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ORIGINAL RESEARCH

Application of polyacrylamide gel electrophoresis of fluorophore-labeled saccharides for analysis of hyaluronan and chondroitin sulfate in human and animal tissues and cell cultures

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ABSTRACT: Hyaluronan (HA) and chondroitin sulfate (CS) are glycosaminoglycans (GAGs) with great importance in biological events, since they participate in and regulate cell adhesion, migration and proliferation. Quantitation and analysis of the fine structure of GAGs are increasingly important for understanding many biological processes, among which are many critical aspects of pathology development and specific phenotype descriptions. Human umbilical cord and human synovial fluid are connective tissues containing high amounts of GAGs and change in the quantity and structure of these macromolecules is described in tissue development and is commonly associated with disease. Moreover, also in *Xenopus laevis* embryo development and chondrocyte cultures, the GAG content and structure play a critical role. A rapid analysis of hyaluronan and chondroitin sulfate Δ-disaccharides derived from the above human and animal samples, derivatized with 2-aminoacridone and analyzed by polyacrylamide gel electrophoresis, is described in this report. Qualitative and quantitative analysis were performed by comparing their migration and the pixel density with standard Δ-disaccharides, running in the same gel. Since this method allows the analysis of large numbers of samples simultaneously in one gel and has a relatively high sensitivity (less than 25 pmol), it is suggested as a cost-effective and useful tool for the fast screening of small amounts of hyaluronan and chondroitin sulfate disaccharides. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: disaccharides; hyaluronan; chondroitin sulfate; polyacrylamide gel electrophoresis

INTRODUCTION

Hyaluronan (HA) and chondroitin sulfate (CS) are linear polymers belonging to the family of glycosaminoglycans (GAGs) (Karamanos, 1999). HA is characterized by the repeating disaccharide [→ *4*GlcA*β1* → *3*GlcNAc*β1* →] and, among the GAGs, it is the only one that does not contain any sulfate group. Even though HA is not covalently bound to a protein core (Laurent and Fraser, 1992), it forms the ligand structure for a family of aggregating proteins, the hyaladherins (Prehm, 1984). It plays an important role in embryonic and tissue morphogenesis (Toole, 2001; Heldin *et al*., 1989), as well as in angiogenesis and in several diseases and malignancies (Asplund *et al*., 1993). CS, known as galactosaminoglycan, consists of the repeating disaccharide [→ *4*GlcA*β1* → *3*GalNAc*β1* →] normally sulfated at the C-4 and C-6 positions of N-acetylated galactosamine (GalNAc), even though any one of the available groups may be sulfated. In living organisms, CS is covalently bound to protein cores forming proteoglycans (PGs), which are considered to be one of the major families of structural glucoconjugates with great importance in biological functions (Kjellen and Lindahl, 1991).

The umbilical cord extracellular matrix is a tissue composed of a large amount of GAGs (Sobolewski *et al*., 1997). GAGs play an important role in many diseases, for instance, in pre-eclampsia an increase in sulfated GAGs and a decrease in HA in umbilical cord arteries are observed (Romanowicz *et al*., 1994). In Down's syndrome, the amount of HA in the Wharton's jelly of the umbilical cord is higher than normal, accompanied by different clinical characteristics (Raio *et al*., 2004).

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Abbreviations used: AMAC, 2-aminoacridone; CS, chondroitin sulfate; GAGs, glycosaminoglycans; HA, hyaluronan; PAGEFS, polyacrylamide gel electrophoresis of fluorophore-labeled saccharides.

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In *Xenopus laevis* embryogenesis, a model organism extensively used in developmental biology, involvement of HA has been proposed. However, detailed information about HA metabolism, content and structure are scant for *Xenopus laevis* embryogenesis (Vigetti *et al.*, 2003).

Synovial fluid contains HA as the main component, with a concentration of 3–4 mg/mL. It increases the viscosity and elasticity of articular cartilage and lubricates the surfaces between the synovium and cartilage. Differences in size and content indicate non-inflammatory or inflammatory diseases, such as rheumatoid arthritis or osteoarthritis (Takahashi *et al.*, 2004).

Chondrocyte culture is a common model for cartilage studies; in fact these cells survive *in vitro*, producing abundant extracellular matrix macromolecules. A common technical approach in cell culture studies is the use of radiolabeled precursors, due to the small amount of material, and therefore it could be very interesting to use non-radiactive material to study the total amount of polysaccharides produced in culture.

Analysis of unsaturated disaccharides derived from intact GAGs after specific enzymic degradation provides a practical approach to the characterization of their structures. Various strategies and methods may be used to determine unsaturated disaccharides, such as HPLC and HPCE (Karamanos *et al.*, 1994, 1995). In a quest for high sensitivities, reactions with fluorophore derivatives, such as dansylhydrazine (Volpi, 2000), 2-cyanoacetamide (Toyoda *et al.*, 1991) and 2-aminoacridone (AMAC; Mitropoulou *et al.*, 2001) were performed using UV or fluorescence detection. Polyacrylamide gel electrophoresis of fluorophore-labeled saccharides (PAGEFS), on the other hand, is a potential method by which many samples can be analysed contemporarily. In our previous study, we described a method of PAGEFS for the analysis of HA- and CS-derived disaccharides (Karousou *et al.*, 2004a).

In this report, PAGEFS is applied to human and animal tissues that contain HA and CS. The biological samples used are from human umbilical cord of Down's syndrome fetuses, embryos of *Xenopus laevis*, synovial fluid from human shoulders and knees affected by osteoarthritis (OA) and medium from a culture of pig hyaline cartilage chondocytes. The Δ-disaccharides, obtained from purified GAGs after enzymic digestion with specific lyases, were derivatized with AMAC and analysed by PAGEFS. Results showed that the main component of these biologic samples is HA, as reported in the literature. Therefore, we suggest PAGEFS as an accurate method with high reproducibility for HA and CS Δ-disaccharides. Since PAGEFS is based on gel electrophoresis, using here mini-slab gels, it is an easy and rapid method, as many samples can be analyzed in parallel in less than 1 h.

MATERIALS AND METHODS

Chemicals and biologic materials. The HA- and variously sulfated CS-derived Δ-disaccharides standard were obtained from Sigma (St Louis, MO, USA). Protease K (EC 3.4.21.64) was purchased from Finnzymes (Espoo, Finland). Hyaluronidase SD (EC 3.2.1.35) and chondroitinase ABC (EC 4.2.2.4) were from Seikagaku Kogyo (Tokyo, Japan). AMAC was obtained from Molecular Probes (OR, USA) and $NaBH_3CN$ from Sigma-Aldrich (Steinheim, Germany). Tris (hydroxymethyl)-aminomethane was from Fluka Chemie and acetonitrile from Merck (Darmstadt, Germany). Acrylamide and *NN′*-methylenebisacrylamide were obtained from Bio-Rad (Richmond, CA), *NNN′N′*-tetramethylethylenediamine (TEMED) from BDH Chemicals (Poole, England) and ammonium persulphate from LKB (Bromma, Sweden). All aqueous solutions were prepared using water filtered through a Milli-Q water system (Millipore). All other chemicals used were of analytical reagent grade.

Isolation and degradation of GAGs from and tissues. Down's syndrome fresh human umbilical cord sample (50 mg), 10 embryos at 30 h post-fertilization of *Xenopus laevis*, human synovial fluid of shoulder and knee with OA (100 μL) and medium from pig chondrocytes primary culture (1 mL, kindly provided by Dr Perretti, University 'Vita e Salute' San Raffaele, Milan, Italy) were digested at 60°C for 2 h in 300 μL of 100 mM ammonium acetate buffer, pH 7.0, containing 20 U/mL of protease K. The enzymic treatment was terminated by boiling for 5 min. Four volumes of 96% ethanol per sample volume were added, and the GAGs in the mixture were precipitated at –20°C overnight.

Ethanol precipitated GAGs were centrifuged at 11,000 ***g*** at 4°C for 15 min. The pellets obtained were dried and dissolved in 100 μL of 100 mM ammonium acetate, pH 7.0, containing 100 mU/mL of hyaluronidase SD and digested at 37°C for 1 h. A 100 mU/mL solution of chondroitinase ABC was added, and the mixture was incubated at 37°C for 3 h. The samples were then frozen at –80°C and then lyophilized. The Δ-disaccharide digested products were then derivatized as described above.

Derivatization. Derivatization of standard HA and CS Δ-disaccharides was performed as described previously by Calabro *et al.* (2001), as modified by Mitropoulou *et al.* (2001). In brief, 10 nmol of each standard Δ-disaccharide in water were completely evaporated in a microcentrifuge tube at 11,000 ***g*** at room temperature. A 40 μL volume of 12.5 mM AMAC solution in glacial acetic acid–DMSO (3:17, v/v) was added, and samples were incubated for 10–15 min at room temperature. A 40 μL volume of a freshly prepared solution of 1.25 M $NaBH_3CN$ in water was added to each sample followed by an overnight incubation at 37°C.

Preparation of polyacrylamide gel. A MiniProtean III cell vertical slab gel electrophoresis apparatus (Bio-Rad) was used with 7.2 cm plates, 0.75 mm spacer and wells of 0.5 cm. Polyacrylamide gels were prepared as described before by Karousou *et al.* (2004a). In brief, a 10 mL volume of T 25%–C 3.75% resolving gel, in 187.5 mM Tris–borate and 187.5 mM Tris–HCl buffer solution (final concentrations), was

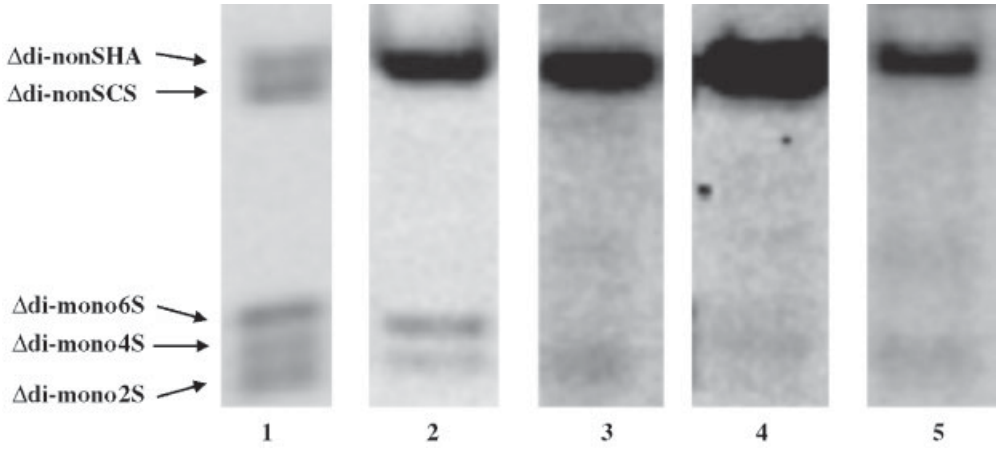

Figure 1. Analysis of AMAC-derivatives of HA and variously sulfated CS Δ-disaccharides of standard mix of 100 pmol of each Δ-disaccharide (lane 1) and from umbilical cord of Down's syndrome fetus (lane 2), embryos of *Xenopus laevis* (lane 3), human knee synovial fluid of OA patient (lane 4) and medium of pig chondrocyte culture (lane 5). Biological samples were treated with proteinase K and then digested with hyaluronidase SD and chondroitinase ABC. The products were derivatized with AMAC and analyzed by PAGEFS. The resolving gel consisted of T 25%–C 3.75% in a mix of 187.5 mM Tris–borate and 187.5 mM Tris–HCl, pH 8.8, and the stacking gel consisted of T 5%–C 1.5% in a 0.36 M Tris–HCl buffer, pH 8.8. Gels were run in 0.15 M Tris–borate, pH 8.8, at 400 V at 4°C for 45 min.

prepared and degassed. A 5 μL volume of TEMED and a 50 μL volume of a freshly prepared 10% (w/v) ammonium persulfate were added. The solution was mixed rapidly and then placed between the glass plates, avoiding air bubbles. The non-polymerized gel was overlaid with butanol. The resolving gel upper surface was rinsed with stacking gel buffer (0.15 M Tris–HCl diluted from the stock solution). A 5 mL volume of T 5%–C 1.5% acrylamide stacking gel in 0.36 M Tris–HCl buffer solution was prepared, followed by an addition of 10 μL TEMED and 50 μL 10% ammonium persulfate.

Electrophoresis and analysis of Δ-disaccharides. A 5 μL volume of each sample, supplemented with glycerol in a final concentration of 20% (v/v), was loaded in each well and a marker sample containing bromophenol blue was also run in an empty well. Electrophoresis was done at 400 V and 4°C and terminated when the marker dye reached 1.2 cm from the bottom of the gel.

Gels were scanned in a UV-light box using a CCD camera (Gel Doc 2000 System) from Bio-Rad Laboratories (Hercules, CA, USA). Analysis of sample bands was performed by comparing their migration and the pixel intensity with standard Δ-disaccharides, running in the same gel.

RESULTS AND DISCUSSION

The identification and quantitation of HA and CS-derived Δ-disaccharides was performed by PAGEFS (Figs 1 and 2), as described previously (Karousou *et al.*, 2004a). A mix of standard Δ-disaccharides was run in the same gel with the samples. Qualitative analysis of bands was performed by comparing the migration with that of the standard mix. As far as the quantitative analysis was concerned, the pixel intensity of each band was measured by densitometric analysis using an appropriate computer program from Bio-Rad and normalized with the intensity of background. Results showed that the dominant disaccharide in these biologic samples was HA (Figs 1 and 2, Table 1). In parallel, analysis of AMAC-labeled Δ-disaccharides was performed with HPLC as described previously (Karousou *et al.*, 2004b), showing the same results as the PAGEFS method (data not shown). The estimated

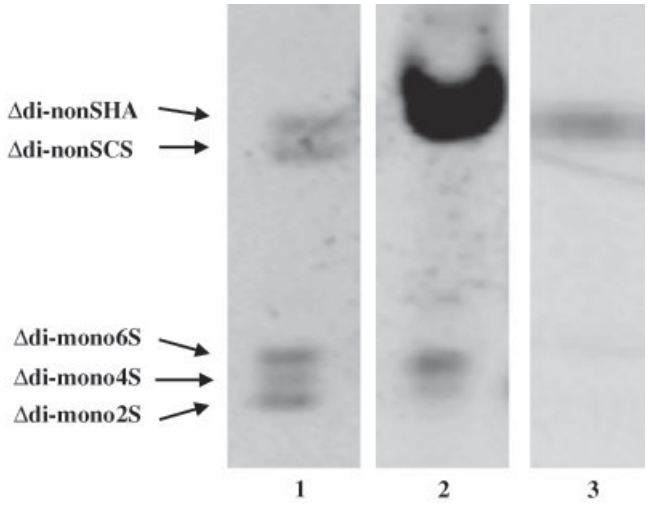

Figure 2. Analysis of AMAC-derivatives of HA and variously sulfated CS Δ-disaccharides by PAGEFS of standard mix of 100 pmol of each Δ-disaccharide (lane 1) and human shoulder synovial fluid of OA patient undiluted and dilute 1:50 (lanes 2 and 3, respectively).

Table 1. Molar ratios of AMAC derivatized Δ-disaccharides from umbilical cord of Down's syndrome fetus, embryos of *Xenopus laevis*, human knee and shoulder synovial fluid and medium of pig chondrocyte culture, determined by PAGEFS

	Disaccharides (%)		
		CS	
Biological sources	HA (%)	4-O-SO^{3-}(%)	6-O-SO^{3-}(%)
Umbilical cord of Down's syndrome fetuses	80.7	8.7	10.6
Embryos of *Xenopus laevis*	91.4	8.6	0.0
Human knee synovial fluid	90.7	0.0	9.3
Medium of pig chondrocyte culture	85.6	14.4	0.0
Human shoulder synovial fluid	97.5	1.0	1.4

disaccharide amounts of the biological samples were in accordance with previous studies, showing that PAGEFS can be accurately applied for the determination of disaccharide composition in biological samples.

In human umbilical cord of Down's syndrome fetus (Fig. 1, lane 2), HA Δ-disaccharides represented 80.7% of the total derived disaccharides. CS-derived Δ-disaccharides accounted for 19.3% of the total disaccharides, in particular 10.6% 4S and 8.7% 6S (Table 1). Indeed, HA disaccharides have proved to be present in higher amounts in Down's syndrome umbilical cord than in normal, and immunohistochemical experiments showed a denser and extended distribution (Raio *et al.*, 2004). It has also been shown recently in our laboratory that, during gestation, a decrease in the amount of HA disaccharides and an increase of CS disaccharides are observed (data not shown).

Since *Xenopus laevis* has been used as a 'model organism' for developmental embryology, the study of HA content which is involved in embryogenesis could be useful. As shown in Table 1, HA accounted for 91.4% of total Δ-disaccharides obtained after specific enzymatic treatments (Fig. 1, lane 3). This result is remarkable considering the extremely low number of embryos used for determination of GAG content. The HA amount has been shown to be critical during embryogenesis of *Xenopus laevis* and the HA content changes can be due to differences in HAS and HYAL gene expression (Vigetti *et al.*, 2003).

HA and CS play a critical role in joint lubrication and cartilage nutrition. Differences in these GAGs levels have been investigated in various joint diseases and for this purpose they have been used as joint markers for inflammations. PAGEFS analysis of HA and CS-derived Δ-disaccharides of human synovial fluid with OA showed an abundance of HA (Fig. 1, lane 4 for knee synovial fluid and Fig. 2, lane 2 for shoulder synovial fluid). Indeed, 97.5 and 90.7% of the obtained disaccharides consisted of HA in knee and shoulder samples, respectively. In shoulder synovial fluid only 1.4 and 1.0% were Δdi-mono6S_{CS} and Δdi-mono4S_{CS}, respectively (Table 1). As shown in Fig. 2, lane 2 the band that indicated the HA amount was oversaturated and measurement of quantity was impossible. For this reason, a 1:50 dilution was performed and sample was analyzed using PAGEFS (Fig. 2, lane 3). The absolute quantitative analysis showed that the concentrations of Δdi-nonS_{HA}, Δdi-mono6S_{CS} and Δdi-mono4S_{CS} in 1 mL of synovial fluid were 2.3, 0.36 and 0.25 μmol/mL, respectively, and the Δdi-mono6S_{CS}–Δdi-mono4S_{CS} ratio was 1.4. In previous studies, it has been reported that the HA concentration in synovial fluid of various joint diseases is lower than in normal (Nakayama *et al.*, 2000; Shibata *et al.*, 1998). In addition, chondroitin sulfation in synovial fluid of knee joints showed a decrease for Δdi-mono6S_{CS} content and a Δdi-mono6S_{CS}–Δdi-mono4S_{CS} ratio of ~1.3 (Lewis *et al.*, 1999). In this report, results on HA and CS content in synovial fluid of OA patients were comparable with previous studies.

In pig chondrocytes medium HA-derived Δ-disaccharides represented the major component and Δdi-mono4S_{CS} was the only detectable sulfated CS disaccharide.

The obtained data suggest that the described method can be accurately used for the determination of disaccharide composition in biological samples containing HA and CS. The AMAC-labeled Δ-disaccharides can be completely separated without any interference from the excess derivatizing reagent, which remains in the stacking gel. If high or low amounts of disaccharides are present, a dilution of the sample or an increase in loading volume in the well can be performed, respectively. The procedure of PAGEFS is simple and rapid. In addition, this technique is of low cost since it does not require expensive equipment, and has relatively high sensitivity (less than 25 pmol of derived Δ-disaccharide). Thus, this technique may be a useful tool for a fast and sensitive screening of multiple samples.

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