COMMUNICATION

Extraction and structural analysis of glycosaminoglycans from formalin-fixed, paraffin-embedded tissues

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Glycosaminoglycans (GAGs) are long, anionic polysaccharides involved in many basic aspects of mammalian physiology and pathology. Here we describe a method to extract GAGs from formalin-fixed, paraffin-embedded tissues and found that they are structurally comparable with GAGs extracted from frozen tissues. We employed this method to structurally characterize GAGs in tissues, including laser-dissected layers of skin and pathological specimens. This method enables the use of the archival paraffin-embedded material for detailed (structural) analysis of GAGs.

Keywords: glycosaminoglycan / laser dissection / paraffin extraction

Introduction

Archiving of clinical tissue samples is generally done by formalin fixation and paraffin wax embedment. Formalin-fixed, paraffin-embedded (FFPE) tissues are valuable samples, typically generated from human specimens, and are used for (patho)histological examination. It has been estimated that worldwide over a billion samples have been stored (Blow 2007). As this material is generally readily available, and patient-related data of many years are usually known, the ability to use this archival material is of great advantage in clinical and molecular research. Much attention has been given to the extraction of DNA and RNA from human FFPE tissues, and this has offered tremendous experimental opportunities, including microarray analysis (Budczies et al. 2011). Little attention has been given to the extraction of (poly)saccharides, let alone the highly heterogeneous, polymeric glycosaminoglycans (GAGs). It has been reported that N-linked protein glycosylation remains preserved in FFPE tissues (Tian et al. 2009). Here, we report a method to extract intact GAGs from FFPE material. GAGs are long (up to 100–150 disaccharides), linear and highly negatively charged polysaccharides and are generally attached to a protein core, forming proteoglycans. They are located at the cell surface and in the extracellular matrix. Based on their disaccharide composition, different family members are distinguished, which include heparan sulfate (HS)/heparin and chondroitin/dermatan sulfate (CS/DS; Esko and Selleck 2002). GAGs are highly variable molecules due to variable degrees of polymerization, epimerization and sulfation at different positions (Supplementary data, Figure S1). They are potentially highly information-dense molecules and recent evidence suggests the presence of defined monosaccharide sequences (Ly et al. 2011). Domain structures within GAGs are crucial for their interaction with a large variety of proteins, including growth factors and regulatory molecules, and these interactions facilitate specific activities in mammalian physiology (Bishop et al. 2007). GAGs and proteoglycans have been implicated in various pathological processes, e.g. amyloid diseases, inflammation and infection and cancer biology (Lindahl and Li 2009). Both qualitative changes (different modification patterns) and quantitative changes have been described.

Results and discussion

To determine the feasibility of GAG extraction/isolation from FFPE tissues, we used rat liver and skin and divided the tissues into several pieces which were either formalin-fixed and paraffin embedded or directly frozen in liquid nitrogen. Optionally, paraffin-embedded tissues were deparaffinized before the start of the procedure (Figure 1A). The procedure comprised papain digestion (at 65°C, a temperature at which paraffin melts), trichloroacetic acid (TCA) precipitation, anion exchange chromatography and methanol precipitation. Deparaffinized, deparaffinized and frozen tissues were compared. Applying agarose gel electrophoresis, which separates the GAGs into the main classes of CS/DS/HS, we found that different types of GAGs could indeed be extracted and isolated from FFPE tissues (Figure 1B). The skin contained an abundance of DS and a smaller amount of HS, whereas the liver...
contained about equal amounts of both. This proportion was similar for samples from FFPE tissues compared with frozen tissues. As the size of the GAG chains could be compromised, as is seen with RNA isolated from FFPE tissues (von Ahlfen et al. 2007), GAGs from frozen and FFPE samples were analyzed using polyacrylamide gel electrophoresis (Figure 1C). The size of isolated GAGs from FFPE tissues was comparable with that of GAGs from frozen tissues, for the liver as well as for the skin. To evaluate if the GAG domain structure was compromised by FFPE treatment, reactivity with seven anti-GAG antibodies, each recognizing different epitopes, was determined using enzyme-linked
immunosorbtent assay (Figure 1D). Similar reactivities were observed for GAGs isolated from FFPE (with or without pre-deparaffinination) or frozen tissues, for both the skin and the liver. Two notable differences between the GAG composition of the liver and skin were the higher reactivity of the skin with the anti-DS antibody LKN1 and with the anti-HS antibody NS4F5. This reflects the abundance of DS and the presence of mast cells in skin. Mast cells contain heparin, a highly sulfated form of HS, which is recognized by the antibody NS4F5. The structural composition of GAGs was further investigated on the disaccharide level by enzymatic depolymerization using heparinases for HS (Figure 1E) and chondroitinase ABC for CS/DS (Figure 1F). The major disaccharide constituents of the liver HS were UA-GlcNAc, UA-GlcNS, UA2S-GlcNS and UA2S-GlcNS6S (for disaccharide abbreviations, see Supplementary data), in agreement with literature data (Lyon et al. 1994), and their proportions were comparable between FFPE and frozen samples (Figure 1E). The major disaccharide (~90%) found in CS/DS from the skin was UA-GalNaC4S, which is abundantly present in DS (Figure 1F). Again, the composition was not altered by FFPE treatment.

As the yield from FFPE tissues when compared with frozen tissues was previously reported to be reduced for DNA (Serth et al. 2000) and N-linked glycoproteins (Tian et al. 2009), we determined the concentrations of isolated GAGs using the metachromatic dye dimethylmethylene blue. For the skin, 266 ± 55 µg GAG/g wet tissue (mean ± SD) was isolated from directly frozen tissue, which was not significantly different (p = 0.09, n = 3) from the amount isolated from deparaffinated FFPE skin tissues (387 ± 77 µg GAG/g wet tissue). However, without deparaffinization, the yield was significantly less compared with frozen tissue (101 ± 33; p = 0.01, n = 3). For the liver, a direct comparison could not be made since frozen material was homogenized, whereas FFPE material was cut into pieces (for the skin both frozen and FFPE material were cut into pieces). The amount of GAGs isolated from FFPE liver material was 20 ± 6 and 24 ± 3 µg GAG/g wet tissue for tissue with/without pre-deparaffinization respectively, and this was significantly less compared with frozen and homogenized material (63.8 ± 24.4; p = 0.04, n = 3, p = 0.05, n = 3 respectively). Combined, these results indicate that, in the case of the skin, deparaffinization is sufficient to quantitatively extract GAGs compared with frozen tissue, but further adaptation may be needed for other tissues.

In general, FFPE tissues from pathological conditions are precious and extraction should ideally be done using minute amounts of material such as present in tissue sections, which has the additional advantage that (immuno)histochemistry can be performed on adjacent sections. Therefore, sections of (deparaffinated) FFPE mouse skin material were probed for GAG isolation. As little as a single 40-µm section of ~10 × 4 mm was sufficient to isolate and characterize GAGs using agarose gel electrophoresis (Supplementary data, Figure S2A–C). Encouraged by these results, we applied this technique in two different ways. First, the GAG composition of two specific layers of the human skin, papillary and reticular dermis was determined using laser-microdissected layers (Supplementary data, Figure S3). For both layers, DS was by far the predominant GAG (agarose gel electrophoresis) and its composition was determined by CS/DS disaccharide analysis (Figure 2A). It was found that these dermal layers have compositional differences, for instance with regard to UA2S-GalNAc4S and UA2S-GalNAc6S. Second, GAGs were isolated from tissue sections from two human skin pathologies: psoriasis and keloid and compared with the normal skin by agarose gel electrophoresis and by the disaccharide analysis of CS/DS and HS (Figure 2B–D). As these sections were generally very small (~3 × 2 mm; Supplementary data, Figure S4), ten 40-µm sections were used for isolation. Although the size of the sections differed, making it difficult to compare the total amount of GAGs, their relative distribution could be determined by agarose gel electrophoresis (Figure 2B). In three of five keloid patients, there was a clear increase in CS compared with the normal skin, in agreement with the literature (Ikeda et al. 2009). In the skin from psoriasis patients, an increased level of HS and CS was noted. Alterations in GAG content and composition have been reported in psoriasis (Smetsers et al. 2004). Immunohistochemical staining of adjacent sections confirmed the increase in CS in these skin pathologies (Supplementary data, Figure S5). In addition, high performance liquid chromatography (HPLC) analysis was performed to evaluate the CS/DS and HS disaccharide composition (Figure 2C and D). For CS/DS, the relative amount of UA2S-GalNAc4S decreased for both pathologies, whereas UA-GalNAc and UA-GalNAc6S were increased. This difference in composition may be explained by the relative increase in CS compared with DS. For HS, differences were noted as well.

To summarize, we present a method to extract/isolate high-quality GAGs from FFPE tissues and show that GAGs are structurally and compositionally comparable with those obtained from frozen tissues. GAGs can be analyzed from as little as one 40-µm tissue section and laser-dissected structures can be evaluated as shown for different layers of the human skin. Pathology-associated differences in GAG classes and disaccharide composition were demonstrated using sections of tissue blocks from the diseased skin. The method described enables the use of archival FFPE material for the structural characterization of GAGs and opens new avenues to analyze GAGs in pathologies and correlate this with patient-related data.

Materials and methods

Biological materials

The liver and skin were derived from 2- to 3-month-old Wistar rats, and tissue was divided into pieces (liver range: 310–1198 mg wet weight, mean ± SD: 720 ± 259 mg; skin range: 118–454 mg wet weight, mean ± SD: 268 ± 105 mg) and either directly frozen in liquid nitrogen or fixed overnight in 4% paraformaldehyde (in 0.1 M sodium phosphate buffer, pH 7.4) at 4°C and embedded in paraffin. For the isolation of GAGs from mouse skin sections, the skin was derived from 2- to 3-month-old BALB/c nude mice and embedded in paraffin as described for liver and skin derived from Wistar rats. Tissue sections of the human (pathological) skin were

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obtained from the archives of the Institute of Pathology of the Radboud University Nijmegen Medical Centre. We used anonymized archival biopsy material left over from previous diagnostic procedures, in accordance with the Dutch code of conduct on the use of human tissue.

Procedure for extraction/isolation of GAGs

Extraction/isolation from tissue blocks
For the isolation of GAGs from rat tissues, FFPE tissue was cut into small pieces (∼5–10 mm³), after removing the excess of paraffin. Pieces were put into 50-mL conical tubes. In case the tissue blocks were deparaffinated, surrounding paraffin was cut away and the blocks were placed in a xylene bath (100 mL) at 60°C for 15 min. This was repeated twice with fresh xylene. Excess xylene was discarded and the tissue was washed with 100% ethanol (100 mL) followed by 96, 70, 50 and 30% ethanol for 3 min each. Subsequently, the tissue was gently rinsed in running tap water for 30s and kept in phosphate buffered saline (PBS) (100 mL) for 30 min for rehydration. After deparaffination, the wet tissue was cut into small pieces (∼5–10 mm³) and put into a 50-mL conical tube.

To both paraffinized and deparaffinized samples, 20 mL of digestion buffer (50 mM sodium phosphate, 2 mM cysteine, 2 mM ethylenediaminetetraacetic acid, pH 6.5) and 25 U of the proteolytic enzyme papain from *Papaya latex* (P3125, Sigma-Aldrich, St Louis, MO) were added per gram of wet tissue weight. For isolation from frozen liver tissues, homogenization by a potter-Elvehjem homogenizer with intervening space of 0.35 mm (5–10 strokes) was performed in 20 mL of digestion buffer (without papain)/gram tissue and the tissue homogenate was transferred into a 50-mL conical tube, and 25 U papain/g tissue was added. For isolation from the frozen skin, a similar procedure could not be used as the skin is too rigid. Instead, ∼5–10 mm³ pieces were cut.

Papain digestion was performed in a 65°C water bath overnight, followed by centrifugation at 5200 × g for 20 min at room temperature. In case FFPE tissues were not first deparaffinized, centrifugation was performed at 37°C. The resulting tissue pellet was digested with papain once more. To both paraffinized and deparaffinized samples, 20 mL of digestion buffer (50 mM sodium phosphate, 2 mM cysteine, 2 mM ethylenediaminetetraacetic acid, pH 6.5) and 25 U of the proteolytic enzyme papain from *Papaya latex* (P3125, Sigma-Aldrich, St Louis, MO) were added per gram of wet tissue weight. For isolation from frozen liver tissues, homogenization by a potter-Elvehjem homogenizer with intervening space of 0.35 mm (5–10 strokes) was performed in 20 mL of digestion buffer (without papain)/gram tissue and the tissue homogenate was transferred into a 50-mL conical tube, and 25 U papain/g tissue was added. For isolation from the frozen skin, a similar procedure could not be used as the skin is too rigid. Instead, ∼5–10 mm³ pieces were cut.

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mL of diethylaminoethyl Sepharose (GE Healthcare, Chalfont St Giles, UK) for further purification of GAGs. In case FFPE tissues were not first deparaffinized, the TCA pellet was not very solid and the supernatant was filtrated after five times dilution, resulting in a clear filtrate which was subjected to the column. An initial wash of the column was performed with 3 mL of 0.2 M NaCl in 10 mM Tris (pH 6.8). GAGs were eluted with 3 mL of 2 M NaCl in 10 mM Tris (pH 6.8) and allowed to precipitate overnight at −20°C by adding five volumes of methanol, followed by 1 h centrifugation at 5200 × g at 4°C. Methanol was discarded quickly and the precipitated GAGs were air-dried, dissolved in Milli-Q and stored at 4°C (the amount of Milli-Q added (in μL) corresponds to the value of half of the wet weight (in mg) of the original tissue material).

Extraction/isolation from tissue sections. For the isolation of GAGs from the mouse and human FFPE skin, sections of 40 μm were made and collected in 2 mL Eppendorf tubes. Deparaffination was performed as described under Extraction/isolation from tissue blocks, using 2 mL Eppendorf tubes, and volumes of 1 mL. Centrifugation steps of 5 min at 14,000 × g were performed after each incubation. The isolation procedure was as described under Extraction/isolation from tissue blocks with the following changes: the amount of digestion buffer was 50 μL/section (with a minimum of 150 μL for one or two sections), and anion exchange chromatography was performed using Vivapure QminiH spin columns (Sartorius-Stedim Biotech, Aubagne, France) with a volume of 450 and 150 μL for washing and elution, respectively. After methanol precipitation, GAGs were dissolved in 10 μL of Milli-Q. All centrifugation steps were performed at 14,000 × g.

Quantification of GAGs by dimethylmethylene blue
To determine the concentration of sulfated GAGs isolated from tissues, a spectrophotometric assay developed by Farndale et al. (1986) was used. In brief, 200 μL of reagent (3.04 g/L glycine, 2.37 g/L NaCl and 18 mg/L 1,9-dimethylmethylene blue dye, pH 3.0) was added to 10 μL of samples in microtiter plates, using 0–100 μg/mL of CS-A and HS (from bovine trachea/kidney, respectively; Sigma-Aldrich) as the standards. Absorption was read at 525 nm.

Agarose gel electrophoresis
Separation of the major classes of sulfated GAGs was achieved by agarose gel electrophoresis, as described (van de Lest et al. 1994). Briefly, 50 ng of isolated GAGs was separated using 1% (w/v) agarose gels in 50 mM Ba(Ac)2 buffer (pH 5.0). After electrophoresis at 30 mA/gel in 50 mM Ba(Ac)2 for 50 min, the gel was fixed and stained with 0.1% (w/v) azure A (Sigma-Aldrich) for 60 min and destained in 10 mM NaAc buffer (pH 5.5) for 30 min. After 30 min washing in Milli-Q, the gel was air-dried, followed by a silver-staining procedure to visualize GAGs.

Enzyme-linked immunosorbent assay
A 96-well microtiter plate was coated overnight with GAGs (1 μg/mL in coating solution), followed by rinsing six times with PBS containing 0.1% (v/v) Tween-20 (PBST) and blocking with 3% (w/v) bovine serum albumin (BSA) in PBS containing 1% (v/v) Tween-20. Plates were incubated with a 2-fold serial dilution in the blocking solution of different single-chain variable fragment (scFv) anti-GAG antibodies for 60 min (for epitope preferences of these antibodies, see Smetsers et al. 2004; Lensen et al. 2006; Wijnhoven et al. 2008). Bound scFv antibodies were detected by 60 min incubation with mouse anti-vesicular stomatitis virus (VSV) tag monoclonal antibody P5D4 followed by 45 min incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark). Plates were washed six times with PBST after each incubation. Enzyme activity was detected using 1 mg p-nitrophenyl phosphate/mL as a substrate, dissolved in 1 M diethanolamine with 0.5 mM MgCl2 (pH 9.8). The absorbance was measured at 405 nm.

Polyacrylamide gel electrophoresis
About 300 ng of isolated GAGs was loaded on a 16.5% acrylamide/bis-acrylamide gel and 1 μg of HS was used as a standard. Electrophoresis was performed in 30 mM Tris/22 mM 2-(N-morpholino)ethanesulfonic acid buffer at 200 V for ca. 2 h. After electrophoresis, the gel was stained with 0.8% (w/v) alcian blue [Sigma-Aldrich, in 2% (v/v) acetic acid] for 30 min and then destained by 10% (v/v) acetic acid for two times 30 min, followed by 30 min neutralization in Milli-Q. To visualize GAGs, the gel was placed in 5% (w/v) Na2CO3 for 10 min, and a silver-staining procedure was employed, as was done for agarose gels.

HPLC disaccharide analysis
For the CS/DS disaccharide analysis of rat skin samples, 200 mIU chondroitinase ABC (from Proteus vulgaris, Sigma-Aldrich) was added overnight at 37°C to 10 μg of isolated GAGs in 100 μL of digestion buffer (25 mM Tris and 2 mM MgAc2, pH 8.0). For the HS disaccharide analysis of rat liver samples, 7.5 mIU each of heparinas I, II and III (from Flavobacterium heparinum, IBEX Pharmaceuticals, Montreal, Canada) were added to 15 μg of isolated GAGs in 100 μL of digestion buffer (100 mM NaAc/0.2 mM Ca(Ac)2, pH 7.2) for 2 h at 37°C, after which 7.5 mIU of heparinas was added again and the reaction was allowed to take place overnight. The reaction was stopped by boiling the samples for 30 s, and disaccharide separation was carried out by ion-pair reversed-phase chromatography with a gradient elution using a Supelcosil LC-18-T column (150 × 4.6 mm; 3 μm). Solvents A and B consisted of 5 and 50% aqueous acetonitrile, respectively, both supplemented with 5 mM tetrabutylammonium phosphate and 10 mM ammonium phosphate monobasic (pH 7.0). Elution was carried out using isocratic elution with 100% A for 2 min, linear gradient elution for 33 min to 100% B, followed by a 5-min wash with 100% B and equilibration by 100% A for 10 min. The flow rate was 1.2 mL/min, column temperature was 25°C and detection took place at 232 nm. Identity of disaccharides was determined by comparison with disaccharide standards (Iduron, Manchester, UK) and the amount of specific disaccharides was calculated from the peak surface area.

For the disaccharide analysis of mouse and human skin sections, 15 mIU chondroitinase ABC (for CS/DS analysis) or
0.66 mIU each of heparinases I, II and III (for HS analysis) was added to 2–4 µL of isolated GAGs for 2 h at 37°C in a total of 10 µL in digestion buffer, as described for disaccharide analysis of rat skin samples, after which the same amount of enzyme was added again and the reaction was allowed to take place overnight. Disaccharides were labeled with 2-aminoacridone and separated and detected by reversed phase-HPLC as described (Ambrosius et al. 2008).

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Conflict of interest

None declared.

Abbreviations

BSA, bovine serum albumin; CS, chondroitin sulfate; DS, dermatan sulfate; FFPE, formalin-fixed paraffin-embedded; GAG, glycosaminoglycan; HPLC, high performance liquid chromatography; HS, heparan sulfate; PBS, phosphate buffered saline; PBS-T, PBS with Triton X-100; PBST, PBS with Tween-20; TCA, trichloroacetic acid; VSV, vesicular stomatitis virus.

References


General histochemistry and immunohistochemistry

Hematoxylin and eosin staining was performed as described (Bancroft and Stevens 1996). For staining of CS and DS, 4-µm sections were deparaffinized in xylene and ethanol and nuclear stained with Mayer’s hematoxylin (1 min) followed by rinsing in tap water. After drying for 30 min, the papillary and reticular dermis were separately dissected from 50 tissue sections. Dissection was performed using a Leica Laser Microdissection System equipped with an UV laser (LMD 6000, Leica Microsystems), as described (Janssen et al. 2011).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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