Isolation, Identification, and Quantitation of Urinary Glycosaminoglycans

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Key Words

Urine · Glycosaminoglycans · Heparan sulfate · Chondroitin sulfate · Dermatan sulfate · Age effects · Human

Abstract

Background: Substantial amounts of glycosaminoglycans (GAGs) are present in the urine of healthy individuals, but the concentration in the serum is very low. This finding suggests that urinary GAGs come from the glomerulus and may reflect the turnover of GAGs in the glomerulus. **Hypothesis:** However, little is known about the physiologic regulation of the urinary GAGs in humans, and so investigations are needed to evaluate the effects of age and sex on urinary GAGs in normal individuals. **Methods:** Eighty-seven healthy subjects were included in this study. Urinary GAGs were isolated and quantified at the nanogram level by combined azure A-silver staining in agarose gels. **Results:** The level of urinary GAGs peaked at 10–19 years in both sexes. The proportion of chondroitin sulfate decreased with age, but the proportion of heparan sulfate increased with age. **Conclusion:** The total amount of GAGs and the proportions of chondroitin sulfate, heparan sulfate, and dermatan sulfate appear to change with age. Therefore, investigations in which urinary GAG is used as a parameter of glomerular

GAG turnover should ensure that control groups are precisely matched for age. Changes in the proportions of each GAG may be more informative than their absolute levels.

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Introduction

The glomerular basement membrane is composed of a central dense layer and two thin layers of the lamina rara externa and the lamina rara interna. Caulfield and Farquhar [1] demonstrated the existence of anionic sites in all of the three layers, which consist of glycosaminoglycans (GAGs), mostly heparan sulfate [2]. GAGs comprise the repeating disaccharide units of hyaluronate, dermatan sulfate, chondroitin sulfate, keratan sulfate, and heparan sulfate. These are formed by polymerization of a specific disaccharide and subsequent modifications including the addition of sulfate groups and inversion of the carboxyl group on carbon 5 of *D*-glucuronic acid to yield *L*-iduronic acid. In the Golgi apparatus, sulfate groups are added to sugars after their incorporation into oligosaccharides. Heparan sulfate chains, which are connected to core proteins by a three-sugar linker, are formed by the sequential addition of glucuronic acid and N-acetylglucosamine [3].

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Recently, Raats et al. [4] elegantly reviewed the mechanisms underlying alterations of glomerular heparan sulfate. They proposed several mechanisms leading to reduced heparan sulfate function: masking by nucleosome-containing immune complexes [5], depolymerization by reactive oxygen species [6], neutral serine proteinases such as elastase and/or cathepsin, and hyperglycemia-induced reduction of heparan sulfate synthesis and sulfation. However, the precise mechanisms of the physiologic and/or pathologic regulation for the synthesis and/or secretion of GAG in glomerular cells are not yet known.

Under normal physiologic conditions, both mesangial cells and epithelial cells are involved in the remodeling (synthesis and degradation) of both the mesangial matrix and the glomerular basement membrane. The main heparan sulfate proteoglycans in the basement membrane are perlecan, agrin, and collagen XVIII. Perlecan and collagen XVIII are most abundant in the mesangial matrix, whereas agrin is a major heparan sulfate proteoglycan in the glomerular basement membrane [4]. Two classes of proteinases have been identified in mesangial cells *in vivo* and *in vitro* [3]: matrix metalloproteinases and serine proteases (plasminogen activators), together with their inhibitors. Catabolism of mesangial cell proteoglycans occurs by two separate routes: (1) limited proteolysis of core proteins and their release into the extracellular environment, and (2) internalization and degradation. Mesangial cells metabolize proteoglycans less actively than do glomerular epithelial cells.

Substantial amounts of GAGs have been shown to be present in the urine of healthy individuals, but the concentration in the serum is very low [7]. A part of urinary GAGs may be derived from the cells lining the renal tubule and urinary tract. However, keeping in mind that approximately 1.6 million glomeruli are present in kidneys and many cells in glomeruli are active in producing GAGs, it seems likely that the main source of the urinary GAGs is glomerulus.

It seems likely that the pool of GAG in the glomerulus is in a dynamic state between synthesis and degradation. This assumption may be extended to the hypothesis that GAG in the urine may reflect the turnover of GAG in the glomerulus. Urinary GAG has been suggested as a clinical marker in various diseases, including lupus nephritis [8, 9], urinary bladder damage and bladder tumors [10–13], mucopolysaccharidoses [14], renal amyloidosis [15], primary glomerulonephritis in experimental animal models and humans [5, 16, 17], proteolytic injury of endothelial and basement membranes which induce capillary leaks in meningococcal septicemia [18], chronic pyelonephritis

[19], chronic obstructive pulmonary disease [20], and diabetic nephropathy [21–25]. An association between heparan sulfate proteoglycan in urine and proteinuria has been reported after renal transplantation [26]. The observations of lower levels of urinary GAG in urolithiasis patients have led to suggestions that GAG are inhibitors of renal stone formation [27, 28].

However, the results of previous studies on urinary GAGs have not been consistent, with some reporting a positive correlation between glomerular disease activity and urinary GAGs [9, 19, 22, 23, 26], while others have reported a decrease in urinary GAG levels during the glomerular injury [15, 17, 21]. Many factors seem to be involved in these past discrepancies. First of all, in many studies the number of subjects was small, and the subjects were heterogeneous in age and sex distributions, and in disease activity. A more serious problem is the lack of information about the physiologic regulation of urinary GAGs. Pons et al. [29] reported circadian changes in urinary GAG excretion in normal rats, but little is known about the physiologic regulation of urinary GAGs in normal humans. It has been reported that urinary GAG excretion decreases with age during the first 15 years and maintains a constant low value between the ages of 17 and 40 years [14]; however, that report did not provide details of the age distribution, and the number of the subjects was small.

Considering that growth and development may have significant effects on the turnover of structural components in organs, we hypothesized that physiologic growth of humans may influence the GAG turnover in the glomerulus, and consequently the levels of urinary GAGs. More information about the physiologic regulation of urinary GAGs is necessary before we can clarify if urinary GAG is a powerful marker of glomerular disease activity. In this regard, fundamental investigations are needed to evaluate the effects of age and sex on urinary GAGs. In the present study, we measured the urinary GAGs in males and females within different age groups, with the subjects ranging in age from 7 to 60 years.

Materials and Methods

Study Subjects

The study subjects were male and female children and adults, recruited from an elementary school, a junior high school, a high school, a medical school, and a university teaching hospital in Chonan, Korea. Inclusion criteria were normal range of growth and development in physical condition assessed by height and body weight (5th–95th percentile for Korean children) [30]. Exclusion cri-

Table 1. Heights and weights of subjects (mean $\pm$ SD)

Age, years	Height, cm		Weight, kg	
	male	female	male	female
<10	127.3 $\pm$ 6.0 (3)	128.2 $\pm$ 6.4 (6)	31.0 $\pm$ 6.5	30.1 $\pm$ 6.2
10–19	160.2 $\pm$ 9.7 (24)	155.1 $\pm$ 8.6 (24)	66.8 $\pm$ 8.7	54.9 $\pm$ 6.2
20–29	172.8 $\pm$ 5.5 (5)	160.2 $\pm$ 4.9 (5)	66.9 $\pm$ 7.3	55.8 $\pm$ 5.4
30–39	170.8 $\pm$ 4.5 (5)	160.3 $\pm$ 5.2 (5)	65.0 $\pm$ 6.7	57.6 $\pm$ 6.6
>39	169.5 $\pm$ 5.6 (5)	159.4 $\pm$ 6.2 (5)	64.0 $\pm$ 7.6	58.8 $\pm$ 6.7

Figures in parentheses are number of subjects.

teria were microhematuria and/or proteinuria in urinalysis, and history of any renal disease. We randomly selected school students after explaining our study design to the class teachers. Participation was voluntary and written informed consent was obtained from participants or their guardians. This study was approved by the Institutional Review Board of Soonchunhyang University Chonan Hospital (Chonan, Korea).

The heights and weights of the subjects are summarized in table 1. The age strata comprised the following age groups: <10 years, 10–19 years, 20–29 years, 30–39 years, and >39 years. All of the subjects were healthy, and urinalysis and blood chemistry were within normal limits. Urine samples collected between 9.00 and 10.00 a.m. were stored in an ice jar and delivered to the laboratory within 1 h. Some urine was subjected immediately to routine urinalysis and quantitative measurement of protein and creatinine levels, and other samples were stored at -70°C until GAG measurement.

Isolation and Quantification of Urine Glycosaminoglycans

Most of the materials were obtained from Sigma (St. Louis, Mo., USA) except for the following: DEAE (diethylaminoethyl) Sepharose fast flow (Pharmacia, Uppsala, Sweden), micro bio-spin column (Bio-Rad, Hercules, Calif., USA), and agarose I (Amresco, Solon, Ohio, USA). Urine GAG was isolated and quantified as described previously [31], with slight modifications for this study. A 0.5-ml aliquot of urine was diluted with 0.75 ml of 10 mM Tris, pH 6.8, and passed through 0.2 ml DEAE Sepharose fast-flow equilibrated with the same buffer. DEAE Sepharose was washed with six column volumes of 10 mM Tris, pH 6.8, containing 0.2 M NaCl. The GAG was eluted with two column volumes of 10 mM Tris containing 2 M NaCl, followed by six column volumes of 10 mM Tris. GAG was precipitated by the addition of five volumes of ice-cold ethanol, incubation overnight at -20°C , and centrifugation for 10 min at -10°C . The precipitates – dried briefly with SpeedVac (Sarvant, Farmingdale, N.Y., USA) – were dissolved in 0.05 ml water, and an aliquot of them was used for quantification. Total amounts of GAGs in urine are expressed here as the number of micrograms per milligram of creatinine.

1% (w/v) agarose gel in 50 mM barium acetate, pH 5.0, supported by gel-bond film (FMC, Rockland, Me., USA) was made by using the mini-IEF gel casting kit (Bio-Rad). One microliter of isolated GAG containing a known amount of chondroitin sulfate, dermatan sulfate, and heparan sulfate as a standard was loaded on the gel for 5 min for sample absorption into it. GAG was separated for 30 vh programmed in PHAST (Pharmacia) at 15°C . The gel was prestained with 0.1%

azure A in 50 mM sodium formate/10 mM MgCl_2 , pH 3.5, destained twice with 10 mM sodium acetate, pH 5.5, and water, and air-dried overnight. The backfilm of gel was cleaned with 96% ethanol and the gel was washed briefly with water. The GAG was then silver stained with 30 mM ammonium nitrate/15 mM silver nitrate/1.75 mM tungstosilicic acid/75 mM formaldehyde/240 mM sodium carbonate until the bands appeared. The reaction was stopped by transferring the gel into water for 2 min, 1% (v/v) acetic acid for 5 min, and again into water for 15 min. The gel was air dried and scanned. The amounts of each type of GAG were calculated by comparison of volumes with known standards [32]. The following standard GAG mixtures were used as controls: S1, chondroitin sulfate and dermatan sulfate 12.5 ng each, heparan sulfate 25 ng; S2, chondroitin sulfate and dermatan sulfate 1.25 ng each, heparan sulfate 2.5 ng (fig. 1).

Statistical Analysis

Data are presented as means $\pm$ SDs. Differences in variables among groups were tested by regression analysis or one-way analysis of variance (ANOVA). Post-hoc analysis was applied if ANOVA was significant. Probability values less than 0.05 were considered significant.

Results

The mean concentration of urinary GAG was 24.0 ± 10.8 $\mu\text{g}/\text{mg}$ of creatinine in normal humans. The level of urinary GAG was significantly different among the different age groups tested by ANOVA. The 10–19 years age groups had significantly higher urinary GAG levels than other groups ($p = 0.007$; fig. 2). It appears that the levels of GAG are low in childhood, reach a peak in the 10–19 years, and then maintain a constant value with increasing age (fig. 2). There was no significant difference in the urinary GAG concentration between male and female groups (data not shown). The proportions of chondroitin sulfate and heparan sulfate were significantly different among the different age groups. The proportion of urinary chondroitin sulfate was significantly decreased with age (β coefficient = -0.47 , $p < 0.001$; fig. 3), whereas that of

Fig. 1. Agarose gel electrophoresis of urinary glycosaminoglycans from normal humans. The glycosaminoglycans were extracted from urinary samples as described in Materials and Methods. The glycosaminoglycans were stained by azure A and silver. Lanes S1 and S2, mixtures of standard glycosaminoglycans (S1: chondroitin sulfate and dermatan sulfate 12.5 ng each, heparan sulfate 25 ng; S2: chondroitin sulfate and dermatan sulfate 1.25 ng each, heparan sulfate 2.5 ng); lane 1, 50 s; lane 2, 40 s; lane 3, 30 s; lane 4, 20 s; lane 5, 10 s. CS = Chondroitin sulfate; DS = dermatan sulfate; HS = heparan sulfate.

Fig. 2. Plots of urinary glycosaminoglycans (micrograms per milligram of creatinine) by age group. Error bar = 95% confidence interval of the mean.

Fig. 3. Proportion of components of urinary glycosaminoglycan (GAG) by age group. Error bar = Standard deviation of the mean. CS = Chondroitin sulfate; DS = dermatan sulfate; HS = heparan sulfate.

urinary heparan sulfate was significantly increased with age (β coefficient = 0.53, $p < 0.001$; fig. 3). The largest proportion of GAG was chondroitin sulfate (62–77%, depending on the age stratum). Dermatan sulfate was less than 5% at all of the age groups. There was no significant difference in the sex distribution within any group, and the proportions of the GAGs did not vary with sex (data not shown).

Discussion

In the present study, GAG concentration in urine was the highest in the 10–19 years age group. The level of GAG in urine increases when weight-gain processes are active. When these reached a plateau, there was no signifi-

cant change of the urinary GAG levels during the observation period. The age strata were only between 7 and 60 years old. In this study, further study is therefore necessary in children younger than 7 years and adults older than 60 years. The proportion of chondroitin sulfate decreased with age, whereas the proportion of heparan sulfate increased with age.

There was no significant difference of GAG levels in urine between males and females within a given age stratum. This finding implies that the turnover of GAG is not under the influence of sex hormones or genes encoded in the Y chromosome.

The exact role of GAG in urine is not yet known. However, considering that its molecular weight is too high for it to pass through the intact glomerular basement membrane and that the serum concentration is negligible, the

GAG in urine should be the turnover product of GAG in the so-called urinary space of the glomerulus which is surrounded by the lamina rara externa, glomerular visceral epithelial cells, mesangium extended to the basement membrane, and parietal epithelium.

Cultured human mesangial cells are known to synthesize and secrete several proteoglycans with chondroitin and dermatan sulfate chains [33–37]. Fetal calf serum, interleukin-1, platelet-derived growth factor, and transforming growth factor- β increase proteoglycan synthesis by cultured mesangial cells in a dose-dependent manner [37]. There is considerable evidence that cytokines are involved in diabetic glomerular injury [21–25] and/or immune-mediated renal injury [5, 16]. Taking these observations together, the level of urinary GAG appears to be a marker reflecting GAG turnover in diabetes mellitus and/or immune-mediated glomerulopathy. Glomerular visceral epithelial cells in culture are also known as a major source of proteoglycans, which are constituents of the glomerular basement membrane that bear anionic charges and thus play an essential role in the permeability of the glomerular capillary wall to proteins [38–41]. Therefore, the urinary GAG turnover is likely to be influenced by the various physiologic and/or pathologic conditions that both mesangial cells and visceral epithelial cells are subjected to.

The pathologic regulation of urinary GAG is beyond the scope of the present study. However, Mitsunashi et al. [7] reported that urine heparan sulfate/creatinine levels decreased during remission and increased markedly during the nephrotic periods in patients with minimal changes in glomerulonephritis. This finding suggests that the level of urinary GAG can be used not only as a very informative parameter in understanding the pathogenesis of glomerular injury, but also it may be a clinical predictor of therapeutic responsiveness in glomerular injuries. How

the synthesis and secretion of GAG in glomerular cells are regulated is not clear. Under normal physiologic condition, both mesangial cells and glomerular epithelial cells synthesize and/or degrade both the mesangial matrix and the glomerular basement membrane. Considering that heparan sulfate is the main GAG of the anionic site in the basement membrane, our results reveal that during the aging process the glomeruli are exposed to environments in which the heparan sulfate turnover rate is stimulated.

One question to be answered in our study is the possibility that urinary GAGs may be derived from the cell surfaces of epithelial cells lining the nephron, ureter, urinary bladder and urethra. Further study is necessary to discriminate the glomerular origin of GAGs from that of the urinary tract.

In conclusion, our results show that the main GAGs in urine are chondroitin sulfate, heparan sulfate, and dermatan sulfate, in that order. The overall levels of GAGs change with age, peaking during adolescence in both genders. Dermatan sulfate was negligible in all of the age groups, and the proportion of chondroitin sulfate decreased with age. In contrast, the proportion of heparan sulfate showed changes that were reciprocal to the changes of the proportion of chondroitin sulfate. When the level of urinary GAG is used as a parameter of the glomerular turnover of GAG, control groups should be precisely matched for age. Moreover, changes in the proportions of each GAG may be more informative than their absolute levels.

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