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ORIGINAL ARTICLE

ANALYSIS OF FUNCTIONAL SUGAR CHAINS OF PROTEOGLYCANS IN THE URINE FROM WOMEN WITH OSTEOPOROSIS

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Abstract The aim of study is to analyze functional sugar chains, glycosaminoglycans (GAGs) in urine from postmenopausal women with osteoporosis. A 24-hour urine was collected from 15 osteoporotic women and normal women. All women were postmenopausal and aged at 60 to 70 years old. Diagnosis of osteoporosis was based on the diagnosis criteria proposed by the Japan Osteoporosis Society after measurement of lumbar spine bone mineral density by dual energy X-ray absorptiometry. The urine was concentrated by dialysis and evaporation, then precipitated with ethanol. The precipitate was then reconstituted into NaCl solution, precipitated again by adding 10 % cetylpyridinium chloride and the precipitation was used as complex GAGs fraction. The yield of complex fraction was significantly higher in osteoporotic subjects than that in normal control. Then, the complex GAGs fraction was analyzed by cellulose acetate membrane electrophoresis and high performance liquid chromatography (HPLC), respectively. Cellulose acetate membrane electrophoresis has revealed that GAGs in urine contained partially desulfated chondroitin sulfate. However, further analysis by HPLC has shown that the excretion of desulfated GAGs was rather less in osteoporotic women, indicating that urinary GAGs fraction from osteoporotic women contains unknown acidic polysaccharides as well as GAGs.

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Key words: Osteoporosis; Urine; Glycosaminoglycan.

原　著

機能的糖鎖の変化からみた骨粗鬆症病態の解明

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抄録　本研究は，閉経後骨粗鬆症婦人の尿中糖鎖を分析する事を目的とした．正常婦人及び骨粗鬆症婦人15人から24時間尿を採取した．対象はすべて60～70歳代で閉経している婦人であった．骨粗鬆症群と非骨粗鬆症群の診断は日本骨粗鬆症学会の診断基準に従った．採取された尿は透析濃縮後，エタノール沈殿を行い，その沈殿物を回収し食塩飽和エタノールと10 % cetylpyridinium chloride を加えて遠心分離して生じた沈殿物を回収した．これを粗複合glycosaminoglycans（以下 GAGs と略す）とした．尿中粗複合 GAGs は正常群に比較し骨粗鬆症群の方が有意に回収されていた．この粗複合 GAGs をセルロースアセテート膜電気泳動及び high performance liquid chromatography（以下 HPLC と略す）で分析にした．尿中に排泄された GAG はセルロースアセテート膜電気泳動では脱硫化されたコンドロイチン硫酸であること，HPLC の分析では正常群に比較し骨粗鬆症群の尿中 GAG の収量は逆に少なくなっており，骨粗鬆症群の尿中粗複合 GAG は GAG の他に酸性多糖が含まれることが明らかになった．

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Introduction

Osteoporosis is a bone disease characterized by the loss of bone strength, increasing the risk of bone fractures. Bone strength depends on bone density and quality, the latter being decided by the bone structure, bone metabolic turnover, microdamage accumulation, and bone tissue mineralization[1]. Bone metabolic turnover is regulated as substances that promote and inhibit the formation of hydroxyapatite[2] are controlled physiochemically and at a cellular level[3]. Generally, the biochemical mechanism of HAP crystal formation in calcified tissues is associated with inhomogeneous nucleus formation induced by extracellular matrix (ECM) noncollagenous proteins. The affinity of ECM components to HAP controls initial mineral deposits, and adjusts the size, shape and direction of formed crystals[4]. In particular, proteoglycan (PG) and its sugar chain, glycosaminoglycans (GAGs), are considered to be important in the regulation of calcification. It has been observed *in vitro* that aglycan, a massive cartilage PG, affects the size, structure and charge density of HAP crystals through growth inhibition[5]. Meanwhile, it has been reported that massive PG promotes calcification due to calcium chelation induced by GAGs[6]. This indicates that PG functions as a cation exchanger[7]. Thus, the importance of PG and GAGs with regards to bone quality, especially calcification, is recognized but there has only been one report published concerning the evaluation of bone quality at a clinical level, in which the excreted amount of GAGs in urine increased in patients with osteoporosis[8]. In this study, we assumed that the metabolism in the body is affected by the quality and amount of GAGs in urine and tried to evaluate the changes in the GAGs level of healthy people and osteoporosis patients by examining the excreted GAGs in the 24-hour urine.

Materials and Methods

Materials

Chemicals

Standard chondroitin 4-sulfate (Ch4S, molecular weight 16,000, whale cartilage), chondroitin 6-sulfate (Ch6S, molecular weight 48,000-80,000, shark cartilage), dermatan sulfate (DS, molecular weight 11,000-25,000, porcine skin), heparan sulfate (HS, molecular weight 11,000, bovine kidney), hyaluronic acid (HA, molecular weight 100,000-150,000, porcine skin), chondroitin, (Ch, molecular weight unknown, shark cartilage) and heparin (Hep, molecular weight 19,000, porcine intestinal mucosa) were purchased from Seikagaku Corporation (Tokyo, Japan). Other reagents and chemicals were obtained from commercial sources.

Preparation of urine samples

A total of 15 outpatients in their sixties and seventies without any history of urological malignant tumors or bony metastasis and metabolic disorders who visited the Obstetrics and Gynecology Department, were selected as subjects. This study was approved by the ethics committee of Hirosaki University Hospital on March 12, 2009 (approval number: 2008168). After informed consent was obtained from all the subjects, 24-hour urine was collected. According to the diagnosis criteria of the Japan Osteoporosis Society, dual energy X-ray absorptiometry of the lumbars was performed. Subjects were classified according to their lumbar young adult mean 9) level into two groups: 10 subjects with a YAM level of 70% and more as the non-osteoporosis group, and 5 subjects with less than 70% as the osteoporosis group.

Extraction of GAGs in urine

Dialysis and concentration : The collected 24-hour urine was dialyzed using 3 L of distilled

water every 12 h for 48 h, employing a dialysis membrane (Spectra/Por RC dialysis tube No. 6). The dialyzed urine was concentrated to about 1/10 of the primitive urine volume by an evaporator. Next, the concentrated urine was centrifuged (3,000 rpm, 10 min) and the centrifuged supernatant was collected. The supernatant was stirred into 4 times its volume of salt-saturated ethanol and was left standing still at a temperature of 4℃ for 2 h. Then it was again centrifuged (3,000 rpm, 10 min) and the deposited fraction was collected. The collected fraction was washed with 80% ethanol and dialyzed for 48 h in the same way as above. After being again centrifuged (3,000 rpm, 10 min), the supernatant was collected, lyophilized, and weighed, and that obtained was labeled as the crude complex carbohydrate fraction.

*Fractionation by cetylpyridinium chlorid*e : After the crude complex carbohydrate fraction was dissolved to 1% in 0.01 M NaCl, 10% cetylpyridinium chloride (CPC) was added dropwise to form deposits, and this solution was left standing still at room temperature for 1 h. The solution was then centrifuged (3,000 rpm, 10 min) to separate deposits from the supernatant. After being washed with 1 mL of 1% CPC under centrifugation, the deposits were dissolved in 1 mL of 2 M $MgCl_2$ and stirred into 4 times its volume of salt-saturated ethanol, before the solution was left standing still at a temperature of 4℃ for 1 h. After further centrifugation (3,000 rpm, 10 min), the obtained deposits were washed with 80% ethanol, then washed with 99.5% ethanol, and the deposits were weighed and labeled as the complex GAGs fraction.

Cellulose acetate membrane electrophoresis : The GAGs fraction was dissolved to 1 % in distilled water and was electrophoresed on a cellulose acetate membrane (Separax) using Ch4S, HS and Ch as standards. Electrophoresis was performed with 1 mA/cm for 20 minutes using a 0.1 M formate-pyridine buffer solution (pH 3.0). Staining was conducted for 10 minutes using 0.1% Alcian Blue 70% ethanol and was washed with 70% ethanol.

Fractionation of GAG fraction by high performance liquid chromatography (HPLC) : The GAG fraction was divided using an anion exchange TSK-gel Q-STAT column (diameter 4.6 mm × length 10 cm, Tosoh Corporation, Tokyo, Japan). Elution was performed by the stepwise concentration gradient method using H_2O as buffer A and 3 M NaCl as buffer B, with a flow rate of 1.0 mL/min. Eluate was monitored with UV at 215 nm, collected by a fraction collector, dialyzed per collection peak in the same way as above and lyophilized. After being fractionated by the TSK-gel Q-STAT column, the GAG was separated by cellulose acetate membrane electrophoresis per collection peak in the same way as above.

Statistical analysis : All data were analyzed with use of GraphPad Software Instat3. The data are presented as mean ±SD . Statistical difference among the study and non-osteoporosis group and osteoporosis group were analyzed with use of t-test. Differences were considered significant for probability<0.05. The Mann-Whitney U-test was applied for the comparison between the fractionation of GAGs fraction by HPLC in non-osteoporosis group and the fractionation of GAGs fraction by HPLC in non-osteoporosis group differences. Differences were considered significant for probability<0.05.

Results

The average age of 10 subjects in the non-osteoporosis group was 66.0 years old and that of 5 in the osteoporosis group was 66.2 years old (Table 1). The average excreted amount of crude complex carbohydrates in 24-hour urine

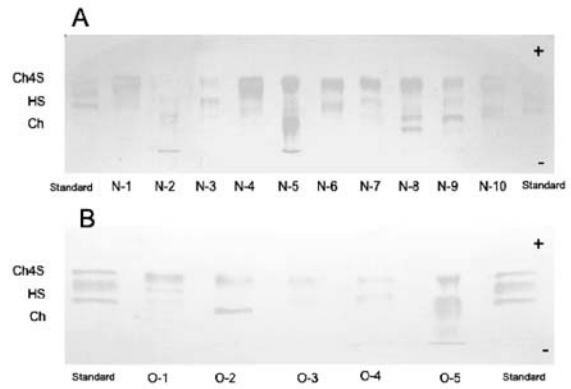

Fig. 1 Expression patterns of urinary GAG by Cellulose acetate membrane electrophoresis. A: 10 urines from non-osteoporotic women, B: 5 urines from osteoporotic women. Electrophoresis was carried out as described in M&M, briefly using 0.47 M formic acid/0.1 M pyridine buffer (pH 3.0) at 1 mA/cm for 20 min. Staining was done with 0.05 % alcian blue in 70 % ethanol. Ch4S, chondroitin 4-sulfate: HS, heparan sulfate: Ch, chondroitin

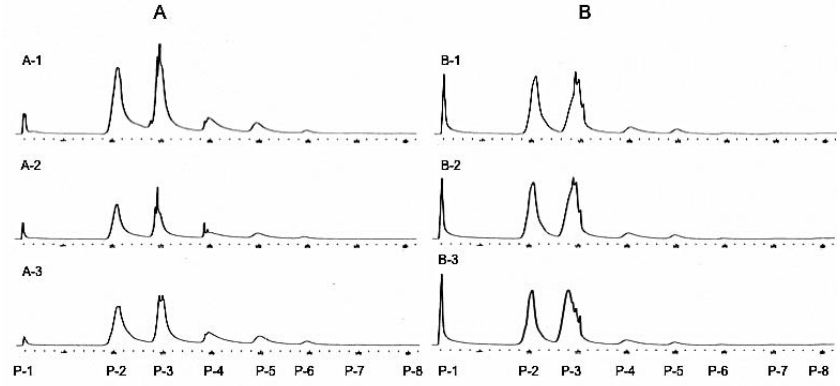

Fig. 2 Elution pattern of urinary GAG by anion exchange column HPLC. Representative elution patterns by an anion exchange TSK-gel Q-STAT column (diameter 4.6 mm × length 10 cm). A : Non-osteoporosis group. B : Osteoporosis group. The elution patterns of both groups were classified into P-1 to P-8 by their elution peak.

in non-osteoporosis subjects was 30.8 mg/L and that in osteoporosis subjects was 37.2 mg/L. Notably, the excreted amount for osteoporosis subjects was larger than that of non-osteoporosis subjects. The complex carbohydrate fraction was compared between the groups using cellulose acetate membrane electrophoresis (Figure 1). The complex carbohydrate fraction included an acidic polysaccharide fraction in both groups. There was no difference between the groups as to the electrophoresis pattern. Next, the GAGs fraction, which was an acidic polysaccharide component, was separated by CPC from the complex carbohydrate fraction of both non-osteoporosis subjects and osteoporosis subjects (Table 1). The average excreted amount of the complex GAGs fraction in the 24-hour urine of non-osteoporosis subjects was 1.6±0.8 mg/L and that of osteoporosis subjects was 3.9±1.6 mg/L, suggesting that the excreted amount of the complex GAGs fraction for osteoporosis subjects was significantly ($p=0.0029$) larger than that of non-osteoporosis subjects. Then, the complex GAGs fraction was further analyzed by

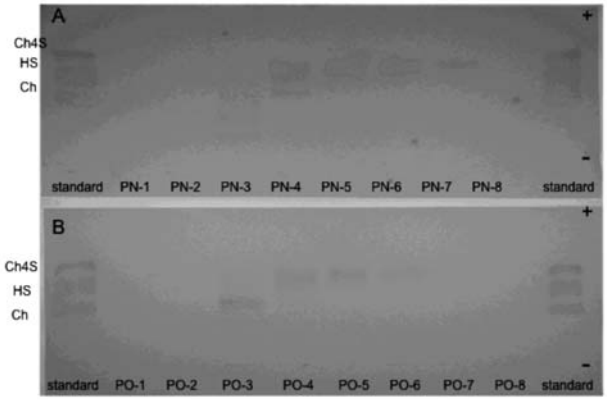

Fig. 3 Expression patterns of HPLC Fractions by Cellulose acetate membrane electrophoresis. Each fraction collected by HPLC was analyzed by Cellulose acetate membrane electrophoresis. A; non-osteoporosis group and B; osteoporosis group. PN-1 to PN-8 is shown a fraction of P-1 to P-8 on fig. 2 in non osteoporosis group. PO-1 to PO-5 is shown a fraction of P-1 to P-8 on fig. 2 in osteoporosis group. Electrophoresis was carried out as described using 0.47 M formic acid/0.1 M pyridine buffer (pH 3.0) at 1 mA/cm for 20 min. Staining was done with 0.05 % alcian blue in 70 % ethanol. Ch4S, chondroitin 4-sulfate : HS, heparan sulfate : Ch, chondroitin.

Table 1 Comparison of 24-hour Urine Results

	No.	Age	24-hour urine volume(mL)	Crude complex carbahydrate (mg)	Crude complex carbahydrate yiedl(mg/L)	Complex GAGs fraction(mg)	Complex GAGs fraction yield (mg/L)
Non-osteoporosis group	N-1	71	950	40.1	41.0	0.5	0.5
	N-2	68	1800	34.2	19.0	1.0	0.6
	N-3	66	1550	54.4	35.1	0.9	0.6
	N-4	70	1750	50.0	51.0	1.8	1.0
	N-5	61	1450	59.6	41.0	2.5	1.7
	N-6	70	1550	46.1	29.0	2.8	1.8
	N-7	64	1800	40.3	22.3	3.8	2.1
	N-8	68	1050	23.3	22.2	2.4	2.3
	N-9	60	900	20.1	22.3	2.3	2.6
	N-10	62	1470	36.9	25.1	4.0	2.7
	Average	66.0 ± 4.0	1427 ± 343.6	40.5 ± 12.7	30.8 ± 10.7	2.2 ± 1.2	1.6 ± 0.8
Osteoporosis group	O-1	62	1600	82.3	51.4	3.3	2.1
	O-2	67	1600	48.0	30.0	5.5	3.4
	O-3	69	1500	40.4	26.9	5.6	3.7
	O-4	63	800	38.0	47.5	3.0	3.8
	O-5	70	1200	36.0	30.0	7.9	6.6
	Average	66.2 ± 3.6	1340 ± 343.5	48.9 ± 19.2	37.2 ± 11.4	5.1 ± 2.0	3.9 ± 1.6*

Difference between acidic polysaccharide yield of non-osteoporosis and that of osteoporosis. *$p<0.05$ compared with non-osteoporosis, analyzed by t-test.

cellulose acetate membrane electrophoresis. As shown in Figure 1A and 1B, the GAGs stained with Alcian Blue was mostly observed between Ch4S and HS and it was considered that these GAGs was from partial desulfation of Ch4S and Ch6S. In order to confirm this, the complex GAGs fractions from each subject were applied to anion exchange HPLC. As shown in Figure

Table 2 Complex GAGs fraction in 24-hour Urine by Anion Exchange HPLC

		P-1	P-2	P-3	P-4	P-5	P-6	P-7	P-8
Non-osteoporosis group	Amount of complex GAGs fraction in 1 L of urine (μg/L)	1.2	3.5	6.0	2.3	0.9	0.3	0.3	0.3
Osteoporosis group	Amount of complex GAGs fraction in 1 L of urine (μg/L)	2.4	4.9	6.7	0.9	0.4	0.1	0.04	0.1

2, the elution patterns of both groups were classified into P-1 to P-8 by their elution peak. The result was that pattern peaks of P-2, P-3, P-4 and P-5 were large. Compared with non-osteoporosis subjects, pattern peaks, especially P-3, of osteoporosis subjects were small but the effusion spread more broadly. Then, the GAGs fraction of both groups was pooled per elution peak, dialyzed, lyophilized and weighed (Table 2). As a result of fractionation by the anion exchange HPLC, collected amounts at P-1, P-2 and P-3 in osteoporosis subjects were large and those at P-4 to P-8 were small, compared with non-osteoporosis subjects. The excreted amount of GAGs in the 24-hour urine of osteoporosis subjects was small at P-4 to P-8, compared with non-osteoporosis subjects (Table 2). There was no difference between the groups in the electrophoresis patterns of P-1 to P-8 using cellulose acetate membrane electrophoresis (Figure 3). However, since P-1 and P-2 were not stained with Alcian Blue, they were not regarded as GAGs, but be acidic polysaccharides. As P-3 to P-8 were electrophoresed between Ch4S (or Ch6S) and HS, which were used as standards, from the viewpoint of the migration distance they were regarded as partially desulfated GAGs. From these results, it is indicated that desulfated GAGs is secreted in urine from menopausal women. Although the present study could not confirm clear difference in GAGs fractions between normal and osteoporotic subjects, it showed that urine from osteoporotic subjects contains significantly large amount of acidic polysaccharides.

Discussion

It is well documented that GAGs is secreted into urine[9), 10)] and it is also shown that urinary GAGs secretion is increased in women after menopause[8)]. However thus far no styudies have been conducted to calarify whether urinary GAGs is associated with osteoporosis. This is the first report that aimed to identify significance of urinary GAG of women with osteiporosis.

Women often suffer from osteoporosis after menopause and some mechanisms have been investigated, such as the decrease in female hormone[11)], but their details are as yet unknown. Osteoporosis is clinically considered as a loss in bone density, but the cause of bone loss is still unknown. Bone is formed by deposits of hydroxyapatite in the network of tissue components, such as proteoglycan and collagen[11)]. We focused on the metabolism of GAG, as GAG is shown to play a direct role in the deposits of hydroxyapatite[12, 13)]. Since osteoporosis is progressed with increased absorption of bone tissues, analysis of GAG fraction is proposed to be clinically useful for detection or diagnosis of the disease. However, it is difficult to study GAG insides bones, and we examined GAG in urine, as urine can be easily collected and served for clinical materials for diagnosis. After subjects were classified into the non-osteoporosis group and osteoporosis group by measurement of bone mineral density, 24-hour urine was collected from all the subjects. Then, GAG in the urine

was fractionated to compare both groups. As a result, it was found that the yield of acidic polysaccharides excreted in 24-hour urine of osteoporosis subjects was significantly larger than that of non-osteoporosis subjects.

With an aim to identify the chemical properties of complex GAG fraction, the complex GAG fraction was fractionated using a cellulose acetate membrane electrophoresis and anion exchange HPLC. Although this procedure has revealed that GAG detected in urine was partially desulfated GAGs, there were no significant differences in amount of GAG or elution profiles on HPLC between two groups. Therefore, it is indicated that a high amount of acid polysaccharide yield obtained from osteoporotic women are other acid polysaccharide except GAG. Further study concerning structures, such as the difference of affinity of GAG in urine to hydroxyapatite in patients with osteoporosis, will be needed.

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