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GLYCOSAMINOGLYCAN IN LIVER AND SPLEEN OF CASEIN-INDUCED EXPERIMENTAL AMYLOIDOSIS OF MICE

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ABSTRACT

Hepatic and splenic amyloidosis was experimentally induced by casein injections in BALB/C mice and examined biochemically for glycosaminoglycan constituents. Heparan sulfate and dermatan sulfate were shown to be major components by electrophoresis on cellulose acetate membrane. It was demonstrated from the studies of DEAE-cellulose ion-exchanging chromatography that the amount of highly sulfated dermatan sulfate was increased in amyloid-laden liver and that the amount of highly sulfated chondroitin sulfate isomer was also increased in amyloid laden spleen. N-Acetyl-D-galactosamine 4-6 disulfate, L-Iduronic acid (chondroitin sulfate G or H) was shown to be increased in amyloid laden-liver by unsaturated disaccharide study with chondroitinase and chondrosulfatase digestion. These changes of glycosaminoglycan constituents may play an important role in the formation of amyloid.

Key words: Experimental amyloidosis, glycosaminoglycans, chondroitin sulfate isomer

INTRODUCTION

Amyloidosis is a disease of unknown etiology characterized by the deposition in tissues of unique amyloid substance. Amyloid substance consists of amyloid-specific substances and amyloid non-specific substances. Amyloid-specific substance is composed chiefly of proteins and has a fibrillar structure. Amyloid non-specific substance includes glycosaminoglycans (GAG), collagen, and reticulin, as well as fibrinogen, complement component and lipoproteins.[1] GAG constitutes the major part of the matrix substances in various tissues. As the pathophysiologic forces that are operative in various tissues and that determine the topographical distribution of the amyloid substance are as yet unknown, it is important to study more precisely the GAG constituents in amyloidosis.

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MATERIALS AND METHODS

Induction of amyloidosis

Inbred male BALB/C mice, 46 to 49 weeks old when sacrificed, were used. A solution of 0.3 ml of 10% (w/v) milk casein (Hammerstin) in 0.01M $NaHCO_3$ was subcutaneously injected in the back of each mouse 6 times per week. One group of mice were injected with casein for 12 weeks and another group of mice were injected for 24 weeks. Mice were sacrificed by cervical dislocation 24 hours after the last injection. The untreated control mice were not injected with casein.

Histochemical examination

A small part of the liver and spleen was fixed in 10% neutral formalin and embedded in paraffin using the routine process. Amyloid was identified by the fact that amyloid was stained with Congo-red and showed characteristic green birefringence under polarized light after staining.

Preparation of crude GAG

After taking a small specimen for histological examination, livers and spleens were rinsed in saline for one second and stored at −20°C. The organs were homogenized in acetone and defatted by repeated exchanges of acetone. The defatted sample was dried in a desiccator, then weighed.

The crude GAG from liver and spleen was prepared by Iwata's method.[2, 3] The acetone-dried sample was suspended in several times volume of 0.05M Tris-HCl buffer, pH 7.3 and was exhaustively digested with Pronase-P (Kaken Kagaku). Alkali elimination was done by incubation for 4 h at 37°C with a NaOH concentration of 0.3N. The mixture was neutralized by adding 6N HCl. Sixty percent trichloroacetic acid was added at a concentration of 5% and the mixture was allowed to stand at 0°C overnight. After centrifugation, the clear supernatant fluid was dialized against running tap water for 2 days. Crude GAG was precipitated by the additoin of 3 times volume of ethanol in the presence of 1% potassium acetate. After 24 hours at 0°C, the precipitate was collected by centrifugation and washed with 70% and 99% ethanol and ethyl ether. The precipitate was dried in a vacuum desiccator overnight.

The quantity of uronic acid was measured by Carbazole[4] and Orcinol[5] reaction.

Electrophoresis on cellulose film

Electrophoresis of GAG was carried out on 10cm square membrane of cellulose acetate (Sepraphore III) at a constant current of 1mA/cm for 90 min. The buffer system used was 0.1M pyridine/0.47M formic acid/water, pH 3.0. The stripes were stained with 1% Alcian blue in 2% acetic acid and washed with 2% acetic acid. Two dimentional electrophoresis was carried out at a constant current of 1mA/cm for 4.5 hours at room temperature, using 0.1M barium acetate solution (pH 8.0) after electrophoresis in 0.1M pyridine/0.47M formic acid.[6, 7]

DEAE-cellulose chromatography

Each GAG sample was dissolved in 0.02M Tris-HCl, pH 7.2 and applied to a DEAE-cellulose column (0.9 × 15.0 cm) equilibrated with the same buffer. After washing with the same buffer, the absorbent was eluted stepwise with 0.3M NaCl, 0.45M NaCl, 0.65M NaCl and 1.0M NaCl in the same buffer.[8] Each fraction was checked for uronic acid by Orcinol reaction. The peak fractions were separately pooled and dialyzed against distilled water. Each dialyzate was frozen and lyophilized. The GAG preparation of each fraction was measured by Carbazole and Orcinol reaction, followed by application to cellulose-acetate membrane electrophoresis. Standard chondroitin 4-sulfate, dermatan sulfate, and chondroitin 6-sulfate were eluted exclusively in the 0.65M NaCl fraction.

Unsaturated disaccharide assay

The crude GAG preparations from liver were applied to the unsaturated disaccharide assay after chondroitinase digestion.[2, 9] Crude GAG preparation was digested by chondroitinase-ABC (CHase ABC) and chondroitinase-AC (CHase AC) (Seikagaku Kogyo). The digests were applied to Toyo No. 51A filter paper and the unsaturated disaccharides were separated by descending development in n-butanol-acetic acid-1N ammonia (2 : 3 : 1, v/v). The disaccharides were stained by $AgNO_3$. The parts containing disaccharide were cut off and the disaccharides were eluted in distilled water, followed by application to the chondrosulfatase assay.

Chondrosulfatase Digestion of Unsaturated Disaccharide

In order to determine the position of sulfate residue, the unsaturated disaccharides were digested by chondro-4-sulfatase, chondro-6-sulfatase (Seikagaku Kogyo Co.) and chondro-4-sulfates plus chondro-6-sulfatase.[9] The reaction mixtures were concentrated and subjected to the descending paper chromatography described earlier.

RESULT

Induction of hepatic and splenic amyloidosis

None of the untreated control group had hepatic or splenic amyloidosis. Out of 19 mice subjected to 12 weeks of casein injections had hepatic amyloidosis and 3 had splenic amyloidosis. Twelve out of 22 mice had both hepatic and splenic amyloidosis after 24 weeks of injections. The mice were divided into five groups, excluding those mice with splenic amyloidosis but without hepatic amyloidosis: (1) untreated control group without hepatic or splenic amyloidosis; (2) group without amyloidosis after 12 weeks of casein injections; (3) group with amyloidosis after 12 weeks of casein injections; (4) group without amyloidosis after 24 weeks of injections; and (5) group with amyloidosis after 24 weeks of injections.

Measurement and characterization of crude GAG

There were no significant differences in the amout of uronic acid of crude GAG among the five groups in either livers or spleens.

Two-dimensional electrophoresis on cellulose acetate membrane showed the presence of two major components in crude GAG from liver in both the amyloid group and non-amyloid groups. One of the components had mobility identical to that of dermatan sulfate and was persistent after chondroitinase-AC digestion prior to electrophoresis but degradated by chondroitinase-ABC digestion. Another component had mobility identical to that of heparan sulfate and was persistent after chondroitinase ABC digestion (Data is not shown.). Electrophoresis in 0.1M pyridine/0.47M formic acid showed the presence of 3 and 4 components in crude GAG from non-amyloidotic and amyloidotic spleen, respectively. One component common to all groups had mobility almost identical to that of heparan sulfate and was persistent after chondroitinase ABC digestion. Another component common to all groups had mobility almost identical to that of dermatan sulfate and was persistent after chondroitinase AC digestion but degradated by chondroitinase ABC digestion. Another component, found only in the amyloid groups, with the greatest mobility was degradated by chondroitinase AC digestion.

Fractionation of crude GAG on DEAE-cellulose

Crude GAG preparations derived from liver and spleen were applied to the DEAE ion-exchanging chromatography. GAG of each fractions was measured for uronic acid and characterized by electrophoresis on cellulose acetate membrane.

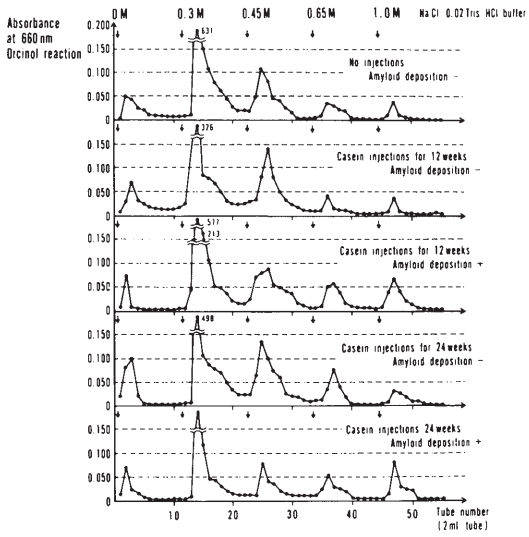

Fig. 1 DEAE-cellulose chromatography of crude GAG fraction from liver. In 1.0M NaCl fraction, the peaks of elution curves were higher in amyloid groups than in non-amyloid groups.

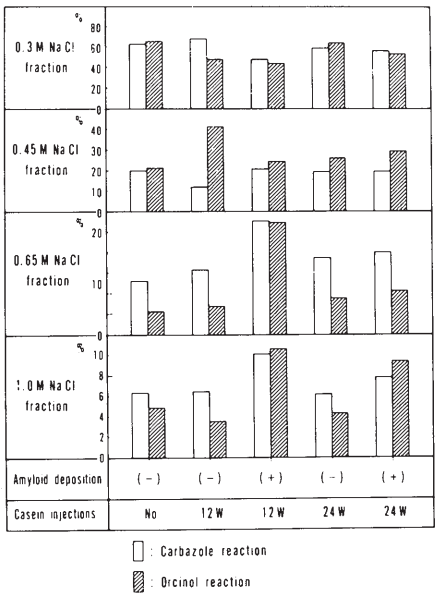

Fig. 2 DEAE-cellulose chromatogram of crude GAG fraction from liver. The relative quantity of GAG in fractions by uronic acid measurement (Orcinol reaction) is shown. In 1.0M NaCl fraction, it was 5.0% in untreated control group, and 3.7% and 4.4% after 12 and 24 weeks casein injection, respectively, in non-amyloid groups. In amyloid groups, it was 10.6% and 9.5% after 12 and 24 weeks injection, respectively.

The peaks of the elution curves for the 0.1M NaCl fraction of the GAG preparations derived from liver were higher in amyloid groups than in non-amyloid groups (Fig. 1). In non-amyloid groups, the relative amount of uronic acid by Orcinol reaction in 1.0M NaCl fraction was 5.0% in the untreated control group, and 3.7% and 4.4% after 12 and 24 weeks of injections, respectively. In the amyloid groups, these values were 10.6% and 9.5% after 12 and 24 weeks injections, respectively (Fig. 2). Electrophoresis on cellulose acetate membrane showed the presence of a component in 1.0M NaCl fractions in all groups with greater mobility than dermatan sulfate. This component was degradated by chondroitinase ABC digestion but not by chondroitinase AC digestion.

For spleen, the peak of the elution curve in 1.0M NaCl fraction was also higher in the amyloid group than in the non-amyloid groups (Fig. 3). In 1.0M NaCl fraction, relative amout of uronic acid by Orcinol reaction was 8.7% in the untreated control group. It was 5.0% and 6.2% after 12 and 24 weeks of injections, respectively, in the non-amyloid groups, whereas, in the amyloid group, was 22.4% after 24 weeks injection (Fig. 4). Electrophoresis on

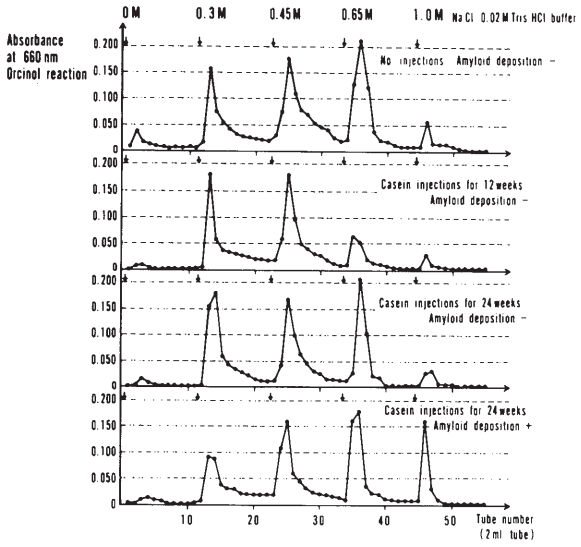

Fig. 3 DEAE-cellulose chromatography of crude GAG fraction from spleen. In 1.0M NaCl fraction, the peak of elution curve was higher in the amyloid group than in the non-amyloid groups.

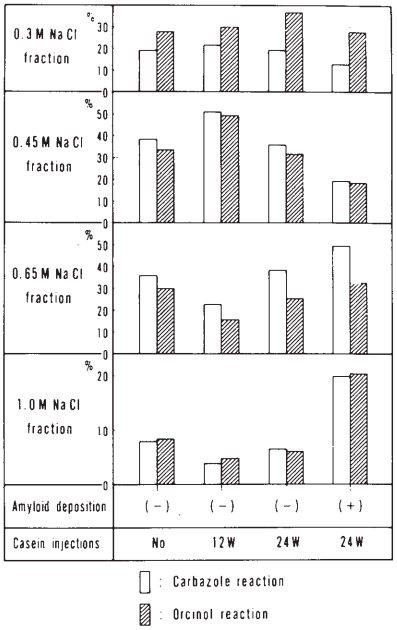

Fig. 4 DEAE-cellulose chromatogram of crude GAG fraction from spleen. The relative quantity of GAG in fractions by uronic acid measurement (Orcinol reaction) is shown. In 1.0M NaCl fraction in the untreated control group, 5.0% and 6.2% after 12 and 24 weeks of casein injections, respectively, in non-amyloid groups. In the amyloid group, it was 22.4% after 24 weeks of casein injections.

cellulose acetate membrane showed the presence of one and two components of GAG in 1.0M NaCl fraction in the non-amyloid groups and in the amyloid group after 24 weeks of injections, respectively. One component common to each group had greater mobility than standard dermatan sulfate and was degradated by chondroitinase ABC digestion but not by chondroitinase AC digestion. Another component detected only in the amyloid group had greater mobility than chondroitin 4-sulfate and chondroitin 6-sulfate and was degradated by chondroitinase AC digestion.

Unsaturated disaccharide assay

The crude GAG preparations from liver were applied to the unsaturated disaccharide assay after chondroitinase digestion. The chromatogram showed the presence of a component moving slower than ΔDi-6S in the chondroitinase ABC digests in each group (A band). This component was not shown in the chondroitinase AC digests. Eluted degradation product in 0.01N HCl from the position of A band was measured in absorbance at 232nm. The value was

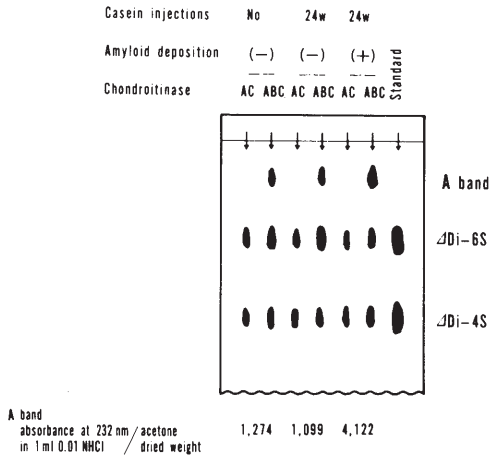

Fig. 5 Unsaturated disaccharide assay of crude GAG from liver after chondroitinase digestion. Paper chromatogram of the chondroitinase digests. A component slower than ΔDi-6S (A band) was recognized afer chondroitinase ABC digestion but not after chondroitinase AC digestion. The absorbance at 232nm of degradation products eluted from the position of A band in 0.01 M HCl was larger in the amyloid group after 24 weeks of injections than in the non-amyloid groups.

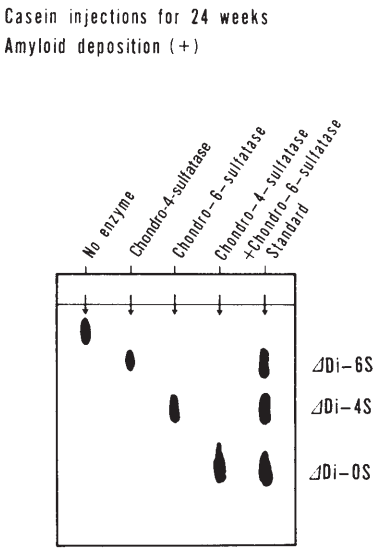

Fig. 6 Paper chromatogram of the chondrosulfatase digests of disaccharide eluted from the position of A band. The disaccharide was degradated into ΔDi-6S by chondro-4-sulfatase digestion, into ΔDi-4S by chondro-6-sulfatase digestion and into ΔDi-0S by condro-4-sulfatase plus chondro-6-sulfatase digestion.

calculated as the degradation product in 1ml of 0.01N HCl per 1g of acetone-dried-liver. In the non-amyloid groups, the value was 1.27 in the untreated control group and 1.10 after 24 weeks of injections. In amyloid group its value was 4.12 after 24 weeks of injections (Fig. 5).

Chondrosulfatase digestion of unsaturated disaccharide

Unsaturated disaccharide eluted from the position of A band was applied to paper chromatography after chondrosulfatase digestion.

Chondro-4-sulfatase digestes moved at the same rate as ΔDi-6S. Chondro-6-sulfatase digests moved at the same rate as ΔDi-4S. Degradation products by chondro-4-sulfatase plus chondro-6-sulfatase had the same mobility as that of ΔDi-0S (Fig. 6).

DISCUSSION

In our study, the amout of GAG did not increase significantly in amyloidotic livers and spleens in the studies of uronic acid measurement. This may be due to the low grade of amyloid deposition.

In regard to the constituents of GAG, it was reported that the increase of GAG was attributable chiefly to heparan sulfate in human amyloidotic liver.[10] Another study proved that dermatan sulfate and heparan sulfate were the major constituents of the liver in human systemic amyloidosis of non-AA type in experimental rabbit amyloidosis induced by casein, and in murine amyloidosis induced by AgNO.[11] In our study, electrophoretic characterization demonstrated that not only heparan sulfate but also dermatan sulfate were included among the major components of GAG from liver as well as from spleen in mice. It was reported, from a study of liver and spleen with human primary amyloidosis, that heparan sulfate, chondroitin sulfate and dermatan sulfate remained closely associatd with highly purified fractions of amyloid fibrils which had resembled immunoglobulin.[10] These findings suggest that not only heparan sulfate but also dermatan sulfate or chondroitin sulfate may play a role in amyloid deposition.

Electrophoresis in 0.1M pyridine/0.47M formic acid showed the presence of unique component of crude GAG in amyloidotic spleen, but not in non-amyloidotic spleen. Enzymatic property of this component to chondroitinase AC and ABC was the same as that of chondroitin 4-S or 6-S, but the component had greater mobility than chondroitin 4-S and 6-S in electrophoresis. That same component was also found in the 1.0M NaCl fraction obtained from DEAE-cellulose chromatography. It was reported that highly sulfated dermatan sulfate was demonstrated in 1.0M NaCl fraction of GAG from rat liver by DEAE-cellulose chromatography.[8] Therefore it is probable that this peculiar component was highly sulfated chondroitin sulfate isomer with glucuronic acid residue. Another component, which was detected in 1.0M NaCl fraction in both amyloidotic and non-amyloidotic spleen, was degradated by chondroitinase ABC digestion but not by chondroitinase AC digestion; this component was thought to be highly sulfated dermatan sulfate. The amount of GAG in 1.0M NaCl fraction was larger in the amyloid group than in the non-amyloid groups. It was suggested, therefore, that highly sulfated chondroitin sulfate isomer increased in amyloidotic spleen.

For the GAG from liver, DEAE-cellulose chromatography showed that the amount of 1.0M NaCl fraction was larger in amyloid groups than in non-amyloid groups. Electrophoretic examination of GAG in 1.0M NaCl fraction revealed a component, whose enzymatic property to chondroitinase AC and ABC was the same as that of dermatan sulfate and whose mobility was shown to be a little greater than dermatan sulfate. It had been reported, as described before, that highly sulfated dermatan sulfate with on N-acetylgalactosamine 4, 6-bissulfate residues had been demonstrated in 1.0M NaCl fraction of GAG from rat liver by DEAE-cellulose chromatography.[8] We think that GAG in 1.0M NaCl fraction was also highly sulfated dermatan sulfate in our study.

Unsaturated disaccharide assay of crude GAG from liver demonstrated a component with slower mobility than ΔDi-6S after chondroitinase ABC digestion but not after chondroitinase AC digestion. This component was thought to be highly sulfated dermatan sulfate. This peculiar component was revealed to contain of N-acetylgalactosamine 4, 6-bissulfate residues by unsaturated disaccharide assay after chondro-4-sulfatase and/or chondro-6-sulfatase. This component was, therefore, identified as N-acetylgalactosamine 4-6 disulfate, L-iduronic acid, so called chondroitin sulfate G or H. Measurement in absorbance at 232nm showed that there was a greater increase of chondroitin sulfate G(H) in amyloidotic liver than in non-amyloidotic liver. It is probable that chondroitin sulfate G(H) component was included in GAG in 1.0M NaCl fraction obtained from DEAE-cellulose chromatography.

GAG constitutes the major part of the matrix substances in various tissues. And it was reported that chondroitin sulfate and dermatan sulfate remained closely associated with highly purified fractions of amyloid fibrils derived from human liver and spleen.[10] Therefore, these changes of highly sulfated chondroitin sulfate isomer may play an important role in amyloid deposition. Further is necessary to clarify this role.

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