Urinary Acid Glycosaminoglycans in a Patient with Oculo-Cerebro-Renal Syndrome

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HAYASHI, S., NAGATA, T., KIMURA, A. and TSURUMI, K. *Urinary Acid Glycosaminoglycans in a Patient with Oculo-Cerebro-Renal Syndrome*. Tohoku J. exp. Med., 1978, 126 (3), 225-234 — An 18-year-old boy with oculo-cerebro-renal syndrome excreted a large amount of acid glycosaminoglycans in urine. The identification and characterization of the acid glycosaminoglycans were carried out by the methods of preparative column electrophoresis, ion exchange chromatography, gelfiltration, paper chromatography of the chondroitinase digests and chemical analysis. On admission to hospital, the main components of the urinary acid glycosaminoglycans were undersulfated chondroitin 4-sulfate of large molecular weight and heparan sulfate. Three months after oral administration of the supplement of alkali, the excretion of heparan sulfate and the molecular size of chondroitin 4-sulfate decreased significantly, although the amount of urinary acid glycosaminoglycans remained at a high level (about 25 mg/day). The decrease of heparan sulfate and the shift to a smaller molecule of chondroitin 4-sulfate were coincident with the improvement in clinical and laboratory findings. These results suggest that the abnormal metabolism of acid glycosaminoglycans is a characteristic manifestation in this case and the studies on ground substance metabolism might be an important approach to the pathogenesis of this syndrome. — oculo-cerebro-renal syndrome; undersulfated chondroitin 4-sulfate; heparan sulfate

The authors have reported a case of oculo-cerebro-renal syndrome in which a large amount of urinary acid glycosaminoglycans (AGAG) was excreted (Hayashi et al. 1978). The urinary AGAG in this patient changed quantitatively as well as qualitatively during the course of therapy. The change of urinary AGAG was coincident with an improvement in bone abnormalities, which was induced by treatment with supplements of alkali and vitamin D. These results suggested that the increase of urinary AGAG resulted from the bone abnormalities.

Mucopolysacchariduria is one of the most characteristic manifestations in patients with mucopolysaccharidoses. With regard to these diseases, the urinary AGAG was studied in detail. It has been demonstrated that the AGAG or its metabolites reflecting the metabolic defects in these diseases are excreted in the urine (Dean and Muir 1973; Coppa et al. 1973). On the other hand, several authors reported an increased excretion of urinary AGAG in patients with many other pathological conditions. In those reports, however, a detailed

characterization of urinary AGAG has not been performed.

The purpose of this study is to characterize the urinary AGAG in a patient with oculo-cerebro-renal syndrome as an approach to the pathogenesis of the bone diseases in this syndrome.

MATERIALS AND METHODS

The preparation of urinary AGAG was carried out as described previously (Hayashi et al. 1978). Twenty-four-hour urine samples were obtained with thymerosal as preservative. Urinary AGAG was precipitated as CPC (cetylpyridinium chloride) complexes. The complexes were dissociated with 1.25 M MgCl_2 . The AGAG was obtained by precipitation with ethanol followed by treatment with alkali (0.5 N sodium hydroxide) and proteolytic digestion (Pronase-P, Kaken Kagaku Co., Ltd, Tokyo). The AGAG obtained was designated as crude AGAG.

Twenty-four-hour samples of urine were obtained from 4 healthy males (aged 18–20 years) as control.

Fractionation of crude AGAG by preparative column electrophoresis. A 2×60 cm column (Mitsumi Preparative Column-Electrophoresis, Mitsumi Scientific Industry Co., Ltd., Tokyo) was packed with Sephadex G-10 (medium) media to a bed height of 55 cm. The column, which was surrounded by a water jacket at 4°C , was connected by two adaptors to two electrode chambers containing 400 ml of 0.05 M barium acetate buffer (pH 5.5). The adaptor at the top of the column was connected to the cathode chamber and the other to the anode chamber. The buffer in the electrode chambers was mixed interchangeably drop by drop at the flow rate of 100 ml/10 min to keep the difference of pH of the buffer solution in both electrode chambers at a minimum. One ml of the sample (about 5% AGAG solution) was applied to the top of the column and allowed to migrate 2 cm into the gel bed. A current of 30 mA (900 V) was applied to the column for 42 hr.

After electrophoresis had completed, the bottom adaptor was exchanged for the flow adaptor and the sample was eluted with the same buffer. Fractions (2.1 ml) were collected and the uronic acid in each tube was determined by the carbazole methods of Bitter and Muir (1962) and Dische (1947), and the orcinol method of Khym and Doherty (1952). From the residue, AGAG was precipitated by the addition of 4 volumes of ethanol.

Fractionation of crude AGAG by Dowex 1 column chromatography. The crude AGAG (450 mg) obtained in the 2nd period was fractionated by the use of a Dowex 1×2 (Cl^- , 200–400 mesh) column (2.7×55 cm). After application of the sample, the column was washed with 500 ml of distilled water and then eluted in succession, with 1,000 ml each of 0.5 M, 1.0 M, 1.25 M, 1.5 M, 2.0 M and 3.0 M sodium chloride. Each fraction was pooled together, dialysed, and brought to dryness.

Gel filtration. A study on the molecular size of the crude and fractionated AGAG was carried out by the use of 0.8×55 cm columns of Sephadex G-200 (medium) and G-50 (fine). Two hundred μl of AGAG solution (0.5 % AGAG solution) was applied to the column equilibrated with 0.2 M sodium chloride. The eluate was collected in 0.5 ml portions and elution profiles were traced by the carbazole reaction. Kd value was calculated with the top of the elution patterns of Blue Dextran and ammonia used as markers.

Paper chromatography. The identification of chondroitin 4-sulfate and chondroitin 6-sulfate was performed by paper chromatography after chondroitinase AC-II digestion according to the method of Saito et al. (1968). Two hundred μl of the AGAG solution containing 0.3 μmole as glucuronic acid was mixed with 30 μl of Tris buffer (pH 8.0) containing 0.5 unit of chondroitinase AC-II (purchased from Seikagaku Kogyo Co., Ltd., Tokyo) and incubated at 37°C for 1 hr. The resulting unsaturated disaccharides were separated by the descending paper chromatography in *n*-butyric acid: 0.5 N ammonia (5:3) for 48 hr. The ultraviolet absorbing spots were cut into small pieces and immersed

in the 0.01 N hydrochloric acid, and determined at 232 nm.

Analytical electrophoresis. Analytical electrophoresis on cellulose acetate sheets was carried out under the following three conditions: in 0.075 M barium acetate at constant current of 1 mA/cm for 1 hr (Kimura et al. 1974); in 0.3 M calcium acetate at 1 mA/cm for 3 hr (Seno et al. 1970); in 0.1 N hydrochloric acid at 1 mA/cm for 2.5 hr (Wessler 1971a).

Chemical analysis. Uronic acid was determined by the carbazole methods of Bitter and Muir (1962), and Dische (1974), and by the orcinol method (Khym and Doherty 1952). Hexosamine was estimated by the method of Blix (1948) after hydrolysis with 2 N hydrochloric acid at 100°C for 14 hr, and the glucosamine/galactosamine ratio was obtained by use of amino acid analyzer (JLC-5H). Sulfate was measured by the method of Dodgson and Price (1962). The measurement of optical rotation was made on an automatic polarimeter (JASCO DIP-180, Japan Spectroscopic Co., Ltd., Tokyo).

Clinical course. The clinical course of the patient was divided into four periods based on the clinical and laboratory findings and the results of urinary AGAG analysis (Fig. 1).

Fig. 1. The clinical course.

Histogram shows urinary excretion of AGAG.

■, Vitamin D₂; ▨, alkali.

RESULTS

Preparative column electrophoresis of urinary AGAG in the 1st and 2nd periods.

The crude AGAG obtained from the two periods was separately fractionated by preparative column electrophoresis. The elution patterns and the results of chemical analysis are shown in Fig. 2 and Table 1, respectively.

The crude AGAG (57 mg) in the 1st period was applied to the column and separated into two peaks (left in Fig. 2). The recovery of AGAG was 94% of the starting AGAG. The AGAG in the tube numbers 1–5 was identified as undersulfated chondroitin 4-sulfate by the following characteristics. These fractions had a molar ratio of glucuronic acid to galactosamine of 1:1 and the carbazole-to-orcinol (C/O) ratios ranged from 0.9 to 1.0. Paper chromatogram of chondroitinase AC-II digests of tube number 1 showed a ratio of Δ Di-OS to Δ Di-4S to Δ Di-6S to Δ Di-diS of 40:53:5:2. On the other hand, the AGAG in the latter hump (tube numbers 8–15) had characteristics of heparan sulfate. These fractions possessed a molar ratio of uronic acid to glucosamine of 1:1 and C/O ratios between 1.3 and 2.8. The optical rotation of AGAG in tube numbers 9–10 and 11–15 was +34° and +28° respectively. The AGAG of these fractions was resistant to chondroitinase gave

ABC digestion. The ratio of chondroitin 4-sulfate to heparan sulfate was 58:42, which agreed with the result of analytical electrophoresis on cellulose acetate sheets (57:43).

Column electrophoresis of the crude AGAG (55 mg) in the 2nd period was carried out in the same manner described above. The elution profile and results

Fig. 2 Preparative column electrophoresis in 0.05 M barium acetate of the urinary AGAG obtained in the 1st (left) and 2nd (right) periods.

●—●, uronic acid (Bitter and Muir); ○—○, C/O ratio.

TABLE 1. Chemical analysis of urinary AGAG fractionated by preparative column electrophoresis

Fraction	Uronic acid (%)	Hexosamine (%)	Sulfate (%)	Molar ratios			$[\alpha]_D^{20}$ (c=1%)
				Uronic acid*	Sulfate*	GlcN : GalN	
1st period							
Tube 1	33.4	39.0	11.4	0.95	0.66	0:100	
Tube 2	33.7	37.9	11.3	0.99	0.67	0:100	
Tube 9-10	34.5	36.2	15.5	1.06	0.96	100: 0	+34
Tube 11-15	31.1	33.6	15.8	1.03	1.06	100: 0	+28
2nd period							
Tube 2	34.5	41.4	13.0	0.93	0.70	0:100	
Tube 3	36.0	40.1	11.1	1.00	0.62	0:100	
Tube 4	36.1	39.9	10.3	1.01	0.58	0:100	
Tube 5	22.8	27.4	8.1	0.93	0.66	0:100	
Tube 6	22.2	26.1	7.7	0.94	0.66	5: 95	

* Expressed as molar ratios based on hexosamine=1.0

of chemical analysis showed that the main component of the AGAG in the 2nd period was undersulfated chondroitin 4-sulfate. A trace amount of heparan sulfate was detected on the analytical electrophoresis.

The molecular weight distribution patterns of the crude and the fractionated AGAG in the two periods are shown in Fig. 3. The crude AGAG in the 1st period

gave a bimodal elution curve on Sephadex G-200 (left in Fig. 3). However, the peak that appeared first from the gel was not detected on the elution profile of the AGAG in the 2nd period. Chondroitin 4-sulfate and heparan sulfate in the 1st period obtained by preparative column electrophoresis were separately applied to the gel and eluted under the same condition. The results are shown in Fig. 3-right. The elution diagrams showed that the molecular size of the chondroitin 4-sulfate was larger than that of the heparan sulfate. Therefore, the large molecular weight AGAG in the 1st period was attributed to chondroitin 4-sulfate molecules. The

Fig. 3. Molecular weight distributions of the crude and the fractionated AGAG on Sephadex G-200.

Left: Elution patterns of crude AGAG in the 1st (●—●) and the 2nd (○—○) periods.

Right: Elution patterns of the AGAG fractionated by preparative column electrophoresis in the 1st period. T-1 and T-2, chondroitin 4-sulfate fraction; T-10, heparan sulfate fraction.

Fig. 4. Molecular weight distributions of crude AGAG on Sephadex G-50.

●—●, elution patterns of urinary AGAG of normal males; ○—○, elution pattern of crude AGAG obtained in the 2nd period (Sep. '73); ●—●, elution pattern of crude AGAG obtained in the 4th period (Feb. '74).

elution pattern from Sephadex G-50 showed that the crude AGAG in the 2nd period contained a smaller molecular size AGAG than that found in normal subjects (Fig. 4).

Fractionation of chondroitin 4-sulfate obtained in the 2nd period. The crude AGAG in the 2nd period was fractionated by Dowex 1 column chromatography. Chemical analysis and electrophoretic identification of AGAG fractionated by Dowex 1 are shown in Table 2. The 1.0 M fraction was the main fraction followed by 1.25 M fraction. The 0.5 M fraction contained a small amount of hyaluronic acid, which migrated the same distance as that of authentic hyaluronic acid and was digested with *Streptomyces* hyaluronidase.

TABLE 2. *Chemical analysis and identification of AGAG fractionated by Dowex 1 column chromatography*

Eluate conc. of NaCl	Distribution of uronic acid* (%)	Molar ratios			Identification by electrophoresis§
		GlcUA: Sulfate†	GlcN: GalN	OS: 4S: 6S: diS‡	
0.5 M	9				(HA)
1.0 M	74	1.01:0.45	4:96	54:39:3:4	C-4/6-S (HS)
1.25 M	12	0.99:1.04	5:95	9:87:3:1	C-4/6-S (DS, HS)
1.5 M	4	1.01:1.46	5.95	1:90:6:3	C-4/6-S (DS)
2.0 M	1	—	—	—	DS, KS (C-4/6-S)
3.0 M		—	—	—	KS (DS)

* Expressed as the percentage of total uronic acid eluted from the Dowex 1 column

† Expressed as molar ratios based on hexosamine=1.0

‡ Obtained from paper chromatography after chondroitinase AC-II digestion (OS, Δ Di-OS; 4S, Δ Di-4S; 6S, Δ Di-6S; diS, Δ Di-diS)

§ Trace amount of AGAG is shown in parenthesis.

Seventy-four per cent of total uronic acid eluted from the column was contained in the 1.0 M fraction. The sulfate content of this fraction was 0.45 mole per glucuronic acid and this low sulfate content was supported by the result of paper chromatography after chondroitinase AC-II digestion. The paper chromatogram of the chondroitinase AC-II digests of the 1.0 M fraction showed that 93% of the total unsaturated monosulfated disaccharide units were Δ Di-4S. From these results, chondroitin 4-sulfate was calculated at about 80% of the total AGAG. On the other hand, chondroitin 6-sulfate was only 3% of total AGAG. Small amounts of heparan sulfate, dermatan sulfate and keratan sulfate were detected by electrophoretic and enzymatic studies, but further investigations of these AGAG were not made.

Urinary AGAG obtained in the 3rd and 4th periods. The amount of urinary excretion of AGAG and the electrophoretic patterns were shown in the previous paper (Hayashi et al. 1978). On electrophoresis in 0.3 M calcium acetate, the amounts of AGAG which had an intermediate mobility between authentic chondroitin 4-/6-sulfate and dermatan sulfate increased with time, but an increase

of heparan sulfate was not detected. The AGAG in these periods were similar to the AGAG in the 2nd period in electrophoretic mobility and in susceptibility to chondroitinase AC-II.

The sulfate content of the crude AGAG gradually decreased with time in the 3rd period (Fig. 5). The elution patterns on Sephadex G-50 of the crude AGAG

Fig. 5. Sulfate content of crude AGAG after the stoppage of the therapy (indicated by an arrow). Histogram shows urinary excretion of AGAG.

in the 4th period (Feb '74) shifted to a region for smaller molecules than that of the AGAG in the 2nd period (Fig. 4).

These results showed the increase of small molecular undersulfated chondroitin 4-sulfate after the stoppage of the therapy.

DISCUSSION

Urinary AGAG in this patient with oculo-cerebro-renal syndrome was changed qualitatively and quantitatively with clinical manifestations. The level of urinary AGAG, the electrophoretic patterns, and the clinical course were discussed previously (Hayashi et al. 1978).

The identification and characterization of urinary AGAG were carried out after fractionation with preparative column electrophoresis and ion exchange chromatography. When the patient was admitted, AGAG was composed of chondroitin 4-sulfate and heparan sulfate with a ratio of 6:4. The chondroitin 4-sulfate had larger molecular weight and lower sulfate content compared with that of normal persons. After the medical treatment, the amount of the heparan sulfate and molecular weight of the chondroitin 4-sulfate decreased, and small-molecular undersulfated chondroitin 4-sulfate was determined as major AGAG.

The composition of various AGAG in normal urine was reported by several authors (Varadi et al. 1967; Wessler 1971b). The major AGAG in normal human urine was chondroitin 4- and 6-sulfate followed by heparan sulfate and dermatan sulfate. Chondroitin 4-sulfate and chondroitin 6-sulfate were detected in almost equal amounts. The chondroitin 4- and 6-sulfate obtained from urine were heterogeneous in sulfate content. Varadi et al. (1967) reported that 65% of AGAG excreted in urine was chondroitin 4- and 6-sulfate and that 25% was so called

"chondroitin". Murata et al. (1973) described the increased distribution of undersulfated chondroitin 4- and 6-sulfate with the advance of age.

In our patient, the sulfate content of each chondroitin 4-sulfate fraction separated by the preparative column electrophoresis was about 0.65 mole per hexosamine. This result showed that the chondroitin 4-sulfate fractions were not a mixture of chondroitin (desulfated chondroitin 4-/6-sulfate) and chondroitin 4-sulfate but undersulfated chondroitin 4-sulfate alone. A small amount of Δ Di-6S was detected on paper chromatography after chondroitinase AC-II digestion but detailed studies on chondroitin 6-sulfate were not made.

The shift of distribution of molecular size in urinary AGAG was demonstrated in the case of mucopolysaccharidosis (Dean et al. 1973). Except for mucopolysaccharidoses, however, it has not been observed. In our case, chondroitin 4-sulfate obtained on admission showed a larger molecular weight than that of normal urine. After the administration of the alkali, the large-molecular-weight chondroitin 4-sulfate disappeared and the elution profile on Sephadex G-50 showed two humps; one indicated a molecular size similar to that of normal subjects, the other showed a smaller molecular size of chondroitin 4-sulfate compared with normal urinary AGAG.

The clinical and laboratory data in this case suggested that the origin of undersulfated chondroitin 4-sulfate was related to bone abnormalities. This suggestion was supported by the study on the distribution of chondroitin 4-sulfate and chondroitin 6-sulfate in hard bone tissue. Mathews (1965) discovered that highly calcified cartilage and bone generally contained a much higher proportion of chondroitin 4-sulfate than did uncalcified skull cartilage. He discussed the possibility that chondroitin 4-sulfate is associated with calcified tissues and also with the processes of calcification. When an increase of urinary chondroitin 4-sulfate was induced by the bone abnormalities, why was chondroitin 6-sulfate not increased? This question cannot be answered exactly. But this fact might suggest the possibility of different functions between chondroitin 4-sulfate and chondroitin 6-sulfate on calcification and metabolism of bone matrix.

The experiment in the 2nd period showed a decrease of heparan sulfate excretion and a shift in molecular size of chondroitin 4-sulfate. At that time the patient received the supplement of alkali alone. Under these circumstances, it is assumed that the supplement of alkali caused a quantitative and qualitative change in urinary AGAG, and also acidosis was observed to play an important role in the development of bone lesions in this syndrome.

It is uncertain whether the large-molecular-weight undersulfated chondroitin 4-sulfate in this case was induced by the abnormal degradation of AGAG or by the deficiency in the activity of sulfotransferase. Mourao et al. (1973) reported four cases of generalized platyspondyly which showed an increase of undersulfated chondroitin 6-sulfate, suggesting the possibility of deficiency in the activity of chondroitin sulfate C-sulfotransferase. Varadi et al. (1967) discussed the possibility that these undersulfated chondroitin sulfate might eventuate from the

excretion of incompletely sulfated intermediates.

On the other hand, it is well known that the degradation of polysaccharide chain of chondroitin 4-sulfate was carried out by hyaluronidase together with the concerted action of β -glucuronidase, β -N-acetylhexosaminidase and sulfatase. Hayashi (1977; 1978) reported the contribution of these enzymes to the degradation of hyaluronic acid, chondroitin 4-sulfate and chondroitin on the basis of a characterization of the degradation products produced by the lysosomal enzymes. Chondroitin 4-sulfate was first degraded only by hyaluronidase. In further degradation, dodecasaccharide derived from chondroitin 4-sulfate or chondroitin serves as the lowest-molecular-weight substrate for hyaluronidase and the decasaccharide as the largest-size substrate for β -glucuronidase in the degradation of chondroitin 4-sulfate or chondroitin by the enzymes of lysosomes. Furthermore, the depolymerization of large-molecular-weight AGAG (hyaluronic acid, chondroitin 4-sulfate and chondroitin) by hyaluronidase was inhibited by the addition of a specific inhibitor of β -glucuronidase (saccharic acid 1, 4-lactone). The inhibition of the action of hyaluronidase was stronger in the depolymerization of chondroitin 4-sulfate than in the degradation of hyaluronic acid. From these results, if this case had abnormality on degradation of AGAG, the excretion of large-molecular undersulfated chondroitin 4-sulfate would suggest a possibility of insufficiency of β -glucuronidase or hyaluronidase. It seems, however, correct that the possibility that only one enzyme in the degradative or synthetic process of AGAG is abnormal, has not been given enough consideration with respect to the relationship between the pathogenesis of the bone lesions and abnormal AGAG metabolism in this case. Further investigations on the role of AGAG in the metabolism of bone tissue formation, arrangement of collagen fibrils and deposition of apatite on collagen fibrils would help to resolve this question.

After 18 months of his discharge, the bone abnormalities were controlled by treatment with alkali and vitamin D₂ but the excretion of small-molecular undersulfated chondroitin 4-sulfate, which had similar characteristics to that of the 3rd period, was not much decreased. Therefore this small molecular and under-sulfated chondroitin 4-sulfate might be related not only to the bone abnormalities but also to renal tubular dysfunction in this syndrome. However, at the present time, we have few available data on urinary AGAG excreted by patients with tubular dysfunction.

At the time of admission, an increase of heparan sulfate was demonstrated. However, not only the origin of heparan sulfate but also the significance of heparan sulfate in this case was obscure. Further investigations on the connective tissue metabolism in this syndrome may be a useful approach for elucidating the cause of bone abnormalities and the pathogenesis.

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