Urinary glycosaminoglycans in patients with systemic lupus erythematosus

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Abstract

Objective

Several investigations indicate that glycosaminoglycans (GAG) are important components of the glomerular basement membrane (GBM) and that they play a remarkable role in the control of charge-selectivity in the glomerular capillary wall. In order to evaluate the possible use of GAG as a marker of glomerular disease, we evaluated urinary GAG excretion in 37 patients with systemic lupus erythematosus (SLE) grouped by disease activity and kidney involvement and in 17 healthy controls.

Methods

GAG were isolated from urine by using ion-exchange chromatography on DEAE Sephacel. GAG composition was determined by cellulose acetate electrophoresis and expressed as relative percentages by densitometric scanning of Alcian Blue stained strips.

Results

Total GAG levels were significantly increased only in active extra-renal SLE patients. Qualitative analysis of urinary GAG revealed the presence of a low sulphated chondroitin sulphate-protein complex (LSC-PG), whose frequency was higher in patients compared to controls. Moreover, inactive SLE was characterized by an alteration of the chondroitin sulphate/heparan sulphate ratio.

Conclusion

These variations suggest the presence of an abnormal permeability of the renal filter in patients without other appreciable signs of kidney alteration. Therefore, qualitative-quantitative urinary GAG analysis could represent an additional diagnostic approach.

Key words

Systemic lupus erythematosus, glomerular basement membrane, glomerular diseases, glycosaminoglycans, proteoglycans, low-sulphated chondroitin sulphate.

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Introduction

Recent studies on the mechanism of protein loss in urine have focused on the role of proteoglycans (PG), a macromolecular complex of glycosaminoglycans (GAG) and proteins, in the control of the anatomic and functional integrity of the kidney. PG, and in particular heparan sulphate proteoglycans (HS-PG), play an important role in the control of charge-selectivity in the glomerular capillary wall, being an important component of the glomerular basement membrane (GBM). Experimental studies in rats have shown that enzymatic removal of anionic sites in GBM was able to induce a loss of HS-PG and a significant increase in albumin excretion by the kidney (1). In congenital and acquired nephrotic syndrome, an increase in HS excretion with a positive correlation with urine albumin excretion has been described. However, no correlation with renal histology could be detected (2). Evaluation of renal biopsy in diabetic nephropathy showed a reduction in HS-PG content, thus confirming the role played by PG in the control of glomerular permeability due to their negative charge (3).

In normal subjects GAG can be detected in considerable amounts in urine, but only in very low concentrations in the serum (4). The main components in normal urine are chondroitin sulphate (CS) and heparan sulphate (HS), while dermatan sulphate (DS) is found only in small amounts (5, 6). Urinary GAG represent a heterogenous mixture of partially depolymerized and partially desulphated products of tissue glycosaminoglycans. Most investigations indicate that urinary GAG originate from the plasma by glomerular filtration, although part of the HS fraction may originate from renal tissue (7). An increase in their excretion and the relationship between GAG excretion, protein loss, tubules and glomerular damage is thought to be a marker of glomerular disease.

We evaluated GAG excretion in patients with systemic lupus erythematosus (SLE) with and without kidney involvement, with active disease and during clinical remission in order to evaluate their possible use as a non-invasive marker of renal disease.

Patients and methods

Thirty-seven patients (35 females and 2 males) entered this study. All were suffering from SLE and 20 of them had lupus nephritis. Their mean age was 36 ± 13 (range 15-60) years, and the average disease duration was 7 ± 4 (range 1-17) years. The diagnosis of SLE was based on the American Rheumatism Association criteria (8). The diagnosis of renal disease was made on the basis of proteinuria, cylindruria and haematuria. All patients with a renal disorder underwent a kidney biopsy in order to assess the type and severity of renal involvement and the course of the prognosis, as defined by the World Health Organization (WHO) (9). Fourteen had membranous proliferative glomerulonephritis (WHO class IV), 4 membranous glomerulonephritis (WHO class V), and 2 mesangial nephritis with proliferative pictures (WHO class III). SLE was considered to be active on the basis of the lupus activity criteria count (LACC) (10). Twelve of the patients with renal involvement had active disease at the moment of the study, whereas 8 had only minimal urinary signs. Seventeen patients had SLE without kidney involvement; 6 had active disease.

All patients were receiving corticosteroids at doses varying on the basis of clinical picture. The average prednisone dose ranged from 0.3 to 0.5 mg/ Kg b.w. every other day, whereas during induction they received 2 mg/ Kg b.w. every other day. In addition, all the patients with kidney disease received i.v. cyclophosphamide at a dose of 20 mg/Kg b.w. every 30 days. No patient had diabetes, chronic renal failure, liver disease or was taking drugs known to interfere with GAG synthesis or catabolism, apart from those being administered to treat SLE.

Seventeen normal subjects, 16 females and 1 male (mean age 38 ± 13 years, range 20-62 years), served as a control group.

Urine was collected over 24 hrs. No preservatives were used and the samples were not refrigerated during col-

lection; aliquots or whole samples were stored at -20°C until analysis. Urinary creatinine was measured by Jaffe's reaction (Boehringer-Mannheim), albumin by an immunoturbidimetric method (Roche).

GAG were isolated from urine by ion exchange chromatography on DEAE-Sephacel (Pharmacia) according to Staprans *et al.* (11). Fifty ml of urine were centrifuged at 5,000 g for 15 min and applied directly to a DEAE Sephacel column (0.7 x 8 cm) equilibrated with 0.15 M NaCl buffered with 0.02 M Tris-HCl, pH 8.6. After extensive washing of the column with the equilibrating buffer, the adsorbed material was eluted with 2 M LiCl and 0.02 M Tris-HCl, pH 8.6. Ten fractions (1 ml each) were collected and analyzed for their uronic acid content by the method of Bitter and Muir (12). All of the hexuronate-containingfractions were pooled and GAG were precipitated with 4 volumes of ethanol at 4°C. The mixture was left overnight and the precipitated was separated by centrifugation at 8,000 g for 15 min, washed twice with ethanol and dried.

The GAG composition was determined, after solubilization with water, by electrophoresis on acetate cellulose strips in a discontinous buffer, according to Cappelletti *et al.* (13). The GAG composition was expressed in terms of relative percentages based on densitometric scanning of Alcian Blue stained strips using a Scan Analysis Program (Thunder Scan-Biosoft). GAG identification was performed by treating aliquots of the samples (containing about 100 g of hexuronate) at 37°C for 18 hrs before electrophoresis with specific eliminases, as previously described (14). All enzymes were purchased from Sigma.

The specificity and efficiency of the enzyme treatment were checked using a standard GAG (Sigma) under the same experimental conditions. In some cases,aliquots of samples were freed of protein by papain treatment (150 g/ mg protein) in 0.1 M sodium acetate buffer, pH 6.2 containing 5.0 mM cysteine and 5.0 mM EDTA at 56°C for 48 hrs, precipitated with ethanol, solubilized with water and submitted to electrophoresis.

In order to evaluate the reliability of the densitometric GAG determinations, we analyzed three solutions prepared by mixing in different proportions standard CS and HS at the same concentration (5 mg/ml): 33.3% CS - 66.7% HS; 50%CS - 50% HS and 66.7% CS - 33.3% HS.

The percentages detected by the densitometer were:

38.6 ± 0.6% CS - 61.4 ± 0.6% HS;
48.9 ± 0.7 % CS - 51.1 ± 0.7% HS;
63.4 ± 1.3 %CS - 36.6 ± 1.3% HS

based on the mean of ten determinations ± SD, respectively.

The results are reported as means ± SD. Data from multiple subgroups were analyzed by a one-way analysis of variance. If differences were found, Student's t-test was used for comparison between two groups. Correlations were tested with the Spearman correlation coefficient test.

Results

No correlation was found between the total urinary GAG concentration and the erythrocyte sedimentation rate (ESC) or antinuclear antibodies, antibodies to DNA,antibodies to non-DNA nuclear and cytosolic antigen, complement components (C3, C4), or gammaglobulin concentrations.

Table I shows the data of diuresis, albuminuria and total urinary GAG levels reported as mg of hexuronate/g of creatinine. GAG levels were higher in patients with extra-renal disease compared to controls, though this difference was not statistically significant.

The electrophoretic patterns of GAG found in healthy urine are shown in Figure 1. Usually only 2 major bands, identified as CS and HS, were detected. However, some control subjects showed an additional component with slower electrophoretic migration than HS. This band did not correspond to any standard GAG and resisted treatment with different specific lyases. The proteolytic treatment of the samples produced a new band with slower electrophoretic migration than CS, that completely disappeared following separate treatments with either chondroitinase AC or ABC (Fig. 2). Therefore, the protein free-GAG component was identified as a low-sulphated chondroitin sulphate (LSC) and the untreated component as a proteoglycan (LSC-PG).

The electrophoretic patterns of urinary GAG from SLE patients (Fig. 3) showed CS, HS, LSC-PG and, sometimes, free LSC. Interestingly, LSC-PG was present in the 47% of control subjects and in almost all the SLE patients, with frequencies ranging from 83.3 to 100% (Table II). When examining subjects positive for LSC-PG excretion (Table II), urinary GAG levels were significantly higher in patients with extra-renal disease compared to controls.

Table III shows the relative percentages of urinary GAG in patients with LSC-PG excretion. Free LSC was found in 19% of all patients. CS/HS ratio was significantly reduced in patients with SLE remission, independently of renal disease.

Table I. Urinary excretion of protein and GAG in SLE patients (mean ± SD).

	No.	Diuresis (ml/24h)	Albuminuria (mg/24h)	Hexuronate (mg/g creatinine)
Controls	17	1159.7 ± 492.1	14.7 ± 10.3	3.68 ± 0.99
Inactive SLE without renal involvement	9	1222.8 ± 345.2	10.9 ± 6.6	4.03 ± 1.04
Active SLE without renal involvement	8	1264.3 ± 506.4	11.8 ± 8.3	5.03 ± 2.03
Inactive SLE with renal involvement	8	1350.0 ± 551.4	92.9 ± 108.9	4.10 ± 1.31
Active SLE with renal involvement	12	1331.7 ± 480.9	676.5 ± 678.0	3.93 ± 1.13

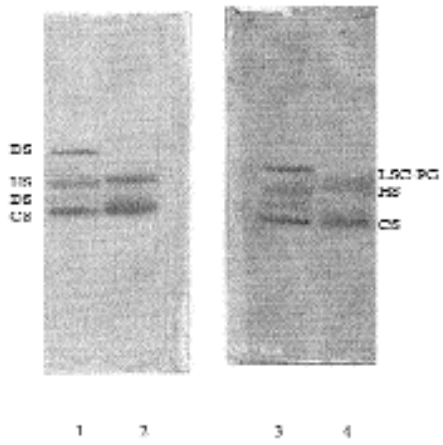

Fig. 1. Electrophoretic pattern of GAG in control urine: (1,3) reference standards; (2) urinary HS and CS; (4) urinary LSC-PG, HS and CS.

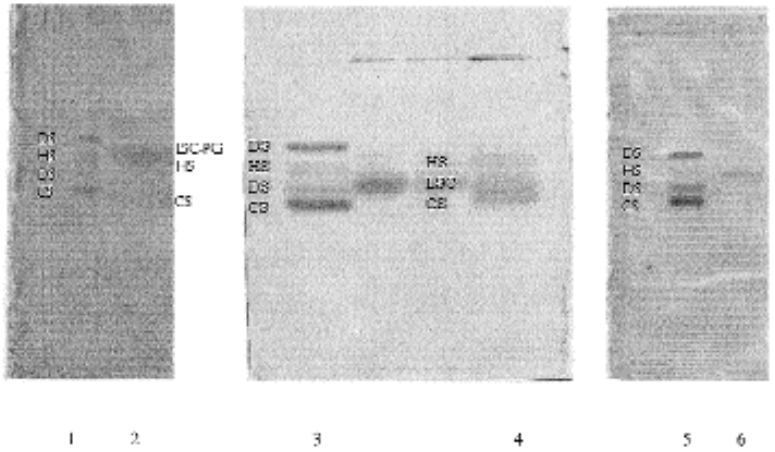

Fig. 2. Electrophoretic pattern of urinary GAG following enzymatic treatments: (1, 3, 5) GAG standard; (2) untreated urinary sample; (4) urinary sample after papain digestion; (6) urinary sample after papain digestion and chondroitinase AC treatment.

Discussion

Since GAG originate from different kinds of connective tissues, their measurement in urine may be useful to evaluate the metabolic state of various organs. Previous reports have shown that GAG concentrations in urine are age-dependent (15) and may be influenced by several diseases. Altered GAG levels have been reported in patients with benign or malignant neoplasia of the liver or kidney, with increases of DS in benign neoplasia, CS in malignant neoplasia and hyaluronic acid (HA) in Wilm's disease (16). Increased GAG excretion has been reported in rheumatoid arthritis (17), diabetes (18) and psoriasis (19). Corticosteroid administration is able to increase GAG excretion by acting on T lymphocytes (20).

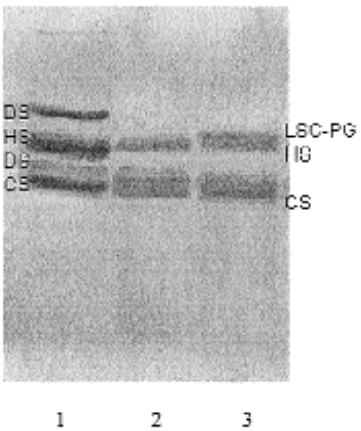

Fig. 3. Electrophoretic pattern of urinary GAG from patients (2, 3). The migration positions of standard GAG (1) are shown.

Glycosaminoglycans, and in particular HS, play an important role in GBM perm-selectivity due to their elevated negative charge, which increases membrane selectivity. Some authors have observed a relationship between albu-

Table II. Urinary GAG excretion in patients with LSC-PG excretion (mean ± SD).

	LSC-PG positive subjects No.	%	Hexuronate (mg/g creatinine)
Controls	8	47.0	3.43 ± 1.05
Inactive SLE without renal involvement	8	88.9	4.16 ± 1.05
Active SLE without renal involvement	7	87.5	5.44 ± 1.79*
Inactive SLE with renal involvement	8	100	4.10 ± 1.31
Active SLE with renal involvement	10	83.3	4.01 ± 1.21

* $p < 0.05$ versus control subjects.

Table III. Relative percentages of urinary GAG in patients with LSC-PG excretion (mean ± SD).

	LSC-PG + LSC (%)	HS (%)	CS (%)	CS/HS
Controls (n = 8)	34.6 ± 10 †	27.9 ± 9.0	37.4 ± 12.6	1.53 ± 0.8
Inactive SLE without renal involvement (n = 8)	40.7 ± 10.6 (LSC-PG 20.6 ± 7.5 LSC 22.2 ± 6.5; n = 2)¶	35.9 ± 11.0	23.3 ± 12.6	0.74 ± 0.5*
Active SLE without renal involvement (n = 7)	34.3 ± 11.8 (LSC-PG 27.9 ± 0.4 LSC 20.1 ± 2.5; n = 2)	34.9 ± 8.3	30.8 ± 9.3	0.94 ± 0.4
Inactive SLE with renal involvement (n = 8)	35.3 ± 9.6 (LSC-PG 29.2 ± 8.3 LSC 20.1 ± 7.0; n = 2)	34.1 ± 4.1	30.6 ± 8.1	0.90 ± 0.2*
Active SLE with renal involvement (n = 10)	39.8 ± 23.4 (LSC-PG 20.4 LSC 31; n = 1)	32.5 ± 6.4	34.4 ± 9.1	1.11 ± 0.4

† Only LSC-PG; *$p < 0.05$ vs control subjects.
¶ Mean values in parentheses indicate LSC-PG and LSC individual percentages in patients showing the additional excretion of free LSC.

min loss in urine and proteoglycan metabolism in patients with congenital nephrotic syndrome; this was suggested to be due to a possible altered incorporation of HS in GBM (21). Others observed a positive correlation between the HS/CS ratio and albumine excretion, but were unable to correlate this finding with the anatomical lesion (2). Data available on SLE patients are quite poor, although relatively increased GAG excretion has been reported (22, 23).

In our study the increased GAG excretion observed in SLE patients with active extra-renal disease could have been due to an activation of T lymphocytes, partly masked by immunosuppressive therapy. In lupic nephritis patients receiving high dose immunosuppressive therapy, we observed GAG levels similar to those measured in the control group. Qualitative analysis of the electrophoretic pattern of urinary GAG revealed the presence of a LSC-protein complex, as previously reported by others in patients with proteinuria due to various diseases (24). Ohkawa *et al.* (6) demonstrated LSC in the deproteinized urine of normal subjects and hypothized that the increase in LSC levels in elderly subjects reflects the aging process. Due to the methods employed, these studies were able to measure only the galactosaminoglycan component of the complex. In fact, the use of precipitating agents induces a co-precipitation of negatively charged urinary proteins and therefore proteolitic treatment is required. Recently (25), it has been proposed that LSC-PG is mainly present as an inter-α-trypsin inhibitor (ITI) in serum and as an urinary trypsin inhibitor (UTI) in urine, thus suggesting a possible plasmatic derivation for the latter. In that study, 10% of the total LSC was detected in urine as free chains.

In our study the percentage of patients with LSC-PG excretion was higher than that found in normal subjects, but no correlation with the progression of the disease was found. Under our experimental conditions, we did not find detectable levels of DS, in agreement with the findings of other authors (11, 24-26). The observed reduction in CS/HS ratio in lupic patients during remission of the disease is difficult to explain and might indicate a tubular or interstitial rather than a glomerular lesion. Lubec *et al.* (27) proposed the use of the CS/HS ratio as a semiquantitative index of tubular damage alternative to urinary protein electrophoresis on polyacrylamide gel. It may be hypothesized that the reduced CS urinary excretion is due to decreased glomerular synthesis of GAG or to increased incorporation in different pathological glomerular structures as observed by Tencer *et al.* (28). These authors reported reduced GAG excretion in patients affected by different kinds of primary glomerulonephritis. Moreover, this reduced CS urinary excretion may be due to an abnormal metabolism of urinary GAG, as evidentiated by the presence of free LSC. The possible influence of the reported tubular damaging action of non-steroidal anti-inflammatory drug (NSAID) administration cannot be excluded in our patients.

The electrophoretic study of the different urinary GAG showed LSC-PG, probably derived from plasma, in most of the patients and free LSC in 19% of them. The presence of this metabolite in the urine of healthy subjects has been described as a reflection of the aging process and in renal diseases with proteinuria it is thought to be due to an altered GBM metabolism. Furthermore, the variations in the percentages of excretion of HS and CS in urine might be a marker of a process of alteration of the renal filter. In conclusion, we believe that urinary GAG excretion is indicative of lupus disease, even if its value can be affected by immunosuppressive therapy. In this regard, there will be the necessity to evaluate the urinary GAG in a group of untreated SLE patients. However, data obtained in this study indicate that the qualitative and quantitative analysis of urinary GAG may represent an additional, non-invasive renal approach because it might indicate the presence of an abnormal permeability of the renal filter in patients without other appreciable signs of kidney alteration.

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