4\)HCO\(_3\)), and the excluded polysaccharide was quantified by the carbazole reaction. A sample of ~5 \(\mu\)g of reduced HS was deaminated with 200 \(\mu\)l of HNO\(_3\) reagent at pH 1.5, and the reaction products were reduced with 0.5 mCi of NaB\(_3\)H\(_4\) (20–30 Ci/mmol; Amersham International) (21). The resultant \(^3\)H-labeled oligosaccharides were recovered by gel chromatography on a...
The yield of HS was determined following enzymatic elimination of other glycosaminoglycans (see "Experimental Procedures") and is given as mg of hexuronic acid per g of dry defatted tissue.

The isolated HS samples were cleaved at N-sulfated GlcN units by treatment with HNO₂ at pH 1.5, and the products were reduced with NaB³H₄. Gel chromatography of the resultant labeled oligosaccharides on Bio-Gel P-10 showed prominent disaccharide fractions recovered for further separation by anion-exchange HPLC. The molecular size of the separated oligosaccharides is indicated by the number of monosaccharide units/molecule, as shown above the peaks. In preparative runs the disaccharide fractions were recovered and lyophilized before further analysis. The patterns pertaining to HS from control (shown in the figure) and from amyloidotic liver were indistinguishable.

**RESULTS**

Sulfated glycosaminoglycans were isolated from tissue specimens of liver and spleen by a procedure involving proteolysis of tissue and anion-exchange chromatography (see "Experimental Procedures"). Galactosaminoglycans (chondroitin sulfates and dermatan sulfate) were eliminated by digestion with chondroitinase ABC, followed by gel chromatography. Quantitative analysis of the residual HS, using the carbazole reaction for hexuronic acid, showed 20–30-fold increased amounts of the polysaccharide in amyloidotic liver compared with control tissue (Table I). Similar although less marked changes were found in spleen tissue.

The isolated HS samples were cleaved at N-sulfated GlcN units by treatment with HNO₂ at pH 1.5, and the products were reduced with NaB³H₄. Gel chromatography of the resultant labeled oligosaccharides on Bio-Gel P-10 showed prominent peaks of disaccharides (derived from consecutive N-sulfated disaccharide units in the intact polysaccharide) and tetrasaccharides (alternating N-sulfated and N-acetylated disaccharide units) and in addition smaller amounts of 6- to ~18-mers (increasing numbers of consecutive N-acetylated disaccharide units) (Fig. 1). Quantitation of N-sulfate/N-acetyl ratios, based on peak areas, showed a slightly higher degree of N-sulfation for spleen HS (~58% of total disaccharide units) than for liver HS (~56% of total disaccharide units). No apparent difference was seen between HS samples derived from amyloidotic and normal tissues of the same type (data not shown).

The same HS samples were analyzed with regard to distribution of O-sulfate groups in N-sulfated block structures as well as in the total polysaccharide chains. The composition of N-sulfated sequences was determined by anion-exchange HPLC of the labeled disaccharides isolated after deamination at pH 1.5 and NaB³H₄ reduction (illustrated in Fig. 2, A and C). The procedure showed excellent reproducibility, the proportions of each disaccharide generally varying by <2% in analysis of different HS preparations from the same tissue specimens (data not shown). The overall disaccharide composition, including also N-acetylated disaccharide units outside the N-sulfated block structures, was obtained by similar analysis of products generated by N-deacetylation followed by deaminative cleavage of all glucosaminidic linkages (Fig. 2, B and D). The disaccharides obtained by total deamination were consistently enriched in the mono-6-O-sulfated species, GlcA-aManR(6-OSO₃) and IdceA-aManR(6-OSO₃) (Figs. 3, A and B), confirming previous findings that 6-O-sulfate groups occur to a large extent outside the N-sulfated blocks (14). Together, the disaccharide distribution profiles generated by the two procedures provide an O-sulfation “fingerprint” pattern for each HS sample, which reflects the total distribution of O-sulfate groups as well as their relation to N-sulfated contiguous domains (Fig. 3).

Remarkably, analysis of HS derived from normal spleen indicated that whereas the various O-sulfated disaccharide units occurred in highly different proportions, the amounts of a given disaccharide varied by only a few percent between samples from two individuals. This agreement applied both to the N-sulfated block structures (Fig. 3A) and to the total chains (Fig. 3B). Similarly, samples of HS from amyloidotic spleen, derived from two different individuals, were almost identical in composition (Fig. 3). However, the O-sulfation profiles of the amyloid HS samples differed clearly from those of the control polysaccharides. Thus, one of the deamination disaccharide products derived from N-sulfated blocks, GlcA-aManR(6-OSO₃) (designated GMS in Fig. 3A; this component corresponds to a -GlcA-GlcNSO₆(6-OSO₃) sequence in the intact polysaccharide), was about twice as abundant as in control material, whereas the most frequent disaccharide component, IdceA(2-OSO₃)-aManR (JSM in Fig. 3A), was decreased by ~15%. The total compositional analysis revealed more striking differences in the HS of amyloidotic liver compared with control tissue.

**Fig. 1.** Gel chromatography of oligosaccharides produced by low pH deamination of liver HS. HS samples were cleaved by reaction with nitrous acid (pH 1.5), and the resultant oligosaccharides were reduced with NaB³H₄ and subjected to mild acid treatment (25 mm H₂SO₄, 80 °C, 30 min) to eliminate products of “anomalous” ring contraction (30). The final samples (500 × 10⁶ dpm ³H) were separated by gel chromatography on a column of Bio-Gel P-10, as described under “Experimental Procedures.” The molecular size of the separated oligosaccharides is indicated by the number of monosaccharide units/molecule, as shown above the peaks. In preparative runs the disaccharide fractions were recovered and lyophilized before further analysis. The patterns pertaining to HS from control (shown in the figure) and from amyloidotic liver were indistinguishable.

| Preparation* | Yield of heparan sulfateb | mg hexuronic acid/g |
|---------------|---------------------------|---------------------|
| Liver         |                           |                     |
| C1 (3)        | 0.2                       |                     |
| A1 (12)       | 6.0                       |                     |
| A2 (13)       | 5.2                       |                     |
| Spleen        |                           |                     |
| C1 (3)        | 0.1                       |                     |
| C2 (35)       | 0.2                       |                     |
| A1 (12)       | 0.4                       |                     |
| A2 (13)       | 1.3                       |                     |

* C, control; A, amyloidosis. The age of each individual is indicated in parentheses.

b The yield of HS was determined following enzymatic elimination of other glycosaminoglycans (see “Experimental Procedures”) and is given as mg of hexuronic acid per g of dry defatted tissue.
and indicates the switch from 0.026 to 0.154 M KH$_2$PO$_4$ elution buffer.

Characterides from amyloid-laden liver and spleen, involving oligo-

results were apparent within as well as outside the block regions (Fig. 3, A and B). It is concluded that the HS species accumulated in the two diseased organs are similar but differ from those normally occurring in liver and spleen.

A previous survey of polysaccharides from amyloid-laden liver and spleen, involving oligo-

ments were less abundant and others more abundant. The differences were apparent within as well as outside the N-sulfated block regions (Fig. 3, A and B). It is concluded that the HS species accumulated in the two diseased organs are similar but differ from those normally occurring in liver and spleen.

DISCUSSION

Increased deposition of glycosaminoglycans, in particular HS, is commonly seen in organs affected by amyloidosis (see the Introduction) and was confirmed in the present study. A previous survey of polysaccharides from amyloid-laden liver and spleen, involving oligo-

saccharide mapping in gradient polyacrylamide gels following partial digestion with specific glycosidases, suggested similar structures for the HSs from the two different organs (24). Indeed, the results of the present, more detailed, structural investigation point to the formation of a “novel,” amyloid-specific HS with a composition different from those of the corresponding polysaccharides in control organs. Analysis of the four samples of HS isolated from amyloid-laden organs, two each from liver and spleen from two different individuals, thus gave strikingly similar compositional data (Fig. 3). These deviated from those relating to all control polysaccharides regardless of tissue source. Notably, the structural properties of the amyloid-associated HS appear unrelated to the underlying primary disease as one of the patients suffered from rheumatoid arthritis and the other from mesothelioma. There are few previous reports of any clinical condition being associated with a change in HS structure, and to the best of our knowledge, there is no previous implication of any HS species of defined composition. An overall decrease in sulfation was observed for HS in experimental diabetes in rats (25). Further, HS preparations obtained from transformed cells in vitro were found to differ in O-sulfation from the corresponding control polysaccharides (26–28).

Previous compositional analysis of HS from human organs pointed to the occurrence of organ-specific structural patterns (13). The present characterization of HS from normal spleen conformed to these previous findings, as the two samples were

![Fig. 2. Anion-exchange HPLC of O-sulfated disaccharides obtained by deaminative cleavage of HS samples followed by reduction with NaB$_3$H$_4$. All four chromatograms relate to liver HS; A and B, control sample (C1; see Table I); C and D, amyloidotic sample (A1). A and C show the disaccharides recovered following HNO$_2$/NaB$_3$H$_4$ treatment at pH 1.5. In this procedure contiguous N-sulfated regions are degraded to labeled disaccharides (with conversion of GlcNOSO$_3$ units in the intact polymer into $[^{3}$H]Man$_R$ residues). B and D show the disaccharides obtained by N-deacetylation of the HS chain, followed by deaminative cleavage of N-sulfated (pH 1.5) as well as N-unsubstituted (pH 3.9) GlcN units. The various peaks are identified by reference to standard disaccharides: GSM, GlcA(2-OSO$_3$)-aMan$_R$; GMS, GlcA-aMan$_R$(6-OSO$_3$); IMS, IdceA-aMan$_R$(6-OSO$_3$); ISM, IdceA(2-OSO$_3$)-aMan$_R$; ISMS, IdceA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$). The asterisk indicates the switch from 0.026 to 0.154 x KH$_2$PO$_4$ elution buffer. For further information see “Experimental Procedures.”](image)

![Fig. 3. Composition of heparan sulfate samples. Samples of HS isolated from human spleen or liver were deaminated, either (A) directly at pH 1.5 or (B) at pH 1.5 and 3.9 following N-deacetylation, and the products were reduced with NaB$_3$H$_4$. Labeled disaccharides were analyzed by anion-exchange HPLC, as illustrated in Fig. 2, and the composition of each sample with regard to O-sulfated disaccharide units was calculated. The designation of the various disaccharides is as described in the legend to Fig. 2. The data for each type of sample, except control liver HS, are derived from two different individuals. The corresponding separate analyses (open circles) are averaged as indicated by the bars; the values for control liver represent a single individual. Abbreviations are defined in the legend to Fig. 2.](image)
highly similar in composition (but differed from spleen amyloid HS; see above). The spleen specimens were obtained from 3- and 35-year-old individuals, suggesting that there was no significant age-related change in HS structure within this age range. The O-sulfation pattern of the HS preparation derived from normal liver deviated markedly from that of any of the other samples investigated from control or amyloidotic tissue (Fig. 3). Since only a single specimen of normal liver tissue was available we cannot draw any conclusion as to the general applicability of this pattern to other individuals. However, it seems reasonable to infer that the same unique amyloid-specific HS occurs not only in spleen but also in liver.

The mechanism behind the selective pathological accumulation of apparently specific HS is unclear. Current information, including immunohistochemical observations (15, 16), suggests that the HS isolated from composite tissues represents a mixture of proteoglycan pools, presumably involving several different core proteins (29). In healthy tissues these pools would seem to occur in essentially constant proportions. Conceivably, the amyloid deposition may lead to selective expansion of one such pool, with HS chains of a particular structure. It is thus notable that perlecan mRNA levels were found to increase significantly during the early stages of experimental murine AA amyloidogenesis (10). Alternatively, the biosynthetic machinery for HS chains in the tissue may be generally perturbed toward the production of a novel polysaccharide species. Yet another possibility is that the amyloid protein(s) may selectively bind to a particular HS structure and thus withdraw this species from normal turnover. The sequestered HS would escape degradation and hence accumulate. Finally, we cannot disregard the possibility that the HS is produced elsewhere in the body and is transported to the site of deposition with the blood. Further studies of the phenomenon will hopefully promote our understanding of the amyloidotic condition as well as of the intricate mechanisms in control of HS metabolism.

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