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Composition of urinary glycosaminoglycans in a patient with relapsing polychondritis

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Abstract

Objectives: Several investigators have reported an increase in urinary glycosaminoglycans (GAGs) in patients with relapsing polychondritis (RP). The aim of this investigation is to analyze the composition and structure of urinary GAGs from a Brazilian patient with RP.
Design and methods: The identification and structural analyses of the GAGs were made by electrophoresis and degradation with specific enzymes and identification of their disaccharides products by HPLC chromatography.
Results: The disaccharide products formed from RP urinary chondroitin sulfate (CS) by action of chondroitin ABC lyase showed a substantial relative increase of nonsulfated disaccharides with a relative decrease of 6-sulfated disaccharides compared to control subjects. In addition, a significant change of the ratio of CS and heparan sulfate was also observed in the RP patient.
Conclusion: The RP patient analyzed has shown a structural anomaly of the urinary CS and this may contribute to the diagnosis of this disease.

Keywords: Glycosaminoglycans; Urinary excretion; Relapsing polychondritis

1. Introduction

Relapsing polychondritis (RP) is a systemic inflammatory disorder characterized by recurrent inflammation of the cartilaginous tissue of the ears and nose, and less frequently of the ribs, larynx and tracheal rings, in association with fever, arthropathy, involvement of the eyes and other laboratory abnormalities [1,2]. Several investigators have reported an increase in GAGs in the urine from patients with RP [1,3], suggesting that an increase in GAGs in the urine from patients with RP might be related to cartilage destruction. On the other hand, Tadaki et al. [4] have reported an increased urinary excretion of GAGs in a Japanese patient with RP, where the major elevated GAGs detected in the urine were found to be dermatan sulfate (DS) and hyaluronic acid (HA), suggesting that these urinary GAGs were derived from the skin, rather than from the cartilage.

Here we report a case of a Brazilian patient who showed the cardinal features of this unusual disease and also a urinary GAG composition different from that reported on the Tadaki's case.

2. Material and methods

2.1. Subjects

All patients participating in this study signed informed consent to a protocol that was reviewed and approved by the Ethics Committee of the University Hospital CFF/UFRJ.

The RP patient, a 41-yr-old Brazilian man, with 6-month history of systemic symptoms of light dyspnoea, chest pains and conjunctival edema was examined in September 2000. Two months later, the patient developed an edematous swelling, redness and warmth with tenderness in both his

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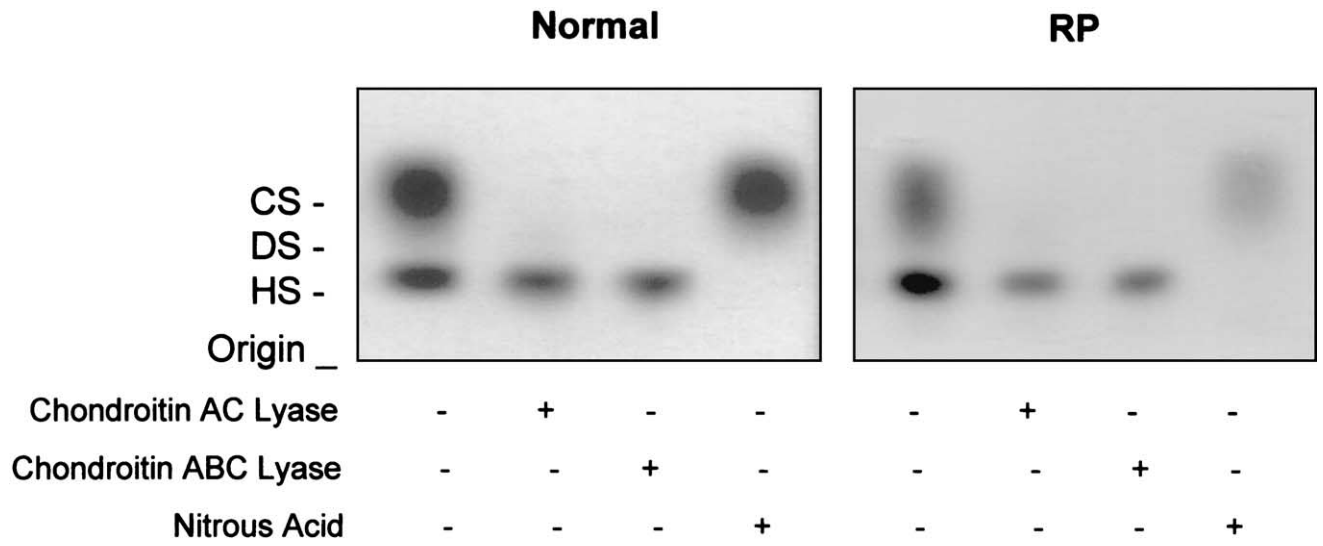

Fig. 1. Representative electrophoretograms of sulfated GAGs from *normal* and from *RP* urines, before and after chondroitin lyase digestion or deamination with nitrous acid. Mono Q-purified urinary GAGs, before and after depolymerization by *chondroitin AC lyase*, *chondroitin ABC lyase* and *nitrous acid*, were analyzed by agarose gel electrophoresis. The urinary GAGs were applied to 0.5% agarose gel and electrophoresis was carried out in 0.05 mol/L 1,3-diaminopropane:acetate (pH 9.0) for 1 h at 120 V. The GAGs in the gel were fixed with 0.1% *N*-cetyl-*N,N,N*-trimethylammonium bromide for 12 h and stained with 0.1% toluidine blue in acetic acid:ethanol:water (0.1:5:5,v/v). Standard GAGs consisted of a mixture containing 10 μg each of chondroitin 4-sulfate (*CS*), dermatan sulfate (*DS*) and heparan sulfate (*HS*) and their electrophoretic mobility are shown on the left.

auricles, except for the part of the lobulus. Ophthalmologic examination revealed ocular inflammation (episcleritis and conjunctivitis). Pulmonary investigation by both, bronchoscopy and expirometry, demonstrated mucosal edema and collapse of the lumen and also an obstructive disturbance. The results of routine laboratory investigations were normal. A skin biopsy specimen taken from the right auricle showed perichondral inflammation with an exhudate composed of neutrophils and lymphocytes. Upon the diagnosis of RP, systemic prednisone 20 mg/day effectively suppressed all the symptoms.

2.2. Extraction of urinary GAGs

Patient's urine was collected during 24 h. Normal urine samples were obtained from three healthy Brazilian adults with age ranging from 30 to 42 yr. The GAGs from the urine were extracted according to the method described by Dietrich et al. [5].

2.3. Identification and structural studies of the GAGs

The urinary GAGs were purified by anion-exchange chromatography on a Mono Q-FPLC column [6] and characterized by agarose gel electrophoresis, digestion with chondroitin lyases and deaminative cleavage with nitrous acid as previously described [7,8]. Analyses of the disaccharides from urinary CS formed by exhaustive digestion with chondroitin ABC lyase were performed on SAX-HPLC analytical column [9].

3. Results

A purified extract of total urinary GAGs from normal subjects was compared to standard sulfated GAGs by electrophoresis on agarose gel, before and after digestion with specific chondroitin lyases or deamination with nitrous acid (Fig. 1). One urinary GAG band migrated on agarose gel as HS standard and disappeared totally from the gel after deaminative cleavage with nitrous acid. Another band migrated between the DS and CS standards. It was totally digested by chondroitin AC or ABC lyases. These experiments characterized these two normal urinary GAGs as HS and CS, respectively. Sulfated GAGs extracted from the RP urine showed a similar electrophoretic pattern to those of normal urine (Fig. 1). Densitometric analysis of the electrophoretic bands (Fig. 1) allowed an estimation of the relative proportions of the two sulfated GAG species detected (Table 1a). These results showed that for normal urine, HS accounted for approximately 30% of total sulfated GAGs and the proportion of CS represented the remaining 70% of total GAGs. It can be seen that there are differences in the proportions of GAGs in the RP urine, with an increase in HS and a decrease in CS, when compared to the figures of normal urine (Table 1a). In addition, no DS GAGs were detected in our analysis, since this GAG species if present, would be resistant to the treatment with chondroitin AC lyase.

The methodology employed in the experiment of Fig. 1 does not allow identification of nonsulfated GAGs (mainly HA), nor structural information on disaccharide composition of CS. Therefore, two complementary protocols for

Table 1
Relative proportions of sulphated GAGs[1] and of disaccharides[2] formed by action of chondroitin ABC lyase on chondroitin sulphate from urine of normal and RP patients.

Compounds	Percentage of the total (%)			
	Normal[3]			RP
	N1	N2	N3	
a) Sulphated GAGs:				
Heparan sulphate	27	30	23	43
Chondroitin sulphate	73	70	77	57
b) Disaccharides from chondroitin sulphate:				
α-ΔUA-1→3-GlcNAc[4]	16	28	16	36
α-ΔUA-1→3-GalNAc($6SO_4$)	36	22	35	19
α-ΔUA-1→3-GalNAc($4SO_4$)	48	50	49	45

[1] Relative values are percents of total and were obtained by densitometry of the electrophoretograms as those shown in Fig. 1.

[2] Proportions of disaccharides were determined by integration of the areas under the peaks on the HPLC-chromatograms.

[3] Values represent three different control subjects (N1, N2 and N3).

[4] The abbreviations used were α-ΔUA, α-$\Delta^{4,5}$ -unsaturated hexuronic acid; GalNAc, N-acetylated galactosamine; GalNAc($4SO_4$), and GalNAc($6SO_4$) derivatives of N-acetylated galactosamine bearing a sulphate ester at position 4 and at position 6, respectively.

identification of urinary GAGs were employed: a) Analysis of the chromatographic pattern of urinary GAGs on an anion-exchange Mono Q-FPLC column (Fig. 2); and b) Analysis of the unsaturated disaccharides from urinary CS formed by chondroitin ABC lyase digestion (Table 1b).

We analyzed urinary GAGs from normal and RP patients by anion-exchange chromatography on a Mono Q-FPLC column previously calibrated with a mixture of standard HA and CS GAGs (Fig. 2A). Using elution with a linear gradient of NaCl, the standard GAG mixture showed two peaks, one eluting at 0.5 mol/L NaCl, composed of HA that was detected by the content of uronic acid (Fig. 2A, open circles), and another that eluted at 1.0 mol/L NaCl, composed of CS that was detected by the content of uronic acid (Fig. 2A, open circles) and by its metachromatic property (Fig. 2A, filled circles). The urinary GAGs from normal and RP patients eluted at 1.0 mol/L NaCl as a single metachromatic peak containing uronic acid corresponding to sulfated GAGs (Fig. 2B and C, respectively). These results showed that no HA was present on both normal and RP urines.

The products formed by exhaustive action of chondroitin ABC lyase on the CS from normal and RP urines were analyzed on a SAX-HPLC column and the results are shown in Table 1b. The disaccharides α-ΔUA-1→3-GalNAc, α-ΔUA-1→3-GalNAc($6SO_4$) and α-ΔUA-1→3-GalNAc($4SO_4$) accounted for approximately 20%, 30% and 50% of the total products derived from CS of normal urine, respectively. Although we did not perform a statistical analysis, due to the difficulty in finding other RP patients, our results revealed differences in the relative proportions of unsaturated disaccharides derived from CS of RP urine, where the relative proportion of nonsulfated disaccharides increased up to 36% with a relative decrease of 6-sulfated disaccharides down to 19%, while the proportion of 4-sulfated disaccharides remained relatively unchanged (45%).

4. Discussion

In the present study, we analyzed the GAG composition in urine from a Brazilian patient with RP using biochemical techniques. The GAGs found in the urine of the patient were composed of HS and CS. Conversely, Tadaki et al. [4] have found that DS (73%) and HA (27%) were the only GAG species present in the urine of a Japanese patient with RP. These conflicting results may either reflect RP patients with different extension of cartilage destruction or be partially due to the different biochemical techniques used by Tadaki and colleagues and by us. In their case, they have used biochemical methods for the detection of GAGs, based exclusively on cellulose acetate electrophoresis and staining of the strips with alcian blue allied with enzymatic digestion of GAGs by testicular hyaluronidase or chondroitin lyases. In our case, we have employed highly sensitive and reproducible biochemical techniques (hexuronic acid and metachromatic assays, agarose gel electrophoresis, chemical and enzymatic GAG degradation treatments, anion-exchange chromatography and HPLC analysis) to determine their qualitative composition and structures.

Histologic and histochemical examinations of biopsies from involved cartilages in RP patients have revealed a complete or relative loss of GAGs [10,11]. Therefore, it is reasonable to presume that the increased urinary GAGs noticed in this disease are induced by cartilage destruction. In fact, in the urine of our RP case the detected GAGs suggest that they may be derived from cartilage. This finding is distinct from that reported in Tadaki's case [4], where the GAG composition detected was suggested as having derived from the cutaneous tissue rather than cartilage. It is possible that the extent of the cartilage destruction in our case was greater than that observed in the Japanese case. The authors have in fact suggested in their work that possibly the extent of the cartilage destruction in their case would be unusually small compared with skin involvements as a case of RP [4]. However, we cannot rule out the possibility that the release of HA and DS, derived from skin tissue destruction caused by inflammation, if present in the urine of our RP patient, might not be large enough to be precipitated and were not measured in this work.

When we compared the relative proportions of GAGs obtained from urine of the RP patient with those of normal subjects, using the same biochemical methodology, the RP patients, contrary to our expectations, excreted relatively less CS as regards to the urinary HS. Thus, the average CS/HS ratio found for the RP patient was 1.3 compared to the average ratio of approximately 2.7 of normal subjects. In addition, CS with a low degree of sulfation was found in the urine of the RP patient. The relative amount of the nonsul-

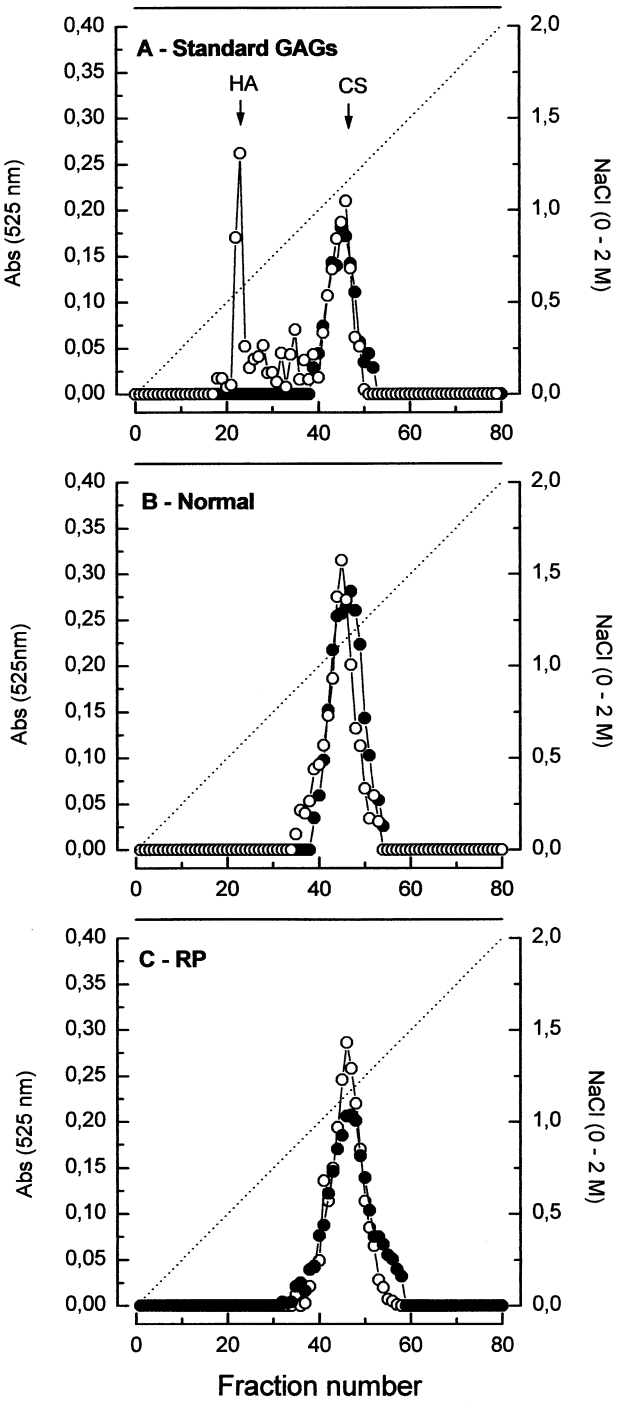

Fig. 2. Anion-exchange chromatography of GAGs from *normal* (B) and from *RP* (C) urines on Mono Q-FPLC. Urinary GAGs were applied to a Mono Q-FPLC column, equilibrated with 20 mM Tris: HCl (pH 8.0). The column was developed by a linear gradient of 0 to 2 mol/L NaCl in the same buffer. The flow rate of the column was 0.5 mL/min, and fractions of 0.5 mL were collected. The GAG fractions were detected by their metachromatic property (●) and by the content of uronic acid (○). The elution patterns of standard hyaluronic acid (*HA*) and chondroitin sulfate (*CS*) GAGs are shown in panel A. In this experiment, a mixture of ~ 200 μg of each standard GAG was applied into the column, eluted with the same NaCl gradient and the presence of GAGs on the fractions was assayed as described above. Note that the fractions containing standard HA were not metachromatic, since this molecule is a nonsulfated GAG.

fated disaccharide formed by action of the chondroitin ABC lyase was significantly higher in the CS of the RP patient when compared with the ones isolated from the urine of normal subjects. Also, most of the CS from the RP patient produced lower amounts of the 6-sulfated disaccharides by action of the enzyme. Interestingly, Tadaki and colleagues in their paper [4] mention that Maekawa and Nagahiro [12] have described that low sulfated CS or glycoprotein was detected in the urine from a case of RP (reference 7 in Tadaki's paper).

Similar structural anomalies of the urinary CS were reported for a genetic syndrome classified as a chondrodystrophy (brachyolmia), where the urine of the patients contained a CS with a low degree of sulfation [13,14]. Dietrich and colleagues [5] have reported similar anomalous structure of urinary CS in cancer patients and suggested that it would be useful in the diagnosis and follow-up of cancer therapy. They have analyzed the GAGs excreted by 44 patients with different types of tumors and 50 normal individuals. The average ratio of CS/HS of cancer patients (58%-CS/42%-HS) was 1.4 compared with 5.1 of normal individuals (83%-CS/17%-HS). In addition, CS with a low degree of sulfation was found in the urine of all cancer patients analyzed.

In conclusion, despite the previous reports of the increase of GAGs in the urine from RP patients, to our knowledge the fine structure and composition of RP urinary GAGs have never been analyzed in details. The two distinguishing differences, e.g., the ratio of CS/HS and low degree of sulfation of CS detected here on the urinary GAGs from the RP patient may contribute to the diagnosis of this disease. However, to assert that urinary GAGs become a reliable marker for disease activity of RP, further data from other patients with this disorder are required.

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