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Increased deposition of glycosaminoglycans and altered structure of heparan sulfate in idiopathic pulmonary fibrosis

Gunilla Westergren-Thorsson a, Ulf Hedström a, b, Annika Nybom a, Emil Tykesson a, Emma Åhrman a, Marie Hornfelt b, Marco Maccarana a, Toin H. van Kuppevelt c, Göran Dellgren d, Marie Wilde a, Xiao-Hong Zhou b, Leif Eriksson a, Leif Bjerner e, Oskar Hallgren a, c, e, f

a Department of Experimental Medical Sciences, Lund University, Sweden
b AstraZeneca R&D, Mölndal, Sweden
c Department of Biochemistry, Radboud University Medical Centre, Nijmegen, The Netherlands
d Department of Cardiothoracic Surgery, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
e Department of Respiratory Medicine and Allergology, Lund University, Sweden.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is characterized by extensive tissue remodelling and aberrant deposition of extracellular matrix (ECM) constituents including collagens, glycoproteins and proteoglycans resulting in stiffer and less compliant tissue that reduces lung function (Bjoraker et al., 1998). Proteoglycans play important roles in the ECM influencing viscoelastic properties, cell differentiation and morphogenesis (Souza-Fernandes et al., 2006). Many of these functions are however properties of covalently attached glycosaminoglycan (GAG) side chains, but these have paradoxically not been fully assessed in IPF. GAGs comprise chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) and hyaluronic acid (HA). The polymers are highly negative due to carboxylate and sulfate groups that mediate binding of a range of proteins such as growth factors and cytokines (Mizumoto et al., 2013; Xu and Esko, 2014). These interactions are not only a matter of charge but also of the position of sulfate groups that creates binding motifs in the polymer chain. HA, the most abundant non-sulfated GAG in the lung, regulates the fluid balance in the
interstitium by its ability to bind and retain water in macromolecular aggregates together with CS-proteoglycans (Allegra et al., 2012; Wight and Hascall, 1983). The CS/DS proteoglycans versican, decorin and CD44 have been reported to increase in IPF, but less is known about their GAG moiety (Bensadoun et al., 1996; Buckley et al., 2011; Venkatesan et al., 2011). CS/DS chains are composed of repeating disaccharides of alternating uronic acid (UA), which can be either β-glucuronic acid (GlcA) or l-iduronic acid (IdoA), and N-acetyl-d-galactosamine (GalNAc), that may be sulfated at the C-2 position of GlcA/IdoA and/or at the C4 and C6 position of GalNAc. The UA in CS is GlcA while in DS a variable number of GlcA residues become epimerized at C-5 to yield IdoA (Malmstrom et al., 2012; Thelin et al., 2013). HS-proteoglycans are also upregulated in IPF and have been suggested to contribute to tissue remodelling and morphogenesis (Jiang et al., 2010; Kliment et al., 2009; Ruiz et al., 2012). These effects may be due to the ability of HS side chains to influence retention and activity of critical mediators including growth factors. HS is for example involved in the coordination of ligand-receptor binding of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and tumour growth factor-beta (TGF-β) that all have been shown to be involved in tissue remodelling processes active in IPF (Abramsson et al., 2007; Takashima et al., 2016; Xu and Esko, 2014). These growth factors are, in addition, targets for Nintedanib and Pirfenidone, the only drugs that have been demonstrated to have clinical efficacy in IPF patients (Jones et al., 2013; Richeldi et al., 2014). HS contains repeating units of UA linked to N-acetyl glucosamine (GlcNAc). During the synthesis in Golgi, the HS chain becomes modified in which GlcNAc residues may become N-deacetylated and N-sulfated, GlcA may be epimerized at C5 to yield IdoA, sulfate groups may be added at C2 on the uronic acid residue and the GlcNAc may be sulfated at C6 and more rarely at C3 (Esko and Lindahl, 2001; Xu and Esko, 2014). The modifications do not go to completion which gives rise to extremely variable structures.

Taken together, the dramatic tissue re-organisation in IPF is accompanied by increased deposition of proteoglycans. However, many of the most important functions of proteoglycans, such as to regulate growth factor binding and activity, reside in the GAG moiety and these structures have not been fully examined in IPF. We hypothesized that GAGs may be involved in this process and the aim of this study was therefore to examine the fine structure of glycosaminoglycans in tissue from dense and less dense areas of the lungs from patients with IPF in comparison with samples from donor lungs.

2. Material methods

2.1. Study subjects

2.1.1. Patients

Patients (n = 10) with severe IPF who were undergoing lung transplantation at the Sahlgrenska University hospital in Gothenburg between 2014 and 2015 were included in the study. The diagnosis of IPF was verified by histological examination of the
explanted lungs. The patients had stopped smoking at least 6 months before the lung transplantation. Written consent was obtained from all subjects before being included in the study.

2.1.2. Control subjects

Lung explants from healthy organ donors (n = 7) with no history of lung disease were included. Lungs were to be used for transplantation but could instead be included in this study as no matched recipient could be identified. In these cases, written consent was obtained from their closest relatives. The study was approved by the Swedish Research Ethical Committee in Lund (FEK 91/2006) and in Gothenburg (FEK675-12/2012).

2.2. Tissue dissection

After crude dissection tissue pieces from distal lungs were kept in Dulbecco’s MEM supplemented with 10% FBS, Gentamycin and Penicillin-Streptomycin (all from Gibco BRL, Paisley, UK) on ice until further dissection. From IFP patients, tissue pieces were taken from locations just underneath the pleura with low compliance and were denoted “IFP dense”. In parallel, tissue pieces were taken at some distance from the pleura from less dense tissue areas, as exemplified in Fig. 1, and named “IPF less dense”. From donor lungs, samples were taken from just below the pleura. Visible airways and vessels and pleura were systematically avoided. All samples were then stored in –80 °C until further analysis.

2.3. Glycosaminoglycan isolation and digestion

A minimum of two separate tissue pieces was analysed from each individual and location. The method to isolate and digest GAG disaccharides has previously been developed in our laboratory (Stacheta et al., 2015). All tissue samples were first lyophilized and weighed. Approximately 2 mg tissue (dry weight) was used from each tissue sample. Proteins were degraded by an overnight incubation at 55 °C with 200 μg/ml pronase (Pronase E, Sigma-Aldrich, St. Louis, MO, USA) in 50 mM Tris/HCl, pH 8, 1 mM CaCl₂, and 1% Triton X-100. After heat-inactivation of the protease, MgCl₂ was added to a final concentration of 2 mM and benzonase (Benzonase® Nuclease, Sigma-Aldrich, St. Louis, MO, USA) was added to a final 20 Sigma units/ml and incubated for 2 h at 37 °C. Benzonase was heat-inactivated, NaCl was added to a final concentration of 0.1 M, and GAGs were purified on an anion spin column (Vivapure Q mini H, Sigma-Aldrich, St. Louis, MO, USA). The GAGs were washed × 5 with 20 mM NaAc pH 5.5, 0.2 M NaCl buffer, and eluted with 20 mM NaAc pH 5.5, 2 M NaCl buffer. Samples were desalted using spin columns with a cut-off size of 3 kDa Amicon® Ultra 0.5 centrifugal filter device. Millipore, Billerica, MA, USA by repeated addition of water. Sample amounts that corresponded to 0.3 mg of the initial dry tissue were used for each digestion. To generate disaccharides from chondroitin sulfate, dermatan sulfate and hyaluronic acid, GAGs were subjected to chondroitinase ABC degradations, overnight at 37 °C in 20 μl 50 mM NH₄OAc and 0.1 mg/ml BSA, containing 10 μl chondroitinase ABC (Chondroitin ABC Lyase, C3667, Sigma-Aldrich, St. Louis, MO, USA). The samples were boiled and the supernatant was dried. As the elimation chondroitinase ABC leaves a double bond C4 = C5, the CS epimeric configuration in the native sugar is lost, therefore it cannot be determined whether the resulting disaccharides originally contained IdoA (dermatan sulfate) or GlcA (chondroitin sulfate). Therefore, all chondroitinase ABC-generated disaccharides are named CS/DS. To degrade HS, the samples were incubated overnight at 37 °C with a mixture of heparinase I, II and III (10 μM of each; overexpressed in E. coli, a kind gift from Jian Liu, University of North Carolina at Chapel Hill) in buffer 20 mM HEPES, 50 mM NaCl, 4 mM CaCl₂, pH 7.

2.4. Glycosaminoglycan disaccharide analysis

Disaccharide analysis was performed as previously described (Stacheta et al., 2015). Briefly, fluorophore-labelling of the resulting disaccharides was performed by adding 10 μl of 20 mM re- purified 2-aminoacridone (AMAC) (Sigma-Aldrich, St. Louis, MS, USA) to lyophilized samples, followed by a 20 min incubation at room temperature before the addition of 10 μl of 1 M NaBH₄CN and incubation at 45 °C for 16 h. AMAC-labelled disaccharides were analyzed with HPLC-fluorescence on a X-Bridge BEH Shield RP18 (2.1 × 100 mm, 2.5 μm) column connected to a Thermo Scientific UltiMate 3000 Quaternary Analytical system with an FLD-3400RS fluorescence detector (excitation λ = 428 and emission λ = 525) set at 30 °C. 20 μl samples were diluted to 100 μl in running buffer (98% A: NH₄OAc, 60 mM, pH 5.6, and 2% B: MeCN) and 2 μl were injected onto the column. Disaccharides were separated using a 39 min gradient run at 0.35 ml/min (0–1 min: 98% A, 1–3 min: 98–96% A, 3–26 min: 96–85% A, 26–28 min: 85–10% A, 28–32 min: 10% A, 32–34 min: 10–98% A, 34–39 min: 98% A). Quantification was done by comparison to known weight of standard disaccharides (Iduron, UK) mock-treated in the same buffers and enzymes as the samples in each series of runs.

2.5. Determination of hydroxyproline

A commercial kit was used to analyse the hydroxyproline content (MAK008, Hydroxyproline assay kit, Sigma-Aldrich, Saint-Louis, MO, US). All samples were analysed in duplicate according to the manufacturer’s instructions. From each subject and location, a minimum of two pieces were analysed. Intact tissue was lyophilized and weighed and samples were hydrolysed in 6 M HCl at 120 °C for 3 h. Tissue homogenates were treated with activated charcoal to reduce soot particles that could influence the colorimetric reaction. The hydroxyproline content in the samples was determined by comparison to a standard curve.

2.6. Sequential extraction of collagen

The method has been previously described (Kalamański et al., 2014). Briefly, collagen was sequentially extracted in 0.5 M acetic acid followed by 0.5 M acetic acid containing 1 mg/ml pepsin acid at 4 °C for 24 h for each step. Supernatants were collected by centrifugation at 14000 × g for 30 min, and pellets were used in the next step. In order to further separate fibrous collagens from other types of collagens were precipitated in 0.9 M NaCl, pH 2.5 overnight at 4 °C on a shaker. After centrifugation at 14000 × g for 10 min, the pellets were washed in 99% Ethanol, 50 mM NaAc at –20 °C for 1 h. The pellets were collected by centrifugation at 14000 × g for 30 min. The hydroxyproline content was measured as described above in all supernatants and pellets.

2.7. Mass spectrometry

HAC-pepsin digested collagens were separated on 7% Tris-Acetate gels and bands were visualized by SimplyBlue SafeStain (both from Invitrogen, Carlsbad, CA, USA). In-gel digestion of individual protein bands was performed using standard protocols described in (Malmstrom et al., 2016). LC–MS/MS measurements of digested peptides were acquired on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). Peptide separation was carried out by an EASY-nLC 1000 liquid chromatography system (Thermo Fisher Scientific) using an Acclaim PepMap® 100 (75 μm × 2 cm nanoViper C18, 3 μm, 100 Å) pre-column (Thermo Fisher Scientific), followed by an EASY-Spray (75 μm × 25 cm) separation column. LC and MS settings are described in (Malmstrom et al., 2016). Briefly a 60 min linear gradient (5–30% over 60 min)
of acetonitrile in aqueous 0.1% formic acid was used at a flow rate of 300 nl/min. For each full MS scan (resolution 70,000 at 200 m/z, mass range 400–1600 m/z) the 15 most abundant ions (TOP15) were selected for fragmentation (resolution 17,500 at 200 m/z). The resulting mass spectra were searched using the Trans-Proteomic Pipeline (TPP, version 4.7) (Deutsch et al. 2010) against the unreviewed Human UniProt FASTA database (version April 2015). Cysteine carbamidomethylation was set as fixed peptide modification and methionine oxidation and hydroxylation of proline were set as variable peptide modifications. A precursor ion mass tolerance of 20 ppm and a fragment ion mass error tolerance of 50 ppm were used. The false discovery rate was set to 1%.

2.8. RNA isolation, cDNA preparation and real-time quantitative PCR analysis

Tissue samples from IPF patients and donor lungs were harvested in RNAlater (Qiagen, Hilden, Germany) and stored in −70°C until further processing. Total RNA was isolated using the mini RNeasy Mini Kit from (Qiagen, cat. No. 74104). For preparation of cDNA, the Quantitate Reverse Transcription Kit (Qiagen, cat. No. 205311) was used. For the qPCR, cDNA preparations were mixed with Quantitect SYBR Green (Qiagen, cat. No. 204143) along with specific primers from Qiagen, N-Deacetylase/N-Sulfotransferase 1, NDS1, (cat. No. QT01002638), Deacetylase/N-Sulfotransferase 2, NDS2, (cat. No. QT00030520), heparan sulfate 6-O-sulfotransferase 1, HSST1, (cat. No. QT0078547), heparan sulfate 6-O-sulfotransferase 2, HSST2, (cat. No. QT01028370), heparan sulfate 2-O-sulfotransferase 1, HS2ST1, (cat. No. QT0010073), 18S ribosomal RNA, RNN18S, (cat. No. QT00199367), and analyzed on a Mx3000P qPCR system (Stratagene, La Jolla, CA, USA) with standard cycling parameters to perform thermo-cycling and real-time detection of PCR products.

2.9. Immunohistochemistry

8 μm cryosections of lung tissue from healthy donors and IPF patients were air-dried and rehydrated. For A04B08V, endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 min, followed by blocking of endogenous biotin using a streptavidin/biotin blocking kit according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA, USA). After blocking with 1% BSA and 0.05% Tween-20 for 20 min, tissue sections were incubated for 1 h at room temperature with a 1:10 dilution of the phage-display antibody fragment A04B08V which had been demonstrated to preferentially recognize 2-O, 6-O- and N-sulfated HS polymers (Kurup et al., 2007). This step was followed by incubation with a 1:800 dilution of mouse anti-VSV-G antibody (clone P5D4) (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h. Sections were then fixed in 4% formalin for 30 min and incubated with a biotinylated goat anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. Following incubation with the avidin/biotin-based peroxidase complex Vectastain Elite ABC (Vector Laboratories, Burlingame, CA, USA), sections were developed with the peroxidase substrate NovaRED (Vector Laboratories, Burlingame, CA, USA), all according to the manufacturer’s instructions. Sections were counterstained with Mayer’s haematoxylin (Sigma-Aldrich, St. Louis, MO, USA), dehydrated, mounted with Pertex mounting medium (Histolab, Gothenburg, Sweden) and photographed using an Olympus BX50F microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP80 camera (Olympus, Tokyo, Japan). To verify the specificity of the stainings, tissue sections were pre-treated overnight at 37°C with a combination of heparinase I, heparinase II and heparinase III, each at 0.1 U/ml, in an enzyme buffer made up of HEPES (20 mM) NaCl (50 mM), CaCl₂ (4 mM), and BSA (0.01%) (pH 7.2). For the

| Table 1 |
| Control subjects and IPF patients in the study. |
| Characteristics | Controls (range) | IPF (range) |
| No. | 7 | 10 |
| Age (range) | 51 (39–65) | 57 (45–69) |
| Ex-smokers/non-smokers | 1/6 | 8/2 |
| Gender, M/F | 4/3 | 4/6 |
| Lung function | | |
| FEV₁ | m | 1.6 (0.8–2.4) |
| FEV₁ (% predicted) | m | 56 (30–90) |
| FVC | m | 2.0 (0.9–3.3) |
| FVC (% predicted) | m | 56 (31–106) |
| TLC | m | 3.1 (1.5–4.7) |
| TLC (% predicted) | m | 49 (31–96) |
| DLCO | m | 2.1 (1.3–2.8) |
| DLCO (% predicted) | m | 27 (16–47) |

* Only values from nine patients.
† Only values from eight patients. m denotes that data is missing.

Heparan sulfate and Perlecan stainings, sections were fixed immediately after air-drying and incubated with the primary antibody at 10 μg/ml of anti-Heparan Sulfate, 10E4 epitope, Amsbio, Abingdon, UK) or 0.5 μg/ml of anti-Perlecan, 785 clone. ThermoFisher Scientific, Rockford, IL, USA) for 2 h at room temperature, followed by incubation with the biotinylated goat anti-mouse IgG antibody and development according to procedures described above. The specificity of the Heparan Sulfate antibody was verified by a pre-treatment overnight at 37°C with a mixture of heparinas III as described for A04B08V and for anti-Perlecan by use of a mouse IgG1 isotype control.

2.10. Statistics

Data are expressed as mean ± SD. Statistical differences between groups were determined by the Mann-Whitney U test. In addition, the Wilcoxon signed rank test was used to determine differences within patients. Differences were considered significant at p < 0.05. All analyses were performed using GraphPad Prism software version 6.0f (GraphPad Software, San Diego, CA).

3. Results

3.1. Study subjects and dissection

The mean age was 51 years (range 39–65) in controls and 57 years (range 45–69) in the IPF group. Among the IPF patients 8 out of 10 were ex-smokers whereas 1 out of 7 of the controls had a history of smoking. The mean FEV₁ was 56% of predicted (range 30–90%) and the mean FVC was 56% of predicted (range 31–106%) in the IPF patients. DLCO was 27% of predicted (range 22–47%) in IPF patients. Demographics of IPF patients and control subjects are shown in Table 1. The diagnosis of IPF patients was verified by histological examination by an experienced pathologist of the explanted lungs. Since the control tissue came from donor lungs that ultimately were not used for transplantation we have no lung function parameters from these individuals. From IPF patients, lung pieces were sampled from two separate locations: from (1) dense tissue areas just below the pleura and from (2) less dense areas more distant from the pleura, while from control subjects only sub-pleural samples were taken (Fig. 1).

3.2. Hydroxyproline determination in lung tissue

To characterize the sampled tissue, hydroxyproline was measured as an indirect measure of the total collagen content. There was a trend that the hydroxyproline content was higher in both less
dense and dense areas from IPF patients compared to control lungs but these differences were not statistically significant (**Fig. 2A**).

The hydroxyproline values corresponded to 95 μg collagen/mg tissue in controls and 128 and 118 μg collagen/mg tissue in less dense and dense areas of IPF lungs, respectively (**Table 2**). Moreover, in adult human lung the majority of fibrillar collagens are heavily cross-linked and the turnover rate is relatively low. In active remodelling processes, such as in IPF, a higher degree of ECM turnover can be expected and this might result in elevated levels of collagen that are not fully cross-linked. To try this hypothesis, collagen was serially extracted from the lung samples and hydroxyproline was measured in all steps. Acid treatment causes a release of collagens with crosslinks mainly in the helix domain. However, this treatment resulted in levels that were below the detection limit of the hydroxyproline measurement in all samples (data not shown). In contrast, 3–8% of the total pool of collagens were extracted by the addition of pepsin that releases collagens with cross-links also in the telopeptide domain (**Fig. 2B**). The absolute majority of collagens in this fraction were fibrillar as they precipitated in 0.9 M NaCl at pH 2.5. The predominating collagens were the fibrillar type-I, III and V collagens but also type-IV and VI collagen (**Table S1**). There was a significant increase in the relative level of pepsin-extracted collagens in less dense areas of IPF lungs compared to dense areas. However, the majority of collagens were acid and pepsin-resistant suggesting that they were further cross-linked (**Fig. 2B**).

**3.3. Increased levels of all GAGs in IPF lungs**

By isolating and digesting GAGs with specific enzymes we could quantify the total pool of CS/DS, HA, HS disaccharides with RP-HPLC (**Figs. 3, S1 and S2**, values for individual patients are shown in **Fig. S3**). With this protocol we were able to analyse the six most common CS/DS disaccharides, HA and the eight most common HS disaccharides with base-line separation. There was a three-fold increase in the total pool of GAGs as well as in CS/DS, HA and HS in both less dense and dense areas in IPF lungs. This corresponded to 1.3 μg of total GAG/mg tissue in controls and 3.4 and 3.6 μg total GAG/mg tissue in less dense and dense areas of IPF lungs, respectively (**Table 2**) suggesting that GAGs are relatively more increased than collagen in IPF.

**3.4. Increased levels of CS/DS in IPF lungs**

Individual CS/DS disaccharides were analysed by HPLC (**Figs. 4 and S1**). All the investigated disaccharides were increased both in less dense and dense areas of IPF lungs compared to donor lungs (**Fig. 4A**). Accordingly, the general increase in all CS/DS disaccharides in IPF resulted in elevated levels of 4-O, 6-O, 2-O sulfated disaccharides and thereby in the total pool of sulfated CS/DS GAGs (**Fig. 4B**). To examine if there were also alterations in the relative abundance of CS/DS disaccharides the disaccharide composition was calculated (**Fig. 4C and D**). In dense areas in IPF lungs the relative degree of sulfation was increased and, as a consequence,
Table 2
Absolute levels of collagen and GAGs.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IPF less dense</th>
<th>IPF dense</th>
<th>(range)</th>
<th>(range)</th>
<th>(range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen(^a)</td>
<td>95</td>
<td>128</td>
<td>118</td>
<td>(60–134)</td>
<td>(74–194)</td>
<td>(79–181)</td>
</tr>
<tr>
<td>Total GAG(^b)</td>
<td>1.3</td>
<td>3.4</td>
<td>3.6</td>
<td>(0.8–1.9)</td>
<td>(1.3–4.8)</td>
<td>(1.8–6.0)</td>
</tr>
<tr>
<td>CS/DS(^b)</td>
<td>0.6</td>
<td>1.5</td>
<td>1.6</td>
<td>(0.3–1.0)</td>
<td>(0.5–2.3)</td>
<td>(0.9–2.3)</td>
</tr>
<tr>
<td>HA(^b)</td>
<td>0.3</td>
<td>0.9</td>
<td>1.0</td>
<td>(0.1–0.4)</td>
<td>(0.2–1.3)</td>
<td>(0.6–1.7)</td>
</tr>
<tr>
<td>HS(^b)</td>
<td>0.4</td>
<td>0.9</td>
<td>1.0</td>
<td>(0.3–0.5)</td>
<td>(0.5–1.2)</td>
<td>(0.3–2.1)</td>
</tr>
</tbody>
</table>

\(^a\) Mean value of the total collagen content in tissue samples (μg collagen/mg dry tissue). The collagen concentration was calculated as (measured hydroxyproline × 7.4) as has been described (Neuman and Logan, 1950).

\(^b\) The mean of individual GAGs or the sum of all measured GAGs (μg GAG/mg dry tissue).

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**Fig. 4.** CS/DS is increased in both dense and less dense areas in IPF lungs.

GAGs were digested with chondroitinase ABC to generate disaccharides that were separated with HPLC and compared to disaccharide standards as shown in Fig. S1. A. Absolute quantification of CS/DS disaccharides. B. Summary of CS/DS disaccharides with specific sulfation. [Total CS/DS]: the sum of all CS/DS; [Sulfated CS/DS]: the sum of sulfated disaccharides i.e. UA-GalNAc-4S (A), UA-GalNAc-6S (C), UA-2S-GalNAc-4S (B), UA-2S-GalNAc-6S (D), UA-GalNAc-4S-6S (E), [4-O-S]: the sum of 4-O-sulfated disaccharides i.e. UA-GalNAc-4S (A), UA-2S-GalNAc-4S (B), UA-GalNAc-4S-6S (E), [6-O-S]: the sum of 6-O-sulfated disaccharides i.e. UA-GalNAc-6S (C), UA-2S-GalNAc-6S (D), UA-GalNAc-4S-6S (E), [2-O-S]: the sum of 2-O-sulfated disaccharides i.e. UA-2S-GalNAc-6S (D), UA-GalNAc-4S (B), [non-sulfate]: non-sulfated disaccharides i.e. UA-GalNAc (O). C, CS/DS disaccharide composition presented as mole% of total. D, Relative abundance of the different sulphation patterns of CS/DS disaccharides presented as the mole% of total. A minimum of two separate tissue pieces was analysed from each subject and location (control subjects n = 7 and n = 10 for IPF patients). *P<0.05, **P<0.01 and ***P<0.001 using Mann-Whitney test.
non-sulfated disaccharides were decreased compared to donor lungs. This data suggests that there is a general increase in CS/DS in dense and less dense areas in IPF lungs and, in addition, in dense tissue areas the relative amount of sulfation is increased.

3.5. Increased levels of HS in IPF lungs

The eight most abundant HS disaccharides found in human tissue was analysed in the lung samples by RP-HPLC (Figs. 5A and S2). All disaccharides were significantly increased both in less dense and dense areas in IPF lungs compared to donor lungs (Fig. 5A and B). This resulted in an increase in the total pool of sulfated HS disaccharides as well as N-, 2-O and 6-O sulfated HS (Fig. 5B).

The triple-sulfated disaccharide UA-2S-GlcNS-6S was increased five-fold and ten-fold in less dense and dense areas of IPF lungs, respectively and was thus the most increased disaccharide. Next, the disaccharide composition was calculated (Fig. 5C and D). In dense areas of IPF lungs there was a significant increase of UA-2S-GlcNS-6S, UA-GlcNS-6S and UA-2S-GlcNAc on expense of UA-GlcNAc and UA-GlcNS that was decreased compared to donor lungs. UA-2S-GlcNS-6S was also increased, while UA-GlcNS was decreased in less dense tissue areas in IPF. In both less dense and dense areas in IPF lungs the relative degree of sulfation was increased and, as a consequence, non-sulfated disaccharides were decreased compared to donor lungs. The increase was dependent on elevated levels of N-, 2-O and 6-O sulfation. We conclude that there was
a general increase in all HS disaccharides in IPF lungs. The disaccharide composition revealed that HS is more sulfated in both less dense and dense areas of IPF lungs compared to donors.

3.6. Expression of enzymes that catalyse the sulfation of HS

We next examined the expression of the sulfotransferases that are responsible for the sulfation of HS. As we did not record a difference in the amount of sulfation of HS between dense and less dense tissue areas from IPF patients we only analysed the tissue expression in less dense tissue samples. We examined Deacetylase/N-Sulfotransferase, NDST1, and Deacetylase/N-Sulfotransferase 2, NDST2, that are responsible for N-sulfation, Heparan Sulfate 6-O-Sulfotransferase 1, HS6ST1 and Heparan Sulfate 6-O-Sulfotransferase 2, HS6ST2, that are responsible for 6-O sulfation and Heparan Sulfate 2-O-Sulfotransferase 1, HS2ST1 responsible for 2-O sulfation. The expression of HS6ST2 was decreased in tissue from IPF patients compared to from donor lungs, while no other enzymes were altered (Fig. 6).

3.7. Location of highly sulfated HS in IPF lungs

To locate the most increased HS disaccharides, UA-2S-GlcNS-6S and UA-GlcNS-6S, in IPF lungs, immunohistochemistry was used with an antibody fragment, AO4B08, developed by phage display that preferentially binds N-sulfated octasaccharides with three consecutive 6-O sulfated disaccharides and an internally located 2-O sulfate group (Kurup et al., 2007). To differentiate positive staining from background, sequential sections were pre-treated with a mixture of heparinase I–III that degrade heparan sulfate and eliminate the epitope. In addition, we used another antibody, 10E4, that prefers HS epitopes that includes N-sulfated glucosamine residues and thus has a broader HS binding specificity (van den Born et al., 2005). As HS is found in basement membranes we also included antibodies against perlecan to elucidate a potential core protein. In donor lungs there was a very limited staining of AO4B08 while 10E4 and perlecan were widely distributed in basement membranes in airways and blood vessels and in smooth muscle cells (Fig. 7). In IPF patients, there was a strong signal for AO4B08 in the border zone between dense and less dense tissue areas while the signal was weaker in areas with dense fibrosis and in normal looking alveoli (Figs. 8 A and B and S4). The staining for 10E4 and perlecan were widely distributed throughout the tissue in basement membranes of airways and blood vessels (Figs. 8 C–F and S4). As a consequence, the three antibodies were located on similar structures in the border zones, suggesting that perlecan may be one core protein that harbours highly sulfated heparan sulfate. This data show that HS is very abundant in the IPF lung, but highly sulfated HS is restricted mainly to the border zones that may represent areas with more active tissue remodelling. Close-ups of the tissue localisation of AO4B08 reveals that it is present on basement membranes and smooth muscle cells of airways and differently sized blood vessels (Fig. S5). Finally, in a majority of IPF patients, immuno-positive spindle-shaped elongated cells were found in the border zones (Fig. S5).

4. Discussion

Our results show that HS, CS/DS and HA are increased in both less dense and dense areas in lung tissue from patients with severe IPF compared to donor lungs. The HS disaccharide composition was altered in IPF with increased proportion of 2-O, 6-O and N-sulfated disaccharides and there was also an increased degree of sulfation of CS/DS. Highly sulfated HS was specifically located in the border zone between denser areas and more normal looking alveoli. Here it was located in basement membranes positive for perlecan as well as on the cell surface of spindle-shaped cells in the alveolar interstitium. As these structures have been implicated in growth factor retention and activity this may have impact on the fibrotic process (Esko and Lindahl, 2001).

In the present study CS/DS, HA and HS were increased 3-fold in IPF patients and there was a trend that the total collagen content was increased in IPF patients compared to control subjects. Subsequently, the GAG to collagen ratio increased from around 1% in controls to more than 3% in IPF patients (weight/weight). This data show that the dramatic tissue remodelling in IPF is accompanied by a higher increase in GAGs than in collagen. Moreover, we observed that the HS composition was modified in IPF as the proportion of N, 2-O and 6-O-sulfated HS disaccharides was increased compared to control. Alterations in the HS sulfation landscape have been demonstrated to influence growth factor retention and activity (Lensen et al., 2005). An increase in the proportion of 6-O sulfated disaccharides and transcripts for HS6ST1 and HS6ST2, the enzymes that are responsible for this specific sulfation has been shown in IPF, and silencing of these gene transcripts in lung fibroblasts resulted in a lower responsiveness to TGF-β1 (Lu et al., 2014). In this study we did not see an increase of the sulfotransferase transcripts, but mRNA only show a snapshot of what is going on in the tissue and extracellular HS may have a slower turnover. Increased proportion of 6-O sulfated HS has also been shown in chronic renal fibrosis and was associated with the ability to mediate FGF-2 signalling (Alhasan et al., 2014). The role of HS in the formation of ternary complex between ligands and receptors in FGF family has been studied extensively and the binding of FGF-1 and FGF-2 to FGR-1 and FGR-2 require N, 2-O and 6-O sulfated HS (MacKenzie et al., 2015; Powell et al., 2002; Settembre et al., 2008). PDGF has also been implicated in the pathogenesis of IPF and the retention and activity in tissue has been demonstrated to be dependent on the interaction with HS (Abramsson et al., 2007; Homma et al., 1995; Maeda et al., 1996). For this interaction N-sulfation was required while it was the overall degree of sulfation rather than the specific amount of 2-O and 6-O sulfation that was important. The altered HS composition that we found in IPF may thus suggest that the ECM landscape in IPF favours growth factor activity and tissue remodelling. Moreover, by immunohistochemistry we found that highly sulfated HS was specifically located in the border zone between dense and less dense areas in basement membranes of blood vessels and airways. These structures were also positive for the proteoglycan perlecan suggesting that this may be one core protein that harbours the highly sulfated HS polymers. We also found highly sulfated HS on the cell membrane of spindle-shaped fibroblast-
like cells in the interstitium in IPF patients. This result confirms data showing that cell bound HS-proteoglycans, including syndecans and glypicans are increased in IPF (Jiang et al., 2010; Kliment et al., 2009; Ruiz et al., 2012).

We observed an increase in CS/DS in both dense and less dense areas of IPF lungs. The increase was due to elevated levels of all observed disaccharides and there was also a difference in the CS/DS composition with a higher proportion of sulfation. Increased levels of 4-O and 6-O-sulfated CS/DS disaccharides have been reported in experimental fibrosis and up-regulation of key enzymes in the CS/DS biosynthesis has been shown in IPF patients (Venkatesan et al., 2011). Moreover, elevated mRNA level and tissue staining of CS/DS proteoglycans including versican, CD44 and decorin has been shown in lungs from IPF patients (Bensadoun et al., 1996; Buckley et al., 2011; Venkatesan et al., 2014; Westergren-Thorsson et al., 2004). Noteworthy, our analysis could not determine the content of iduronic acid in the CS/DS chains.

In the present study the collagen content in IPF patients tended to increase compared to control subjects, without reaching statistical significance. Although this is consistent with one study the majority of studies suggest that the total collagen content as well as the predominating collagen isoforms increase in IPF patients compared to control subjects (Fulmer et al., 1980, 1979; Kirk et al., 1986, 1984; Parra et al., 2006; Rahhu et al., 1985; Selman et al., 1986). However, the increase is sometimes subtle because of the heterogeneous distribution of collagen in lung tissue and the patchiness of fibrotic areas in IPF lungs, that may introduce sampling errors (Laurent, 1986). This problem may be minimized by increasing the sample size from each individual and to include more patients which was not possible in the present study. The increased tissue stiffness in IPF may also be a matter of collagen cross-linking (Chien et al., 2014; Last et al., 1990; Neuman and Logan, 1950). We approached this issue by sequential extraction of collagen. The majority of collagens were resistant to acid and pepsin extraction both in IPF patients and control subjects suggesting that fibrillar
collagens are heavily cross-linked in adult individuals regardless of disease as has been shown in other studies (Seyer et al., 1976). However, as we did not examine pepsin-insensitive collagens that were further cross-linked the possibility that they may still be alterations cannot be excluded. Our analysis revealed an increase in the proportion of fibrillar collagens sensitive to pepsin in less dense compared to dense tissue areas in IPF when using paired analysis. This suggests that there are areas within the IPF lungs with increased collagen turnover and less cross-linked collagen, as has previously been shown (Decaris et al., 2014).

One of the major roles of glycosaminoglycans, and HA in particular, is to regulate fluid content in tissue. Mature tissue has a lower concentration of water than in early life, but during tissue repair higher water content is crucial to rebuild the matrix scaffold, which fits well with active tissue remodelling in IPF (Turino and Cantor, 2003). How this alteration influences compliance and tissue stiffness remains to be elucidated. In addition, HA has been shown to influence the phenotype and especially the invasiveness of fibroblast/myofibroblasts. This property is dependent on HA and its receptor CD44, as the invasive capacity of fibroblasts from IPF lungs is dependent on expression of HA synthase 2 (Li et al., 2011).

More material than can be achieved by lung biopsies were needed for the analysis presented in this study, and we therefore used lung explants from severe IPF patients and donors and could not examine patients in less advanced disease states. IPF is considered to be a progressive disease characterized by dense fibrotic tissue areas that expand over time. With this knowledge we studied tissue samples from dense or less dense areas of IPF lungs. The selection of dense and less dense tissue samples was based on morphology and location in relation to the pleura. There was, however, a great deal of heterogeneity between and within IPF patients. Despite this, we were able to record differences that reflect disease activity suggesting that the division of sample groups still may be relevant. Our cohort of IPF patients was younger than what can be expected but to our knowledge there are no cases of familiar disease but we cannot exclude that possibility. The fact that we only have

Fig. 8. Highly sulfated HS co-localize with perlecan in the border zone between dense and less dense areas of IPF lungs. Serial cryosections from IPF lungs were stained with A04B08, a phage display-derived antibody fragment, that recognizes highly sulfated regions in HS that contain N-, 2-O and 6-O sulfated disaccharides, with 10E4, that are reactive against less sulfated stretches within HS polymers and for the HS-proteoglycan perlecan. A Staining for A04B08. B. Pre-treatment with Heparinase I, II and III decreases the reactivity of A04B08. C. Staining for 10E4. D. Pre-treatment with Heparinase I, II and III eliminates the staining of 10E4. E. Perlecan staining that requires Heparinase pre-treatment. F. Isotype control. Structures that are stained by all three antibodies are shown indicated by solid arrowheads. Open arrowheads show structures that are positive for 10E4 and perlecan but not for A04B08. Scale bars = 200 μm.
patients that were undergoing lung transplantation may favour a younger cohort.

5. Conclusion

In conclusion, our data show for the first time, that the aberrant tissue remodelling in IPF is accompanied by an increased deposition of GAGs. In addition, the fine structure of HS is altered with increased sulfation, which may influence growth factor binding and activity and thus impair the fibrotic process.

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Appendix A: Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.biocel.2016.12.005.

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