Diabetes Results in Structural Alteration of Chondroitin Sulfate in the Urine

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ABSTRACT – Purpose. The assessment of the clinical significance of chondroitin sulfate in patients with type 2 diabetes mellitus (T2DM) and diabetic nephropathy (DN) for the detection of the relationship between chondroitin sulfate (CS) structure and disease. **Methods.** Healthy control (n=15), type 2 diabetic patients with normalbuminuria (n=12), and patients with microalbuminuria (n=13) were enrolled in the study. Total sulfated glycosaminoglycans (GAGs) concentration in the first morning urine was evaluated by 1,9-dimethylmethylene blue method and the composition was determined by agarose gel electrophoresis. Urinary chondroitin sulfate was quantified by a combination of treatment with specific lyase digestions and separation of products by SAX-HPLC. **Results:** GAGs concentration significantly increased in diabetic patients with microalbuminuria compared to diabetic patients with normalbuminuria. Qualitative analysis of urinary GAGs revealed the presence of chondroitin sulfate, heparan sulfate, and low-sulphated chondroitin sulphate-protein complex (LSC-PG). There was a decrease in CS and an increase in LSC-PG in the urine of patients with diabetes compared to healthy controls. Moreover, in diabetic patients, chondroitin sulfate contains more 6-sulfated disaccharide and less 4-sulfated disaccharide. There was a statistically significant difference in ratio of 6-sulfated disaccharide to 4-sulfated disaccharide among the three groups. **Conclusions:** GAGs were significantly increased in diabetic patients with microalbuminuria. The levels of urinary GAGs, ratio of LSC-PG/CS, as well as ratio of 6-sulfated to 4-sulfated disaccharides could be useful markers for diagnosis of patients with diabetic nephropathy.

INTRODUCTION

Chondroitin sulfate (CS) belong to a unique class of molecules call glycosaminoglycans (GAGs), which also include heparan sulfate (HS), dermatan sulfate (DS), keratan sulfate and hyaluronic acid (1). These molecules are important parts of plasma membranes and extracellular matrix (ECM). In most cases, GAGs are attached to core proteins forming proteoglycans (PGs) (2), which play an important role in cell proliferation, differentiation, cell migration, organ morphogenesis, and bacterial/viral infections (3-5).

CS is composed of disaccharide units [glucuronic acid (GlcA) (β1→3) N-acetyl-galactosamine (GalNAc) (β1→4)]$_n$ which could be sulfated at different positions of GalNAc residues and/or uronic acid (6). The differences of the urinary CS, 4-sulfated/6-sulfated ratio and its related degree of sulfation, could be useful for monitoring the progression of the disorder, such as pseudoxanthoma elasticum (7) and bladder pain syndrome (8).

Diabetic nephropathy (DN) is one of the most common diabetic complications characterized with ECM accumulation and glomerulosclerosis (9). Abnormal metabolism of PGs in the kidney has been reported to play an important role in the development of DN. Although HS constitutes 80-90% of total sulfated GAGs in the kidney (10), and was considered as a major component in charge selectivity of the glomerular basement membrane (GBM) (11), there was no correlation between

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alteration of glomerular HS structure and microalbuminuria in human diabetes and animal model of diabetes (12). Moreover, degradation of HS in the GBM did not result in proteinuria (13). However, CS has been found to be associates with an alternation of GBM in diabetic rats (14). In addition, diabetes resulted in structural alteration of CS in the kidney and altered their binding to extracellular matrix components (15). However, the structure of CS chains of DN patients during the disease progression has not been rigorously studied.

Normal human urinary GAGs contain mainly CS, HS (16) and low-sulfated chondroitin sulfate (LSC) proteoglycan (LSC-PG) (17). The relationship of urinary GAGs with albuminuria has been investigated both in patients with type 1 and type 2 DM (18, 19). Some studies observed that GAGs could be useful biomarkers for diagnosis. However, there are no reports available with respect to investigation of the GAGs compositions and structure of CS/DS in the urine during DN development. Therefore, in the current study, we investigate whether GAGs compositions and modification of CS structure in urine might reflect development of DN and be used as a biomarker to predict renal outcomes in type 2 diabetic patients.

MATERIALS AND METHODS

Study design

We conducted the study in accordance with the ethical principles of the Declaration of Helsinki, the Good Clinical Practice guidelines of the International Conference on Harmonization and local regulatory requirements. The study protocol was approved by the ethics committee of the hospital.

Subjects: Patients were recruited (aged from 40 to 75 years old) that suffered from type 2 diabetes mellitus over 5 years with normal kidney function or renal dysfunction. Those patients with other acute or chronic complications, malignant tumor, other nephropathy, infection, stress state, cardio function defined as NYHA class III and above or positive hepatitis B virus surface antigen were excluded. Total 25 qualified patients were then grouped in accordance with the degree of urinary albumin excretion (UAE) into DM with normoalbuminuria (<20μg/min) and DM with microalbuminuria (20-200μg/min). Patients had been treated with oral hypoglycemic agents (sulfonylureas, metformin, and acarbose). Angiotensin converting enzyme (ACE) inhibitors and/or Ca^{++}-channel blockers were used for hypertension treatment. HMG coenzyme A reductase inhibitors and statins were used in the regulation of blood lipids. Fifteen age and sex matched healthy volunteers were employed as control subjects. The demographic data and clinical characteristics of the study groups are summarized in Table 1. There were no significant differences in gender, age, body mass index (BMI) and diastolic blood pressure between healthy volunteers and patients. Systolic pressure values in DM with or without albuminuria group were within the normal range, but were significantly higher than that of healthy controls.

Urinary specimens: First void of morning urine samples were collected from healthy volunteers and patients. The specimens were centrifuged at 3000 rpm for 10 minutes and supernatants were stored at -80°C until assessment. Prior to assessment, the specimens were thawed and centrifuged. All urine specimens were assayed in a blinded manner to researchers. All patients had given their informed consent before the study.

Laboratory methods: For each urinary sample, the creatinine concentration was measured by Jaffe's method and other clinical parameters were determined by an automatic biochemistry analyzer. Serum levels of cholesterol, triglyceride, creatinine, high-density lipoprotein and low-density lipoprotein were measured using a CD-1600CS hematology analyzer (Abbott Labs, USA). Glycated hemoglobin (HbA1C) was measured by a turbidimetric inhibition immunoassay technique (Roche Diagnostics, USA).

Extraction and purification of GAGs from human urine: GAGs were isolated from 10mL of urine using ion-exchange chromatography on DEAE-Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden). After extensive washing of the column with the equilibrating buffer, the adsorbed material was eluted with 2M NaCl and 0.02M Tris-HCl, pH 8.6. All of the hexuronate-containing fractions were pooled and mixed with four volumes of ethanol at 4°C. The mixture was left overnight and GAGs were precipitated by centrifugation at 8,000 g for 15 min, washed twice with ethanol, and dried.

After solubilization with water, total sulfated GAGs concentration were estimated by the 1,9-dimethylmethylene blue (DMB) method (20) and the composition was determined by agarose gel electrophoresis in barium acetate/1,2-diaminopropane buffer and sequentially stained with toluidine blue and Stains-All as reported in previous publication (21).

Table 1. Clinical characteristics of the study groups.

	Healthy controls	Diabetes mellitus Normoalbuminuria	Microalbuminuria
N (male/female)	15(7/8)	12(5/7)	13(7/6)
Age (years)	54.1±4.3	61.6±7.4	58.3±8.7
Diabetes duration (years)	--	10.4±4.5	12.2±6.0
BMI (kg/m^2)	24.7±2.5	24.3±3.1	25.6±3.7
Systolic pressure (mmHg)	115±14	134±12[a]	130±15[a]
Diastolic pressure (mmHg)	77±9	80±8	80±9
Fast blood glucose（mmol/L）	5.29±0.48	7.70±2.56[a]	7.72±3.12[a]
HbA1C(%)	4.89±0.92	8.37±1.46[a]	8.89±2.58[a]
Urinary albumin excretion (μg/min)	ND	9.4±2.3	82.44±18.9
Serum creatine (μmol/L)	86.47±9.75	82.22±19.20	89.03±23.80
Urea nitrogen (mmol/L)	5.63±1.45	5.45±1.56	6.41±2.12
Cholesterol (mmol/L)	5.23±1.03	5.15±0.81	5.27±1.01
Triglyceride (mmol/L)	2.06±1.78	1.74±0.76	1.97±1.23
High-density lipoprotein (mmol/L)	1.15±0.23	1.12±0.30	1.29±0.36
Low-density lipoprotein (mmol/L)	3.17±0.88	3.20±0.61	3.12±0.82

[a] Statistically significant when compared to healthy subjects group at $p<0.01$.

LSC-PG were identified by comparison with co-migrating standard of urinary trypsin inhibitor (UTI). The gels were scanned and bands were analyzed by Image J program.

Qualitative and quantitative evaluation of unsaturated CS disaccharides from GAGs: Ten microliters of urinary extract was incubated with 25mU of chondroitinase ABC (Sigma-Aldrich, USA) at 37^{o}C for 18 h in 50mmol/L Tris-HCl buffer (pH 8.0). The reactions were stopped by boiling for 3 min. The disaccharide products of urinary CS were identified by strong-anion-exchange (SAX) HPLC as reported by others (8). Constituent disaccharides were identified by unsaturated disaccharides standard (Iduron Corporation, UK) and quantified by specific calibration curves. Charge density (the sulfate-to-carboxyl ratio) was calculated considering the presence and the percentage of carboxyl and sulfate groups for each disaccharide.

STATISTICAL ANALYSIS

Values are reported as mean±SD or median (range). One-way ANOVA was used to assess the difference between the study groups at $p<0.05$.

RESULTS

Effect of diabetes on basic parameters: As shown in Table 1, fasting blood glucose (FBG) levels in DM patients were significantly higher than that in healthy control. HbA1c (%) levels in patients with DM were higher than the normal range (4-6%), but there was no significant difference between patients with normoalbuminuria and with microalbuminuria. The serum concentrations of cholesterol, triglyceride, high-density lipoprotein and low-density lipoprotein did not show significant difference between diabetic patients and healthy control.

Effect of diabetes on total sulfated GAGs, HS and CS in the urine: GAGs excretions in healthy control and patients with normoalbuminuria and microalbuminuria were 1.05±0.62, 1.74±0.96, 3.12±2.09μg/μmol creatinine, respectively (Fig 1). There was a progressive increase in GAGs excretion from healthy controls to patients with normoalbuminuria to patients with microalbuminuria. GAGs excretion in patients with microalbuminuria was significantly increased compared with that in healthy volunteers and patients with normoalbuminuria ($p<0.05$).

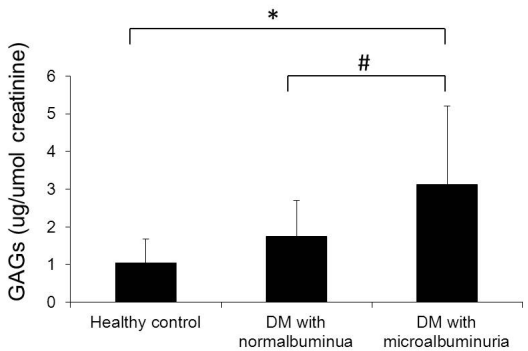

Figure 1. Urine GAGs in healthy controls and diabetic patients. Data are presented as mean±SD. * indicates $p<0.05$ between DM with microalbuminuria and healthy control. # indicates $p<0.05$ between DM with microalbuminuria and DM with normoalbuminuria.

However, there was no significant difference of GAGs excretion between healthy volunteers and patients with normoalbuminuria.

Urinary GAGs from healthy volunteers and patients are composed of CS, HS and LCS-PG as shown in Table 2 and Figure 2. Patients with microalbuminuria excreted less CS (23.61% versus 36.79%) but more LSC-PG (36.70% versus 24.28%) compared to healthy subjects. The ratio of LSC-PG to CS was 0.67, 1.15, and 1.55 for healthy volunteers, patients with normoalbuminuria and patients with microalbuminuria, respectively. There was a statistically significant difference among the three groups. Urinary content of HS was 38.73%, 41.96% and 40.69% for healthy control and patients with normoalbuminuria and microalbuminuria respectively. There was no statistically significant difference among these groups.

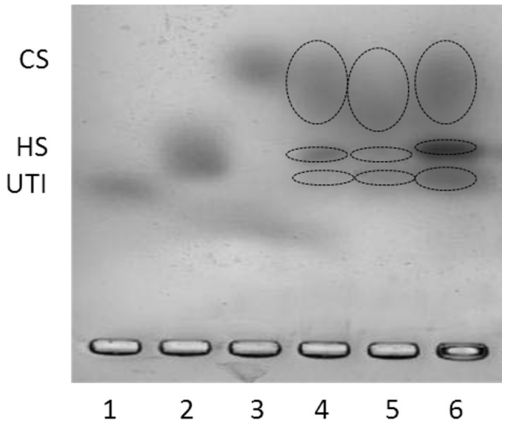

Figure 2. Agarose gel electrophoresis stained with toluidine blue and Stains-All of GAGs extracted from 10mL urine of healthy controls and diabetic patients. Lane 1: chondroitin sulfate (CS) standard; lane 2: heparan sulfate (HS); lane 3: UTI, urinary trypsin inhibitor; lane 4: GAGs extracted from healthy controls; lane 5: GAGs extracted from diabetic patients with normalbuminuria; lane 6: GAGs extracted from diabetic patients with microalbuminuria.

Effect of diabetes on disaccharides composition of CS in the urine: Urinary CS from healthy and patients were digested with chondroitin lyase, and unsaturated disaccharide products were analyzed as shown in Figure 3. Profile of non-sulfated disaccharide (ΔDi-0s), and monosulfated disaccharides, C-6 position of the galactosamine unit (ΔDi-6s) and C-4 position of the galactosamine unit (ΔDi-4s) in healthy and patients was shown in Table 3. Except for the decrease in ΔDi-6s in patients, ΔDi-0s and ΔDi-4s were all increased in patients with either normoalbuminuria or microalbuminuria. These changes in the percentages of urinary CS disaccharides are indicative of a different kind of polysaccharide amongst the groups, in particular for a greater charge density. There were no significant differences amongst these groups. However, when ratio of ΔDi-6s to ΔDi-4s was calculated, there was a progressive increase in ΔDi-6s/ΔDi-4s from healthy to patients with normoalbuminuria to patients with microalbuminuria (0.69 to 1.21 to 1.78, respectively). The differences were statistically significant amongst these groups.

DISCUSSION

GAGs are important components of GBM in the kidney. Therefore, urinary GAGs and their metabolites have been investigated in different kidney diseases (19, 22, 23).The present study demonstrated that urinary GAGs consist of CS, HS and LSC-PG. Compared with healthy control, urinary CS in patients with diabetics was relatively low compared to urinary LSC-PG. Thus, the ratio of LSC-PG/CS is quite different among these groups. Therefore, the ratio of LSC-PG/CS could be used as a diagnostic marker for diabetic nephropathy. It was reported that CS proportion is above 2/3 in the urinary GAGs and decreased with age (16, 24). LSC-PG occurs in blood and urine as the major chondroitin sulphate PG (25). It is an acidic glycoprotein composed of a core protein of 143 amino acids and two carbohydrate side chains: an oligosaccharide and a GAG chain consisting of a relatively low-sulfated chondroitin 4-sulfate chain (26-28). In urine, its concentration is very low in normal health controls and increases in inflammatory diseases, malignant diseases, pregnancy, post-surgical states and immune disease (23, 29-31). De Muro et al reported the ratio of LSC-PG/CS increased in DM condition (32), which are consistent with results in our current study. However, they did not report the condition of LCS-PG/CS in patients with nephropathy.

In the present investigation, we investigated the modifications of CS in urine from patients with diabetic nephropathy for the first time. The finding of CS unsaturated disaccharides such as ΔDi-0s, ΔDi-6s and ΔDi-4s in urine of healthy controls have been reported previously (7, 33), which are consistent with our findings in healthy controls. However, there is no report on these structural modifications of CS in diabetic patients. The profile of CS unsaturated disaccharides in diabetic patients with normoalbuminuria and microalbuminuria exhibited a quite different pattern from that of healthy controls.

Table 2. The relative concentrations of GAGs compositions obtained from the urine of healthy control, DM patients.

	Healthy controls	Diabetes mellitus	
		Normoalbuminuria	Microalbuminuria
CS%	36.79 (31.91-40.01)	26.99 (16.09-38.76)	23.61 (15.66-44.18)[a]
HS%	38.73 (32.89-43.96)	41.96 (35.35-48.48)	40.69 (32.04-47.76)
LSC-PG%	24.48 (13.87-29.54)	31.05 (19.66-38.74)	36.70 (26.78-44.89)[a]
LSC-PG/CS	0.67 (0.38-0.82)	1.15 (0.41-1.15)[a]	1.55 (0.51-1.62)[b,c]

Results are given as median (range). [a] Statistically significant when compared to healthy subjects group at $p<0.05$. [b] Statistically significant when compared to healthy subjects group at $p<0.01$. [c] Statistically significant when compared to DM patients with normoalbuminuria group at $p<0.05$.

Table 3. Relative percentages of nonsulfated (ΔDi-0s), 6-sulfated (ΔDi-6s) and 4-sulfated (ΔDi-4s) disaccharides for CS from diabetic patients and healthy subjects.

	Healthy controls	Diabetes mellitus	
		Normoalbuminuria	Microalbuminuria
ΔDi-0s, %	48.9% (23.9%-60.3%)	37.32% (23.1%-48.7%)	34.7% (19.7%-44.5%)
ΔDi-6s, %	21.0% (9.4%-41.8%)	41.4 (27.1%-52.8%)	39.5% (31.3%-49.7%)
ΔDi-4s, %	30.1% (16.6-37.3%)	21.3 (16.4%-59.8%)	25.6 %(10.7%-43.8%)
ΔDi-6s/ΔDi-4s	0.69 (0.31-1.12)	1.21 (0.98-1.68)[a]	1.78 (1.02-3.01)[a,b]
Charge density	0.51 (0.33-0.71)	0.63 (0.47-0.91)	0.65 (0.44-0.93)

Results are given as median (range). [a] Statistically significant when compared to healthy subjects group at $p<0.05$. [b] Statistically significant when compared to DM patients with normoalbuminuria group at $p<0.05$

There were more monosulfated disaccharide units and a lower percentage of non-sulfated disaccharide units. Moreover, the structural modifications generate CS that is sulfated to a greater extent in position 6 than in position 4 compared with healthy subjects. To date, there is little information available about CS unsaturated disaccharides in the kidney of diabetic patients. For example, Joladarashi and coworkers observed that percentage of CS/DS sulfation was reduced in diabetic rats (15). In addition, abnormal structures of urinary CS were determined in pseudoxanthoma elasticum patients (7) and the data are in agreement with those described with abnormal GAGs isolated from skin lesions of pseudoxanthoma elasticum patients. These results indicate that abnormal urinary CS might reflect abnormalities of CS-PG in pathological organs.

CS chains have been considered to participate only in structural stabilization and attracted less attention until recently, which indicates an important biological function. Sugar backbones of CS can mainly be sulfated at C2 position of uronic acid residues and/or at C4 and/or C6 positions of GalNAc residues to form various disaccharides. In addition to these ordinary units, non-sulfated GlcA-GalNAc (Di-0s) and rare disulfated D-unit [GlcA(2S)-GalNAc(6S)] (2S stands for 2-O-sulfate) and E-unit [GlcA-GalNAc(4S, 6S)] are often found in small proportions in mammals (34). Particular functional structures formed by combinations of these various disaccharide units may participate in specific binding to bioactive molecules (35) and hence may greatly influence overall functions of these molecules (36). For example, several CS-PGs have been characterized in the kidney such as neural/glial cell 2 (NG2)(37). NG2 is a high molecular weight and integral membrane chondroitin sulfate proteoglycan, which can be associated with collagen XV, leprecan and bamacan. Moreover, members of small leucine-rich proteoglycans such as decorin and biglycan also contained mixed CS/DS chains. Decorin and biglycan could bind type I collagen and TGF-β; therefore, they can inhibit TGF-β signal transduction and modulate fibrogenesis (38, 39). Alternation of CS/DS structures in the kidney resulted in a decreased binding toward type IV collagen and laminin, and in an increased binding to fibronectin in STZ induced diabetic rats (15).

In conclusion, our observation of total sulfated GAGs in urine of diabetic patients is consistent with previous reports. However, the profile of GAGs and

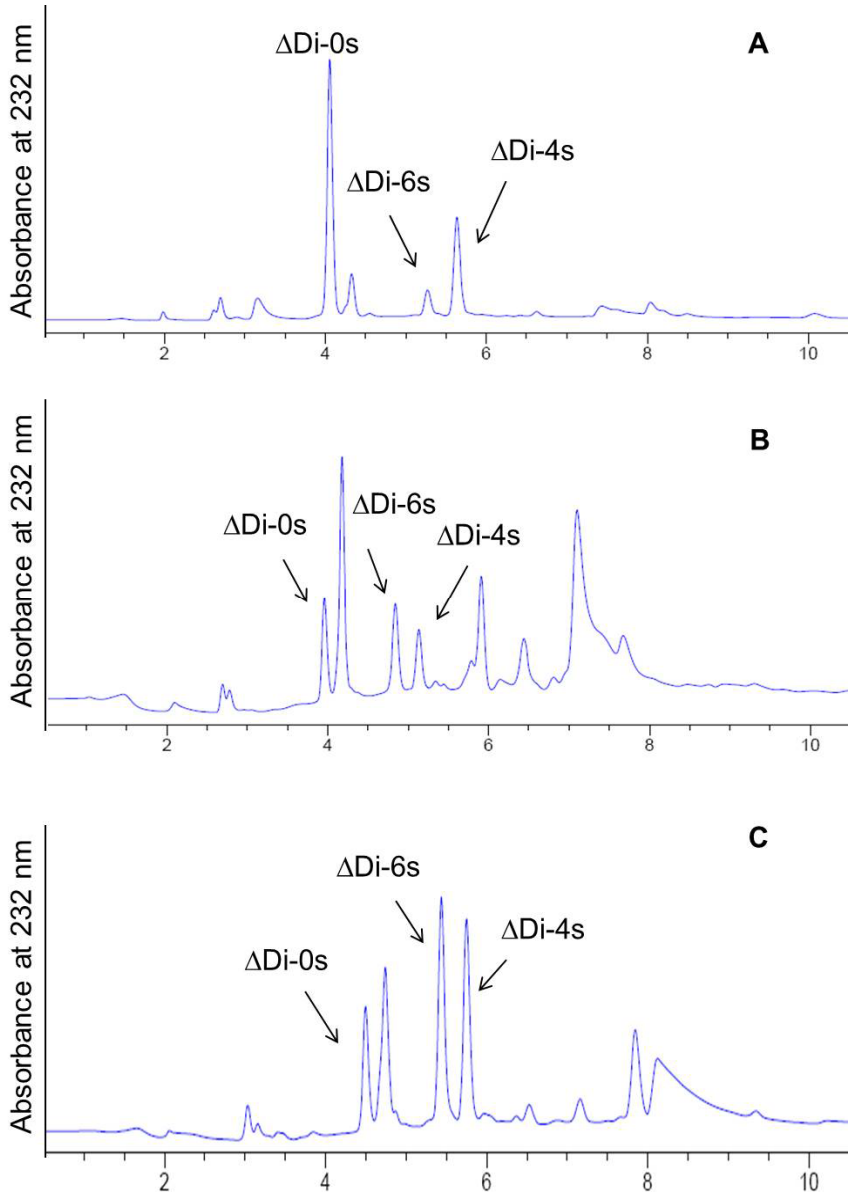

Figure 3. HPLC separation of unsaturated nonsulfated and variously sulfated disaccharides of CS after treatment with chondroitinase ABC. Unsaturated disaccharides of CS from normal urine (*A*), unsaturated disaccharides of CS from DM with normoalbuminuria patients urine (*B*), unsaturated disaccharides of CS from DM with macroalbuminuria patients urine (*C*). ΔDi-0s represents HexA-GalNAc; ΔDi-4s represents ΔHexA-GalNAc(4-OSO_3); ΔDi-6*s* represents ΔHexA-GalNAc(6-OSO_3).

CS modification in urine of healthy and patients with diabetic nephropathy has never been investigated. Therefore, ratios of LSC-PG/CS and ΔDi-6s/ΔDi-4s in the urine of patients with diabetic nephropathy could be used as diagnostic markers for diabetic nephropathy or markers for follow-up of a therapy.

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