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Profiling of the eye aqueous humor in exfoliation syndrome by high-performance liquid chromatographic analysis of hyaluronan and galactosaminoglycans

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Abstract

The concentrations of hyaluronan and galactosaminoglycans - i.e., chondroitin sulfate and dermatan sulfate - were measured in the aqueous humor of the eye from patients with exfoliation syndrome and from healthy persons. The glycosaminoglycans/proteoglycans were almost completely precipitated (>97%) with ethanol in the presence of dextran as carrier and, following enzymic digestion, hyaluronan and galactosaminoglycans, were quantitatively converted to $\Delta^{4,5}$-disaccharides. Non-degraded heparan sulfate and proteins/glycoproteins were removed by ultrafiltration using a Centricon 3 membrane. Separation and determination of hyaluronan- and galactosaminoglycan-derived Δ-disaccharides were performed by ion-suppression HPLC. For an accurate analysis in triplicate, as little as 50 μl of aqueous humor is required. Application of this method to the analysis of samples from six patients with exfoliation syndrome and three healthy persons showed that hyaluronan levels in patients (6.65–16.15 $\mu g\ ml^{-1}$) were significantly higher (3–8 times) than in healthy persons (2.0–2.24 $\mu g\ ml^{-1}$). There was no significant alteration in the galactosaminoglycan concentration. The obtained data open a new area in the deeper understanding of the exfoliation syndrome pathophysiology and in establishing a highly sensitivity and accurate HPLC method for its diagnosis and patient's follow-up.

Keywords: Exfoliation syndrome; Hyaluronan; Galactosaminoglycan

1. Introduction

The term exfoliation syndrome (XFS) was suggested in the late 1980s [1]. It is now well recognized that XFS is an entity, not only confined to the eye, but most probably reflecting a systemic disorder [2]. A number of organs, including skin, myocardium, lung and liver, were found to have exfoliation material mainly in their connective tissues [3,4]. From the ophthalmological point of view, XFS is a common specific entity which may lead to the development of glaucoma [5].

Many studies on XFS have focused on biologically important macromolecules, such as glycosaminoglycans (GAGs) and proteoglycans (PGs) in the trabecular meshwork [6,7]. Much of the discussion on outflow of these macromolecules to the aqueous humor is based on histological examination follow-

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ing conventional methods of fixation and embedding. Most PGs and GAGs, however, are lost during these procedures and the histochemical reactions therefore have low validity. It has recently been proposed that the main biochemical characteristic of XFS is a primary hyaluronan (HA) overproduction in the anterior chamber and the cornea [8]. All GAGs, except HA, are covalently bound into a protein core forming PGs. Via interactions with effector macromolecules, such as collagen and laminin, GAGs play a main role in the organization of the extracellular matrix. The GAGs and PGs form a macromolecular network which is largely responsible for the physicochemical properties of the tissue, such as tissue hydration and resistance to pressure [9,10]. The trabecular meshwork contains HA (10–15%), galactosaminoglycans (GalAGs) (35–70%), keratan sulfate (4–15%) and heparan sulfate (HS) (14–22%). It should be noted that the extracellular matrix network of the trabecular meshwork is the most likely site of resistance to aqueous humor outflow [11,12]. The outflow may be affected in several ways by these trabecular GAGs and PGs. It therefore seems probable that HA and GAG/PGs play a major role in regulating the aqueous humor circulation and, thus, the intraocular pressure.

The fine biochemical structure of the exfoliated material remains unknown. In view of the finding that XFS material is rich in GAGs, it has been suggested that an abnormal metabolism of GAGs/PGs may precede the formation of the exfoliation precipitate [13]. However, little is known about the HA concentration and the type and amount of GAG in the aqueous humor. Digestion of HA and GalAGs with chondroitinases ABC and AC in the presence of chondosulfatases, followed by ion-suppression HPLC analysis, has been reported [14] to be one of the most selective and accurate determinations of these GAGs in biological samples.

The aim of this study was, therefore, to develop a simple biochemical procedure that permits accurate determination of the content of HA and GalAGs in the aqueous humor of patients with XFS and comparison with the findings in healthy persons. Application of this procedure could help in understanding XFS in more detail and in detecting this disorder in earlier stages of the patient's life.

2. Experimental

2.1. Chemicals and biological material

Dextran 40, commercially available as Rheomacrodex 10%, came from Kabi-Pharmacia (Stockholm, Sweden). Standard preparations of Δdi-nonS_{HA} [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose] and Δdi-non$S_{CS/DS}$ [2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose] were purchased from the Seikagaku Kogyo (Tokyo, Japan). Chondroitinases ABC (EC 4.2.2.4) and AC (EC 4.2.2.5) were also obtained from Seikagaku. Chondro-4- (EC 3.1.6.9) and 6-sulfatases (3.1.6.10), CSA from whale cartilage (grade I) and HA from human umbilical cord were obtained from the Sigma (St. Louis, MO, USA). The non-sulfated CS (chondroitin) was prepared from squid skin, as previously described by Karamanos et al. [15]. Centricon 3 membranes were obtained from Amicon (Beverly, MA, USA). All other chemicals used were of analytical reagent grade.

The collection of biological material and the procedure followed were in accordance with the ethical standards of the Helsinki Declaration of 1975 and its latest version. Samples of aqueous humor were collected in test tubes without preservatives. Aqueous humor (0.1 ml) was aspirated by inserting a 25-gauge needle into the anterior chamber. All aqueous humor samples were obtained just before the initiation of surgery for cataract extraction. Immediately after sample collection and before transport to the laboratory, five volumes of 95% (*v*/*v*) aqueous ethanol containing 2.5% (*w*/*v*) sodium acetate and 10 μl of dextran solution were added.

2.2. Sample treatment and preparation of standards

The precipitation of GAGs/PGs in the sample–ethanol mixture was completed, keeping the mixture overnight at 4°C. The precipitate was recovered by centrifugation in a Beckman microfuge (11 000 *g* for 5 min) and the HA and GalAGs present were digested by chondroitinases ABC and AC and chondro-4- and 6-sulfatases (0.1 unit ml^{-1} of each

enzyme) in a 50 m*M* Tris-HCl (100 μl) solution, buffered at pH 7.5, for 90 min at 37°C [16,17]. Removal of the non-degraded HS, proteins/glycoproteins and dextran was performed using ultrafiltration of the digestion mixture by centrifugation (11 000 *g* for 10 min) using a Centricon 3 membrane (cut-off 3 000 Da). A portion of 10–20 μl from the filtrate was then taken for direct determination of HA and GalAGs-derived Δ-disaccharides by HPLC.

Standard preparations of HA, CSA and non-sulfated chondroitin were digested under the same conditions and the final solutions used for analysis had concentrations of 1.0 and 10.0 $\mu g\,ml^{-1}$.

2.3. Analysis of HA and GalAGs by HPLC

Chromatography was performed on a Pharmacia LKB gradient pump 2249 equipped with a reodyne model 7125 injector unit having a 50 μl loop and LDC spectromonitor 1204 A UV–VIS absorbance detector, set to 231 nm, with an 8 μl flow cell. Separation and analysis of HA and GalAGs were carried out on an Econosphere-NH_2 (250×4.6 mm I.D., Alltech, IL, USA), 5 μm particle size, connected to an amino precolumn (30×4.6 mm I.D., Brownlee Labs., Santa Clara, CA, USA). For optimal separation, the chromatography was performed under conditions previously described [14], using 5 m*M* sodium phosphate, pH 2.55, as eluting buffer, at 1.0 $ml\,min^{-1}$. Peak areas were recorded and evaluated, using the Dapa chromatographic software system V1.4. Quantitation of Δ-disaccharide contents in samples was performed using precisely known amounts of Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ dissolved in chondroitinase digestion buffer to give working standard solutions of 0.05, 0.1, 1.0, 2.5, 5.0, 10, 25 and 50 $\mu g\,ml^{-1}$. Before use, the elution buffer was degassed by vacuum filtration through a 0.2 μm membrane filter, followed by agitation in an ultrasonic bath.

2.4. Statistical analysis

The significant differences among glycosaminoglycan levels in patients and healthy persons were determined by *t*-test.

3. Results and discussion

3.1. Resolution and quantification of HA and GalAGs by HPLC

The determinations of HA and GalAGs in aqueous humor were based on the analysis of Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ produced by digestion of HA and GalAGs, respectively, with chondroitinases and chondrosulfatases. It has been previously reported [14,18,19] that the utilization of chondro-4- and 6-sulfatases has the advantage of converting all sulfated chondroitin sulfate (CS)/dermatan sulfate (DS) -derived Δ-disaccharides into non-sulfated ones and, therefore, the total amount of GalAGs- i.e. CS and DS- can easily be determined by recording the Δdi-non$S_{CS/DS}$ peak area. As shown in Fig. 1A, a complete separation between commercially available Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ was obtained within 10 min. Analysis of standard HA and non-sulfated chondroitin preparations as well as of the commercially available $\Delta^{4,5}$-disaccharides showed the absence of contaminating substances, suggesting a high purity of the preparations used. The appearance of Δdi-non$S_{CS/DS}$ as a double peak is due to partial separation of α- and β-anomeric forms which, however, do not interfere with the separation from Δdi-nonS_{HA}. Quantification was based on the area of both anomeric peaks. Following treatment with chondroitinases and chondrosulfatases and subsequent centrifugation using Centricon 3 membranes, the presence of various amounts of HS and glycoproteins, such as collagen and mucin type I-S, did not interfere with the analysis of HA and GalAGs (Fig. 1B).

The sensitivity and linearity of the method were tested by measuring the peak areas of standard Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ at various concentrations. Peak areas were linearly related to the amount of Δ-disaccharide up to 10 μg of each Δ-disaccharide injected into HPLC - i.e., within the entire interval tested. The detection limit for Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ (molar absorptivity of 5500 $M^{-1}\,cm^{-1}$), estimated as a signal to noise ratio of 3, corresponds to approximately 75 $ng\,ml^{-1}$ (150 $pmol\,ml^{-1}$). To obtain accurate determinations by performing three injections within a 95% confidence

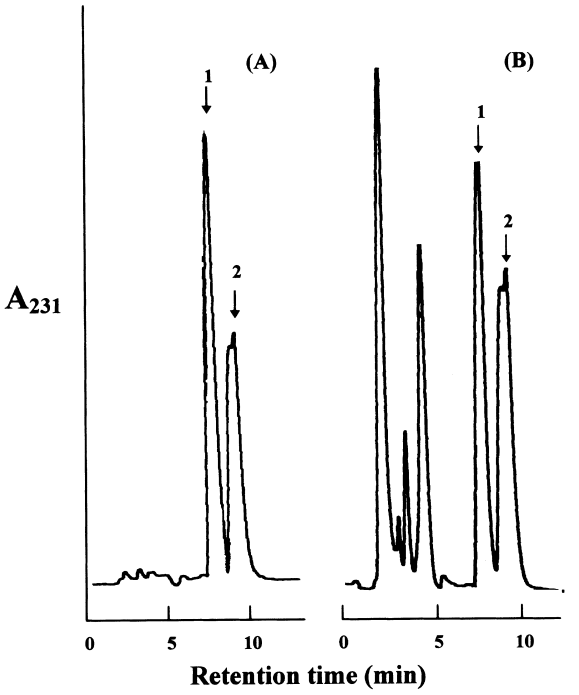

Fig. 1. Typical chromatograms showing the resolution of commercially available Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ (0.2 and 0.1 $\mu g\,ml^{-1}$, respectively) (A) and of those produced by digestion of HA and non-sulfated chondroitin (0.2 and 0.14 $\mu g\,ml^{-1}$, respectively) with chondroitinases ABC and AC and chondro-4- and 6-sulfatases (B). Elution was performed with 5 m*M* phosphate buffer, pH 2.55, at a flow-rate of 1.0 $ml\,min^{-1}$. Peaks were recorded at 231 nm.

interval and an injection volume of 10 μl, as little as 100 ng ml^{-1} of each of HA and GalAGs is required. The entire procedure was accurate and reproducible (1.4–2.8% of the target values), even when it was used to analyze samples with very low concentrations of glycosaminoglycans (0.1 $\mu g\,ml^{-1}$).

3.2. Applications to the analysis of HA and CS/DS in aqueous humor

Direct precipitation of GAGs, without addition of carrier, showed varying degrees of recovery (60–80%), especially when HA and CS/DS are present in low amounts (0.2–2 $\mu g\,ml^{-1}$). This problem was overcome by adding 10 μl of commercially available sulfated dextran as carrier. Analyzing various concentrations of HA, CSA and non-sulfated chondroitin, before and after precipitation in the presence of dextran and comparing with peak areas obtained by direct analysis of standard solutions we found that when dextran was used as carrier almost all GAGs tested were recovered (>97%) in the precipitate. This high degree of recovery also suggested that HA and GalAGs were quantitatively converted to $\Delta^{4,5}$-disaccharides in accordance with previous results [14,18,19]. Moreover, dextran was removed by ultrafiltration on the Centricon 3 membranes and possible minute remainders do not interfere with the analysis of Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$. Taking into account that no interference with the analysis of HA and GalAGs was recorded from the UV-background of the eye aqueous humor it is concluded that this approach is useful for analyzing HA and GalAGs in aqueous humor.

In this study, aqueous humor from the eyes of six patients with XFS and from three healthy persons has been analyzed by HPLC. Representative chromatograms are shown in Fig. 2. The secretion of HA

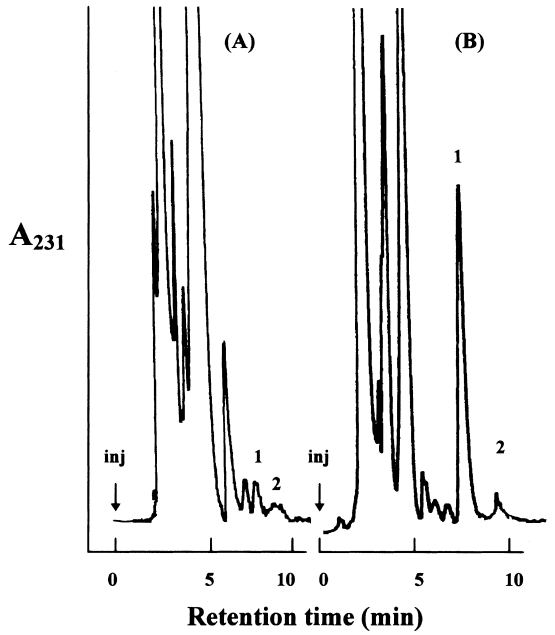

Fig. 2. HPLC analysis of Δdi-nonS_{HA} and Δdi-non$S_{CS/DS}$ in the aqueous humor of the eyes of a healthy person (A) and a patient with XFS (B). The concentration of HA in case A was 1.2 $\mu g\,ml^{-1}$, but 14.4 $\mu g\,ml^{-1}$ in case B. The CS/DS content was 0.35 in case A and 0.6 $\mu g\,ml^{-1}$ in case B. For separation conditions see Fig. 1.

is characteristic only of patients with XFS (peak 1, Fig. 2A,B). The CS/DS content, however, was not significantly increased (peak 2, Fig. 2A,B). Results from analysis of six XFS patients and three healthy persons are summarized in Fig. 3. Healthy persons had an HA content of 2.1 ± 0.1 $\mu g\,ml^{-1}$, whereas XFS patients had a 3–8-fold increase in HA levels ($6.65–16.15$ $\mu g\,ml^{-1}$). CS/DS comprise a minor proportion in both healthy persons as well as XFS patients (≤ 0.5 and 0.93 ± 0.41 $\mu g\,ml^{-1}$, respectively).

The significantly higher HA levels in the aqueous humor of XFS patients than in healthy persons, may suggest a degradation of the connective tissue in the trabecular meshwork. Since abnormal metabolism of GAGs/PGs has been thought by Baba et al [13] to precede the changes in XFS patients, the secretion of HA and GAGs in the aqueous humor is, most probably, due to systemic damage of the extracellular matrix interacting network including HA, PGs, collagen and glycoproteins. The existence of HA, as the only GAG, in aqueous humor may further indicate either an increased synthesis of this macromolecule or disruption of the HA–PG aggregate, due to the action of proteinases, acting on the PG protein cores and link proteins essential for stabilizing the HA–PG complex. Further studies of this problem will improve our understanding of the XFS pathophysiology. Such studies are proceeding in our laboratories.

In this report we describe the utilization of HPLC for the analysis of HA and sulfated GalAGs in aqueous humor with high accuracy. The chemical sensitivity obtainable with this HPLC procedure is so high as to permit the analysis of HA and GalAGs present in minute amounts (≥ 50 $ng\,ml^{-1}$) even when UV absorbance detection is used. Pulsed amperometric detection may be also of importance since is it has been reported to be quite sensitive as well [20]. Exfoliation syndrome is currently the commonest identifiable entity leading to the development of glaucoma. The results of this study suggest that the HA levels may well be used for the routine diagnosis of XFS. Further studies according to age, sex and nationality may be of great importance in characterizing XFS in early stages.

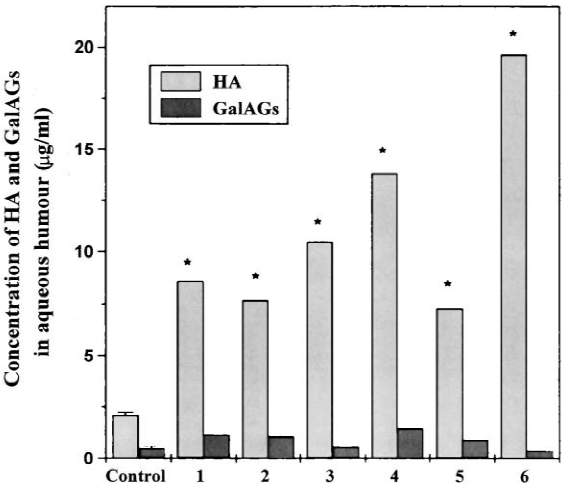

Fig. 3. Histograms showing differences in the levels of HA and GalAGs in aqueous humor of the eye from six XFS patients versus the average value±SD obtained for three healthy persons (control). Significant alterations were recorded only in HA levels (3–8-times higher) but not in GalAGs content. Asterisk indicates significant differences, $P\leq 0.05$.

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