



Increased hyaluronic acid content in idiopathic pulmonary arterial hypertension

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ABSTRACT: Idiopathic pulmonary arterial hypertension (IPAH) is a fatal disease characterised by elevated blood pressure in the pulmonary circulation. Initial vasoconstriction, proliferation of pulmonary arterial smooth muscle cells (PASMC) and increased deposition of extracellular matrix (ECM) contribute to pathological remodelling of pulmonary arterioles in IPAH. Glycosaminoglycans (GAGs), components of the ECM, control cellular proliferation and differentiation, but their expression in IPAH remains elusive.

In the present study, GAG expression was investigated in the lungs of patients with IPAH or control transplant donors, and expression and localisation of GAG-metabolising enzymes were analysed *in vivo* and *in vitro*.

A significant increase in the expression of hyaluronic acid (HA) was detected in IPAH lungs, associated with increased hyaluronan synthase (*Has*)1 and decreased hyaluronoglucosaminidase 1 gene expression, as assessed by quantitative RT-PCR and Western blotting. HAS1 protein localised to PASMC *in vivo* and increased HA deposition was observed in remodelled pulmonary arteries in IPAH. Transforming growth factor- β 1, a profibrotic growth factor, led to increased HA secretion and HAS1 expression in primary PASMC.

The results demonstrate an increased hyaluronic acid content in idiopathic pulmonary arterial hypertension lungs, associated with increased hyaluronan synthase 1 and decreased hyaluronoglucosaminidase 1 gene expression. Synergistic regulation of glycosaminoglycan-metabolising enzymes in favour of accumulation may, thus, regulate pathological vascular remodelling in idiopathic pulmonary arterial hypertension lungs.

KEYWORDS: Hyaluronic acid, pulmonary arterial hypertension, pulmonary arterial smooth muscle cells, transforming growth factor- β 1, vascular remodelling

Idiopathic pulmonary arterial hypertension (IPAH) is a rare but fatal disease characterised by elevated blood pressure in the pulmonary circulation due to increased vascular resistance of pulmonary arterioles [1, 2]. If untreated, IPAH leads to right ventricular hypertrophy and failure and subsequent death. Early in disease pathogenesis, endothelial cell dysfunction triggers increased vasoconstriction and *in situ* thrombosis. This is followed by pathological vascular remodelling, a process characterised by intimal fibrosis and thickening of the medial and adventitial layers due to uncontrolled proliferation of pulmonary arterial smooth muscle cells (PASMC) and perivascular fibroblasts [3–5]. In parallel, enhanced cellular activation of PASMC and fibroblasts leads to excessive extracellular matrix (ECM) deposition, which potentiates the increased stiffness of pulmonary arteries in IPAH [6, 7].

Altered ECM turnover is a hallmark of several pulmonary diseases, including adult respiratory

distress syndrome, asthma, idiopathic pulmonary fibrosis, or chronic obstructive lung disease, which underlines the importance of ECM homeostasis for proper lung function [7–9]. In the lung, the ECM is subjected to a daily turnover of ~10% of total ECM, indicating that subtle changes in turnover rates accumulate to produce large changes in total ECM composition with time [10]. The ECM is largely composed of collagens, fibronectin, vitronectin, proteoglycans and glycosaminoglycans (GAGs) [11]. GAGs are linear acidic polysaccharides of variable length and composition that are grouped into four major categories: hyaluronic acid (HA); heparin and heparan sulphate (HS); chondroitin and dermatan sulphates (CS and DS, respectively); and keratan sulphate [12, 13]. GAGs have previously been shown to control lung inflammation, as well as the phenotype of systemic vascular smooth muscle cells [14–16].

HA is a major component of the basement membrane and constitutes ~10% of all proteoglycans [17].

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HA is synthesised at the inner surface of the plasma membrane by one or more hyaluronan synthase (HAS) isoforms (HAS1, -2 or -3), which exhibit distinct enzymatic properties and synthesise HA chains of various average chain length [18]. After synthesis, HA chains are extruded through pore-like structures into the extracellular space. Alternatively, HA can be degraded by one of four hyaluronoglucosaminidase (HYAL) isoforms (HYAL1–4). HYAL1, the main hyaluronoglucosaminidase, exhibits the highest hyaluronidase activity and, thus, represents the major enzyme for HA degradation [19].

HA can influence cellular responses in several ways. First, the interaction of HA with pericellular macromolecules leads to softening of the ECM, thereby facilitating cell shape changes that are required for cell division, migration or plasticity [20–22]. As a result, increased levels of HA have been implicated in inflammation, morphogenesis, regeneration, wound healing, tumour invasion or cancer metastasis [21–23]. Secondly, HA can directly interact with the cell surface receptors CD44 and the receptor for hyaluronan-mediated motility (RHAMM), which directly transduce intracellular signalling cascades *via* extracellular signal-regulated kinase 1/2 [12, 24, 25].

CD44 and HA expression was increased after lung injury induced by the DNA-damaging chemotherapeutic agent bleomycin and mediated pro-inflammatory mediator release by alveolar macrophages [26]. Increased HA content was detected in atherosclerotic regions [20, 27, 28] and HA has been shown to induce smooth muscle cell migration [29]. Overall, this suggests the potential involvement of HA in remodelling of the systemic vasculature, but its contribution to pulmonary vascular remodelling in IPAH remains elusive. In the present study, GAG expression was investigated in lung tissues of IPAH patients and control transplant donors, and the expression and localization of HA metabolizing enzymes were analysed in human lung samples and PASMC *in vivo* and *in vitro*, respectively.

MATERIALS AND METHODS

Lung tissues and primary PASMC

Lung tissue samples were obtained during lung transplantation from 12 patients with IPAH (mean \pm SD age 32 ± 10 yrs, seven females, five males; table 1) and nine control subjects (organ donors, age 38 ± 14 yrs, five females, four males). None of the IPAH patients exhibited bone morphogenetic receptor type II (BMPRII) mutations. For tissue processing, all samples were immediately placed in 4% (w/v) paraformaldehyde or liquid nitrogen after explantation. The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (Giessen, Germany; AZ 31/93). Informed consent was obtained from each subject for the study protocol. Primary PASMC were generated from arteries of IPAH or donor lungs derived from explanted lung tissues, as indicated, and prepared for isolation of PASMC, as previously described [30]. Cells were cultured in smooth muscle cell growth medium 2 (PromoCell, Heidelberg, Germany) at 37°C in a 5% CO₂, 95% O₂ atmosphere. Passages three to seven were used for the experiments.

GAG isolation and purification

Lung tissue specimens were homogenised at 4°C using a Polytron homogeniser in 25 mM Tris-HCl, pH 7.6 (10 mL per g

of tissue). Homogenised tissues were delipidated in chloroform/methanol (1:2 v/v). Organic solvents were removed by centrifugation (at $3,200 \times g$ for 20 min at 4°C) and the pellet was washed with 10 mL ethanol, centrifuged as before and dried at 40°C for 4 h. The remaining pellet was then resuspended in 0.1 M Tris-HCl, pH 8.0, 1 mM CaCl₂, and GAGs were isolated and purified following digestion with pronase (from *Streptomyces griseus*; EMD Chemicals Inc., San Diego, CA, USA), DNase I (EC 3.1.21.1; Calbiochem, EMD Chemicals Inc.) and β -elimination in the presence of 1 M NaBH₄ [20]. Total GAGs were precipitated with four volumes of ethanol in the presence of 0.1 volumes of 3 M sodium acetate overnight at -4°C, recovered with centrifugation (20 min at $2,000 \times g$), dissolved in double-distilled H₂O and stored at 4°C. Colorimetric determination of uronic acids was performed as previously described [31].

Cellulose acetate electrophoresis

Samples of 2 μ L of GAG solution, containing 4 μ g of uronic acids, were subjected to cellulose acetate electrophoresis in 100 mM pyridine, 470 mM formic acid, pH 3.0, using 7 mA constant current, at room temperature for 70 min. Commercially available GAG standards used as markers included HA, HS, DS and CS (all from Sigma-Aldrich, St Louis, MO, USA). After electrophoresis, the cellulose acetate strip was stained with 0.2% Alcian blue (w/v) in 0.1% acetic acid (v/v) for 10 min and washed with 0.1% acetic acid (v/v) for 20 min [20]. Staining intensity was quantified using a computer-assisted image analysis programme according to the manufacturer's recommendation (Eastman Kodak, Rochester, NY, USA).

GAG characterisation

Speed-dried GAGs (5 μ g of uronic acids) were incubated in a final volume of 15 μ L with the following enzymes. 1) Heparinase: samples were dissolved in 100 mM Tris-HCl buffer, pH 7.0, containing 3 mM CaCl₂, and incubated with 4×10^{-4} U heparin lyase I (EC 4.2.2.7, *Flavobacterium heparinum*;

TABLE 1 Characteristics of idiopathic pulmonary arterial hypertension (IPAH) patients

Patient	Diagnosis	Sex	Age yrs	Ppa mmHg	Treatment
1	IPAH	F	26	NA	NA
2	IPAH	M	21	NA	Prostacyclin
3	IPAH	F	28	95	NO, Bosentan
4	IPAH	M	44	83	Prostacyclin
5	IPAH	F	52	92	Prostacyclin
6	IPAH	M	28	80	Prostacyclin
7	IPAH	F	43	NA	Bosentan
8	IPAH	F	40	56	Bosentan
9	IPAH	M	45	81	Sildenafil
10	IPAH	M	30	95	Remodulin
11	IPAH	F	20	NA	Sildenafil
12	IPAH	F	12	NA	NA

None of the patients exhibited bone morphogenetic receptor type II mutations.
Ppa: pulmonary arterial pressure; F: female; M: male; NA: not available; NO: nitric oxide.

Seikagaku, Tokyo, Japan) for 15 h at 30°C. 2) Heparitinase: samples were similarly dissolved and incubated with 4 × 10⁻⁴ U heparan sulphate lyase (heparitinase: EC 4.2.2.8, *Flavobacterium heparinum*; Seikagaku) for 16 h at 43°C. 3) Chondroitinase ABC: samples were dissolved in 100 mM Tris-HCl buffer, pH 8.0, containing 50 mM sodium acetate, and incubated with 2 × 10⁻⁴ U chondroitin ABC lyase (EC 4.2.2.4, *Proteus vulgaris*; Sigma-Aldrich) for 16 h at 37°C. 4) Chondroitinase B: samples were dissolved in 100 mM Tris-HCl buffer, pH 7.4, and incubated with 0.1 U chondroitin B lyase (*Flavobacterium heparinum*; Seikagaku) for 16 h at 37°C. 5) Keratanase: samples were dissolved in 50 mM Tris-HCl buffer, pH 7.4, and incubated with 0.05 U keratan sulphate endo-β-D-galactosidase (EC 3.2.10.3, *Pseudomonas* species; Sigma-Aldrich) for 16 h at 37°C. 6) Hyaluronidase: samples were dissolved in 20 mM sodium acetate, buffered with acetic acid to pH 5.0, and incubated with 4 U hyaluronate lyase (EC 4.2.2.1, *Streptomyces hyalurolyticus*; Seikagaku) for 14 h at 60°C. Incubation times and enzyme concentrations were as required for complete degradation of standard substrates, as previously described [32].

HA measurements

To analyse secreted HA, primary PASMCM were grown to subconfluence in 24-well plates. Cells were then stimulated with transforming growth factor (TGF)-β1 (0.2, 2 or 10 ng·mL⁻¹, as indicated) for 6, 12 or 24 h. At the end of the incubation time, aliquots of culture media were removed and HA content measured by ELISA according to the manufacturer's instructions (Corgenix, Westminster, CO, USA). Similarly, HA content in lung specimen and cell lysates were determined by ELISA. To measure *de novo* GAG synthesis, subconfluent primary PASMCM were incubated for 24 h in the presence or absence of TGF-β1 (0.2 or 2 ng·mL⁻¹, as indicated), bone morphogenetic protein (BMP)-2 (10 or 20 ng·mL⁻¹, as indicated), the type I TGF-β receptor kinase inhibitor SB431542

(10 μM), the p38 inhibitor SB203580 (10 μM) or platelet-derived growth factor (PDGF)-BB (10 ng·mL⁻¹). The [³H]-glucosamine (0.5 μCi·mL⁻¹; Amersham Corp., Little Chalfont, UK) was then added to culture media for another 24 h. Culture media and cell layers were then collected and digested with 0.1 kU pronase. Total GAGs were precipitated by adding a mixture of ethanol (80% final concentration) containing 1.3% (w/v) sodium acetate. The samples were stored at -20°C overnight and centrifuged at 10,000 × g, the pellets were dissolved in 0.5 M NaOH and total GAG synthesis was measured by liquid scintillation counting, as previously described [16].

Immunohistochemistry

Human paraffin-embedded lung specimens (3 μm) from healthy donors or IPAH patients were stained with biotinylated hyaluronan-binding protein (HABP; Seikagaku), smooth muscle α-actin (α-SMA; Sigma-Aldrich), HYAL1 (Novus Biologicals, Littleton, CO, USA) or HAS1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, using the Histostain Plus Kit (Zymed, San Francisco, USA) as previously described [30]. Sections pre-incubated with 50 U·mL⁻¹ of hyaluronate lyase (EC 4.2.2.1, *Streptomyces hyalurolyticus*; Seikagaku) for 3 h at 37°C served as negative controls for HABP, and sections incubated with isotype-matched pre-immune serum served as negative controls for α-SMA, HYAL1 and HAS1 immunostaining.

Quantitative RT-PCR

Total RNA was extracted using QIAGEN extraction kits according to the manufacturer's protocols (QIAGEN, Hilden, Germany) and cDNAs were generated by reverse transcription using SuperScript™ II (Invitrogen, Carlsbad, CA, USA). Quantitative (q) PCR was performed using fluorogenic SYBR Green and the Sequence Detection System 7700 (PE Applied Biosystems, Foster City, CA, USA) [30]. Human hydroxymethylbilane synthase, a ubiquitously and equally expressed

TABLE 2 Primer sequences and amplicon sizes used for the analysis of human lung tissues and pulmonary arterial smooth muscle cells						
Gene	Accession No.	Forward primer		Reverse primer		Amplicon size bp
		Sequence	Length bp	Sequence	Length bp	
Has1	NM001523	gcgatactgggtagccttca	20	ggtgtaccaggcctcaaga	20	131
Has2	NM005328.1	acagacaggctgaggacgac	20	ctgtgattccaaggaggag	20	126
Has3	NM005329.2	gtcatgtacacggccttcaa	20	cctactggggatcctcctc	20	125
Hyal1	NM007312	gtgctgccctatgtccagat	20	atlttcccagctcaccacaga	20	132
Hyal2	NM033158	tctaccattggcgagagtg	19	gcagccgtgtcaggaat	19	119
Hyal3	NM003549	gatctgggaggttctgtcc	20	agagctggagaggctcaggt	20	110
Hyal4	NM012269	tgaggatctccaccatgaca	20	ggcagcactttctctatgg	20	134
Cd44	NM000610.3	cccagatggagaaagctctg	20	gtgtttgtctgcacagatgg	20	113
Rhamm	NM012484.1	gttgtgcaccatctccaggt	20	agctgaagcaggcaaggtag	20	152
Hmbs	NM000190.3	gcacccacacacagcctac	19	gtaccacgcgaatcactct	20	108
All primer sets were designed to work under identical real-time PCR cycling conditions and simultaneous amplifications were obtained in the same run. Sequences were according to GeneBank reference sequences. Primer sequences are given in 5'–3' orientation. Has: hyaluronan synthase; Hyal: hyaluronoglucosaminidase; Rhamm: receptor for hyaluronan-mediated motility; Hmbs: hydroxymethylbilane synthase.						

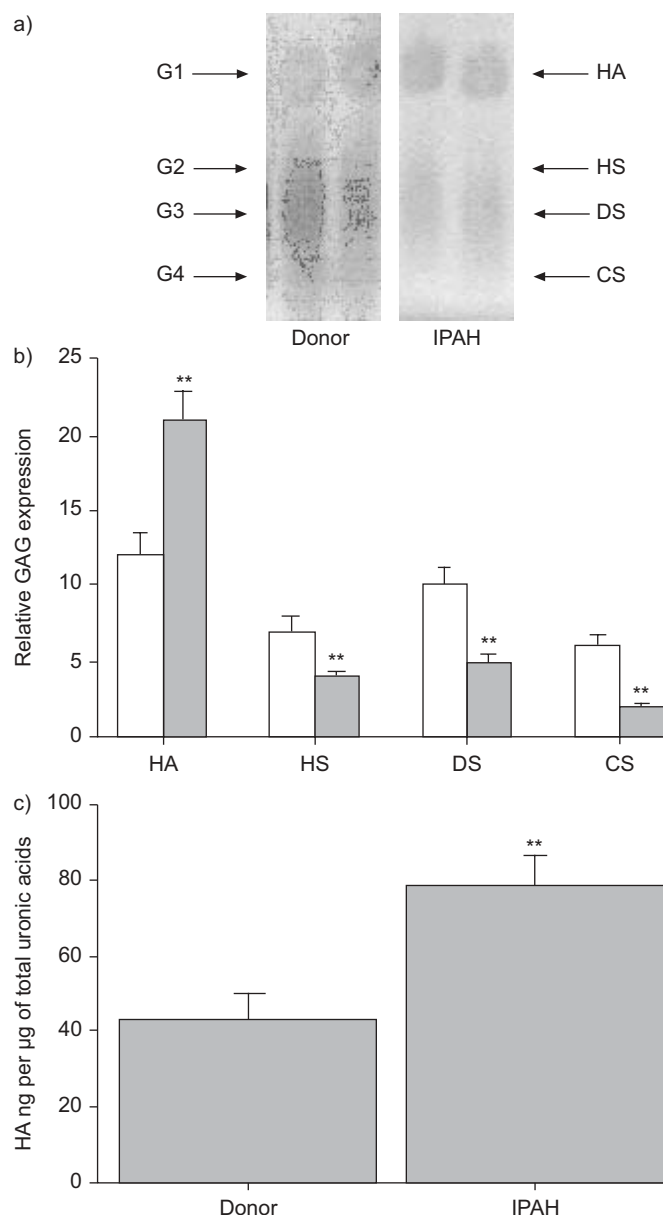


FIGURE 1. Glycosaminoglycan (GAG) expression in idiopathic pulmonary arterial hypertension (IPAH) and control lung tissue samples. a) Representative cellulose acetate membranes demonstrating the electrophoretic separation of total GAG in donor and IPAH lung specimens. G1–G4: four detectable GAG peaks in lung tissues. Commercially available GAG standards were as follows. HA: hyaluronic acid; HS: heparan sulphate; DS: dermatan sulphate; CS: chondroitin sulphate. b) Densitometric quantitation of mean \pm SEM values of the electrophoretic separation of GAGs. \square : donor; \blacksquare : IPAH. c) Measurement by ELISA of the relative content of HA in aliquots of total GAG containing 0.15 μ g of uronic acids. Data are presented as mean \pm SEM. For all investigations, samples from four donor and five IPAH lung tissue specimens were used. **: $p < 0.01$.

gene free of pseudogenes, was used as a reference gene in all qRT-PCR reactions. PCRs were performed using the primers listed in table 2 at a final concentration of 200 nM. Relative transcript abundance of a gene was expressed as the change in threshold cycle (C_t) values (C_t for reference minus C_t for target), as previously described in detail [30].

RESULTS

Expression of GAG in IPAH

Electrophoretic analysis of total GAG isolated and purified from lung tissue specimens from IPAH patients or healthy donors revealed the presence of four distinct GAG populations (G1–G4) in donor and IPAH lungs (fig. 1a). Enzymatic treatment with specific GAG-degrading enzymes (table 3), as well as comparison with the electrophoretic mobility of commercially available GAG standards (fig. 1a), identified G1 as HA, G2 as HS, G3 as DS and G4 as CS. Densitometric analysis of alcian blue staining of these GAGs revealed a significant increase in HA content and a significant decrease in the content of HS, DS and CS in IPAH tissue specimens, compared with donors (fig. 1b). In order to further quantify the increase in HA, the HA concentration was measured within total GAG in lung specimens by ELISA. As shown in figure 1c, the relative amount of HA in the lungs of IPAH patients was significantly increased, compared with lung tissues from transplant donors (mean \pm SEM 78.6 \pm 7.83 versus 43.175 \pm 6.87 ng per μ g of total uronic acids for IPAH and donor lungs, respectively).

Localisation of GAG in IPAH

To further localise HA and its main metabolizing enzymes HAS1 and HYAL1 in the lungs of IPAH patients or transplant donors, immunohistochemical staining was performed. As depicted in figure 2, HA expression could be detected throughout the alveolar interstitium, in the perivascular and peribronchiolar adventitia and in the endothelium. In remodelled lesions in IPAH, HA also frequently localised to subendothelial areas that stained negative for α -SMA (fig. 2i and j), indicating that it is expressed in areas of de-differentiated PASC. In contrast, HAS1 and HYAL1 expression was dominant in PASC, suggesting that HA is synthesised by PASC and subsequently secreted towards the adventitia. While HYAL1 expression was attenuated in IPAH, significant HYAL1 expression was retained in bronchial and alveolar epithelial type II cells (fig. 2h). Staining using tissue sections pre-digested with hyaluronidase or species-matched isotype controls exhibited no positive staining, indicating specificity of the antibodies used (data not shown).

Expression of GAG-metabolising enzymes in IPAH in vivo and PASC in vitro

Since the expression of HA was increased in IPAH, *Has* and *Hyal* mRNA expression was quantified in lung tissue specimens and primary PASC using qRT-PCR. As depicted in figure 3a, *Has1–3*, *Hyal1–3*, *CD44* and *Rhamm* were all expressed in the human lung, while *Hyal4* mRNA was expressed only at very low levels. When comparing expression levels in donor and IPAH lungs, significantly increased expression levels of *Has1* and *CD44* but decreased levels of *Hyal1* were detected in IPAH (fig. 3a). Increased HAS1 protein expression in IPAH lungs was confirmed by Western blot analysis (fig. 3b).

As the immunohistochemical data (fig. 2) indicated that HAS1 and HYAL1 were localised to PASC *in vivo*, and PASC represent a key pathogenic cell type in IPAH, primary PASC were isolated from IPAH and donor patients and *Has1* and *Hyal1* mRNA expression was assessed by qRT-PCR (fig. 4). While no differences were observed in *Has1* mRNA expression,

TABLE 3 Enzymatic treatment of total glycosaminoglycans (GAGs) isolated and purified from human lung tissue specimens with GAG-degrading enzymes

Substrate	GAG-degrading enzyme					
	Chondroitinase ABC	Chondroitinase B	Hyaluronidase	Heparinase	Heparitinase	Keratanase
G1	-	-	+	-	-	-
G2	-	-	-	-	+	-
G3	+	+	-	-	-	-
G4	+	-	-	-	-	-
CSA	+	-	-	-	-	-
DS	+	+	-	-	-	-
CSC	+	-	-	-	-	-
H	-	-	-	+	-	-
HA	-	-	+	-	-	-
HS	-	-	-	-	+	-
KS	-	-	-	-	-	+

Total GAGs isolated and purified from human lung tissue specimens of donors (n=5) or idiopathic pulmonary arterial hypertension patients (n=5) were treated with the indicated GAG-degrading enzymes. The digestion was monitored by cellulose acetate electrophoresis. Data represent observations from five different lung tissue specimens. G1–G4: GAG populations obtained following cellulose acetate electrophoresis, numbers correspond to those of figure 1a; CSA: chondroitin sulphate A; DS: dermatan sulphate B; CSC: chondroitin sulphate C; H: heparin; HA: hyaluronic acid; HS: heparan sulphate; KS: keratan sulphate; +: 100% degradation; -: no detectable degradation.

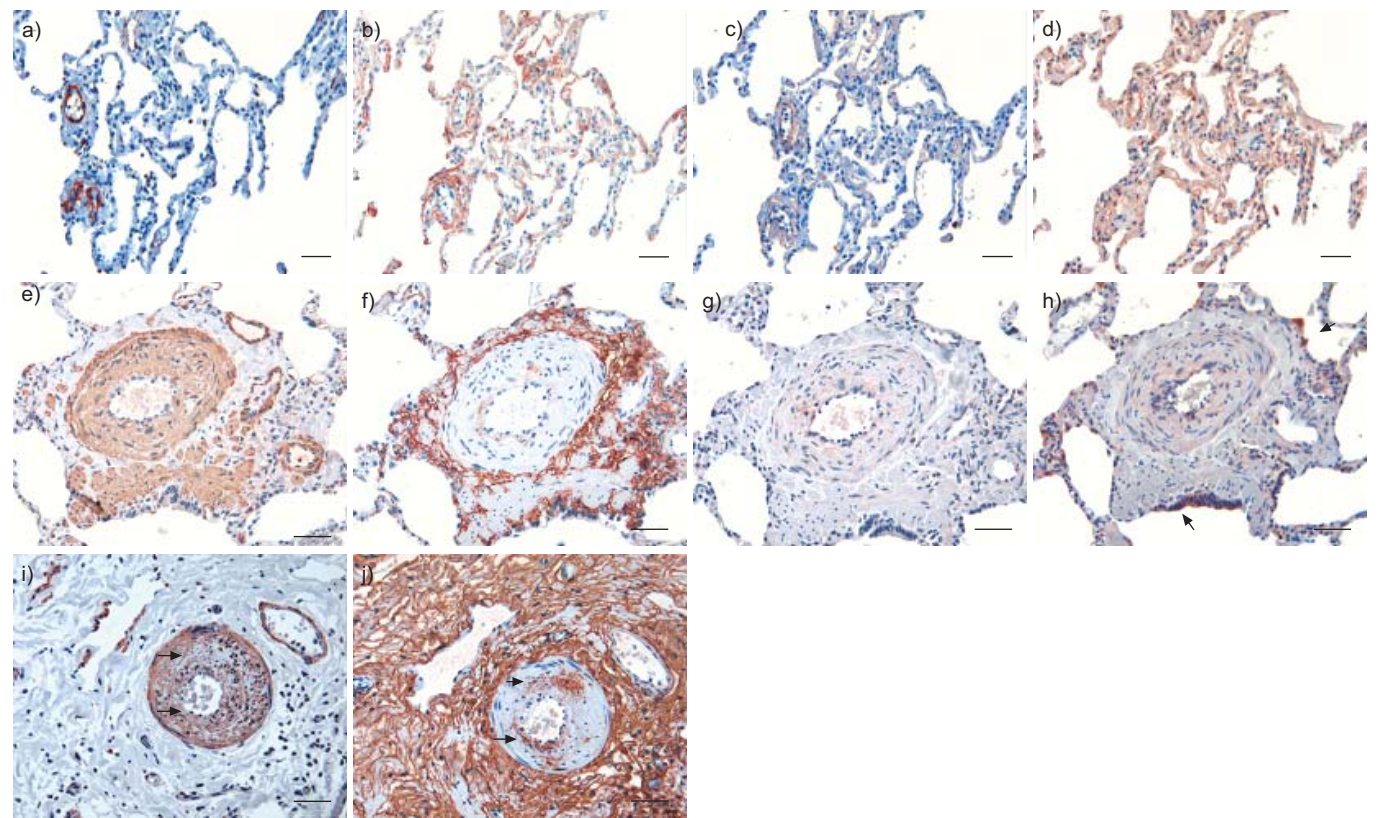


FIGURE 2. Localisation of smooth muscle α -actin (α -SMA; a, e and i), hyaluronan-binding protein (HABP; b, f and j), hyaluronan synthase (HAS)1 (c and g) and hyaluronoglucosaminidase (HYAL)1 (d and h) in pulmonary arteries of control transplant donors (a–d) and idiopathic pulmonary arterial hypertension (IPAH) patients (e–j) was assessed by immunohistochemistry. Hyaluronic acid (HA) was visualised by staining with HABP, while α -SMA, HAS1 and HYAL1 were stained using specific antibodies. Sections are representative for at least four different donors or IPAH patients. h) Arrows indicate HYAL1 expression in bronchial and alveolar epithelial type II cells in IPAH. i and j) Arrows indicate HA expression in pulmonary arterial smooth muscle cells in remodelled lesions that are negative for α -SMA. Scale bars=50 μ m.

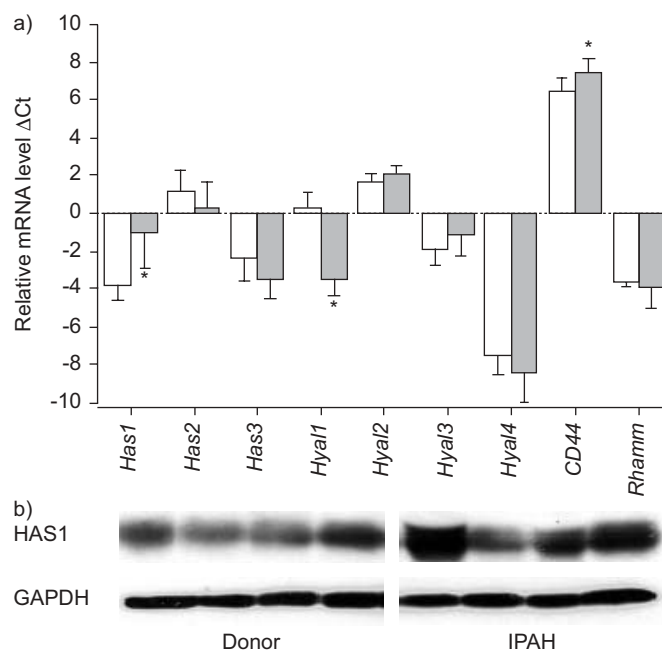


FIGURE 3. Differential expression of hyaluronan synthase (*Has*)1, hyaluronoglucosaminidase (*Hyal*)1, *CD44* and the receptor for hyaluronan-mediated motility (*Rhamm*) in lungs of idiopathic pulmonary arterial hypertension (IPAH) patients. a) mRNA was extracted from lung specimens obtained from IPAH patients (■, n=12) and donors (□, n=8) and the relative expression levels of *Has*1–3, *Hyal*1–4, *CD44* and *Rhamm* were measured by quantitative RT-PCR. Data are presented as mean \pm SEM relative expression level, as change in threshold cycle (ΔCt) values. *: $p < 0.05$. b) HAS1 protein levels (66 kDa) were investigated in lung homogenate samples from donors or IPAH patients by Western blotting. Reduced glyceraldehyde phosphate dehydrogenase (GAPDH; 38 kDa) served as a loading control. Data are representative for at least three independent experiments.

*Hyal*1 expression levels were significantly attenuated in primary PASMC obtained from IPAH patients compared with donors, thus reflecting the changes observed upon analysis of lung homogenates (compare figs 3a and 4).

Secretion and deposition of GAG by PASMC

Since 1) IPAH is characterised by increased ECM deposition by PASMC, 2) TGF- β 1 is a key regulator of ECM deposition, and 3) increased HA staining was observed in vessels of IPAH patients (fig. 2), the effect of TGF- β 1 on total GAG secretion and deposition in primary PASMC was investigated using [3 H]-glucosamine incorporation studies. As shown in figure 5a, both PDGF-BB and TGF- β 1 significantly enhanced secretion of total GAG by PASMC. TGF- β 1 at 2 ng·mL $^{-1}$ also significantly induced deposition of GAG into the provisional ECM deposited by PASMC. In contrast, BMP-2 did not affect GAG synthesis by PASMC (fig. 5a).

In order to further elucidate TGF- β 1-induced signal transduction pathways controlling GAG synthesis, PASMC were pre-incubated with specific inhibitors of the TGF- β type I receptor (SB431542) or of p38 mitogen-activated protein kinase (MAPK; SB203580), and TGF- β 1-dependent [3 H]-glucosamine incorporation was assessed (fig. 5b). Pre-treatment with either SB431542 or SB203580 led to a significant inhibition of basal and TGF- β 1-induced GAG secretion. In addition, SB431542 or

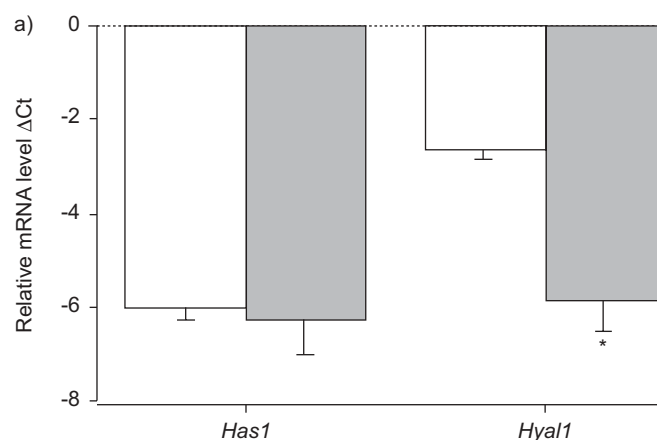


FIGURE 4. Differential expression of hyaluronoglucosaminidase (*Hyal*)1 in pulmonary arterial smooth muscle cells (PASMC) derived from the lungs of idiopathic pulmonary arterial hypertension (IPAH) patients. The hyaluronan synthase (*Has*)1 and *Hyal*1 mRNA levels were assessed in primary PASMC isolated from donors (□, n=3) and IPAH (■, n=6) lungs by quantitative RT-PCR. Data are presented as mean \pm SEM relative expression level, as change in threshold cycle (ΔCt) values. *: $p < 0.05$.

SB203580 pre-treatment inhibited TGF- β 1-induced but not basal GAG deposition by PASMC (fig. 5b).

Secretion of HA by PASMC

In order to further investigate whether the TGF- β 1-dependent increase in [3 H]-glucosamine incorporation was in part a result of increased HA secretion, HA secretion by PASMC was measured by ELISA. As depicted in figure 6, PASMC secreted considerable amounts of HA under baseline conditions, and this secretion was significantly enhanced by TGF- β 1 in a time-dependent manner. While high TGF- β 1 concentrations (10 ng·mL $^{-1}$) led to a significant increase in HA secretion already after 12 h, physiological concentrations of TGF- β 1 (0.2–2 ng·mL $^{-1}$) significantly increased HA secretion by PASMC only after 24 h (fig. 6).

Expression of GAG-metabolising enzymes in PASMC

The time-course of TGF- β 1-induced HA secretion indicated that *de novo* gene expression of *Has*1–3 or repression of *Hyal*1–3 may be required for the increase in HA. Therefore, *Has* and *Hyal* mRNA expression was assessed in primary human PASMC that were stimulated with TGF- β 1 for ≤ 6 h. Using qRT-PCR, a significant increase in *Has*1 gene expression was detected as early as 2 h after TGF- β 1 stimulation of PASMC, and there was an even further increase after 6 h (fig. 7). In contrast, TGF- β 1 had no significant effect on the expression of *Has*2 or -3, *Hyal*1–3, *CD44* or *Rhamm* (fig. 6). Interestingly, *Hyal*4 was not expressed in PASMC and was, therefore, not included in fig. 7.

DISCUSSION

Despite major recent advances in the pathophysiology, diagnosis and therapy of IPAH, the molecular mechanisms underlying endothelial and smooth muscle cell dysfunction in this disease remain to be fully elucidated [4]. Initial endothelial dysfunction contributes to impaired vasoregulation and is accompanied by dysregulated release of soluble mediators.

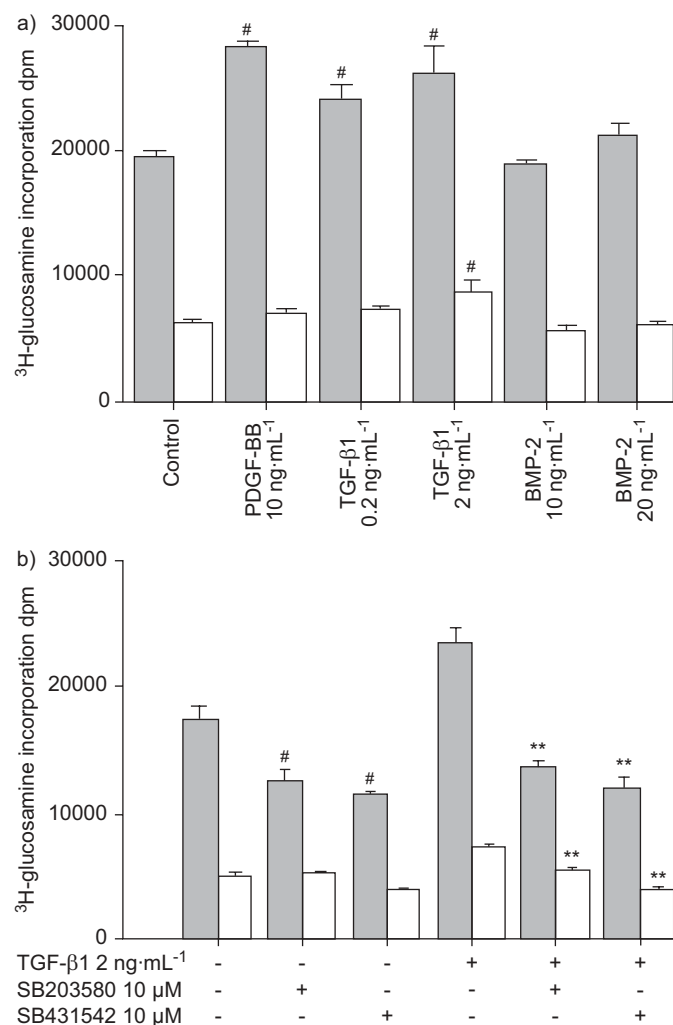


FIGURE 5. Effect of transforming growth factor (TGF)-β1 on glycosaminoglycan (GAG) secretion and deposition by pulmonary arterial smooth muscle cells (PASMC). a) Subconfluent primary human PASMC were incubated with platelet-derived growth factor (PDGF)-BB, TGF-β1 or bone morphogenetic protein (BMP)-2 for 24 h, in the presence of [^3H]-glucosamine at 0.5 μCi·mL⁻¹. Incorporation of [^3H]-glucosamine was then assessed in supernatants (representing secreted GAG; ■) and cell layers (representing deposited GAG; □). b) Subconfluent primary PASMC were stimulated with TGF-β1 in the presence or absence of the p38 kinase inhibitor SB203580 or the type I TGF-β1 receptor kinase inhibitor SB431542, and the incorporation of [^3H]-glucosamine was assessed. Data are presented as mean ± SEM, n=4 for each treatment. #: p<0.005 compared with control values; **: p<0.01 compared with TGF-β1-stimulated values.

Later stages of IPAH are dominated by vascular remodelling and characterised by enhanced proliferation and ECM synthesis of PASMC, with increased endothelial cell apoptosis [4, 5]. GAGs, major constituents of the lung ECM, have recently been shown to play a significant role in inflammatory and noninflammatory lung diseases, exhibiting spatiotemporally distinct effects on epithelial or mesenchymal cell types [8, 9]. GAGs regulate hydration and water homeostasis, maintain cell and tissue structure and function, modulate inflammatory responses and influence tissue repair and remodelling [8, 13]. This led the current authors to hypothesise that differential

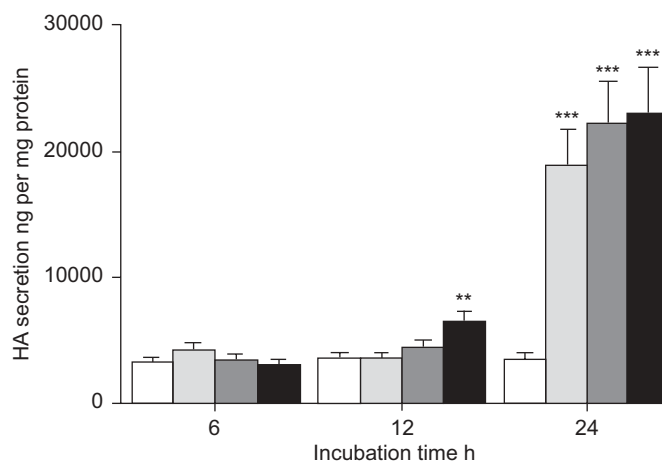


FIGURE 6. Effect of transforming growth factor (TGF)-β1 on hyaluronic acid (HA) secretion by pulmonary arterial smooth muscle cells (PASMC). Subconfluent primary PASMC were incubated without TGF-β1 (□), or with 0.2 ng·mL⁻¹ (■), 2 ng·mL⁻¹ (■) or 10 ng·mL⁻¹ (■) for 6, 12 or 24 h. HA was measured in cell culture supernatants by ELISA. Data are presented as mean ± SEM of four independent experiments. **: p<0.01; ***: p<0.001.

secretion of GAG by PASMC is associated with vascular remodelling in IPAH.

In the present study, IPAH lung tissues exhibited a significantly increased HA content, the major GAG produced by PASMC. While the relative amount of HA was significantly increased, the levels of the sulphated GAGs heparan, dermatan and chondroitin sulphate were decreased, indicating an increased ratio of nonsulphated to sulphated GAG. The increased HA content of IPAH lung tissues was associated with increased and decreased gene expression of *Has1* and *Hyal1*, respectively. While no relationship was sought between the HA amount and the severity of IPAH (e.g. assessed by pulmonary arterial pressure or pulmonary vascular resistance), the current authors are confident that HA exerts a pathophysiological role, at least in the late and/or severe stages of IPAH that transplant patients exhibit.

Similar changes were observed in primary human PASMC cultured from the lungs of IPAH patients, which demonstrated a significant decrease in *Hyal1* mRNA levels compared with PASMC obtained from transplant donor lungs. In addition, stimulation of PASMC with TGF-β1, a growth factor involved in the pathogenesis of IPAH [3, 4], led to increased *Has1* gene expression as early as 2 h after stimulation. Since increased HA staining was observed in actively remodelled pulmonary arteries, it is tempting to speculate that HA secretion by PASMC directly influences endothelial and smooth muscle cell proliferation and may control vasoreactive responses in IPAH.

This is substantiated by the fact that selective overexpression of *Has2* in smooth muscle cells in transgenic mice resulted in significantly increased HA content in the tunica media, enhanced mechanical stiffness and strength and accelerated development of atherosclerosis [28]. Similar effects were reported for the HA receptor CD44, using CD44-null mice. CD44 expression promoted susceptibility to atherosclerosis, recruitment of macrophages and smooth muscle cell activation

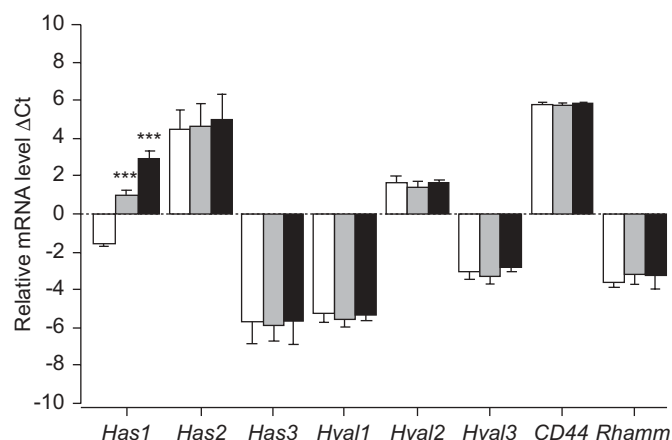


FIGURE 7. Induction of hyaluronan synthase (*Has*)1 gene expression in transforming growth factor (TGF)- β 1-stimulated pulmonary arterial smooth muscle cells (PASC). mRNA was extracted from primary human PASC (n=3) without TGF- β 1 (□) or from PASC stimulated for 2 h (▒) or 6 h (■) with 2 ng·mL⁻¹ TGF- β 1. The relative expression levels of *Has*1–3, hyaluronoglucosaminidase (*Hyal*)1–3, *CD44* and the receptor for hyaluronan-mediated motility (*Rhamm*) were determined by quantitative RT-PCR. Data are presented as mean \pm SEM relative expression level, as change in threshold cycle (Δ Ct) values. ***: $p < 0.001$.

and proliferation [27]. While no pulmonary vascular changes have been investigated in either previous study, it is highly likely that similar changes in HA and CD44 content in human pulmonary arteries, as reported in the present study, also affect vascular stiffness and strength, hence further contributing to the increased resistance observed in IPAH.

How are the biological effects of HA induced? First, it is known to bind 1000 times its own weight in water, hence contributing to tissue hydration and water homeostasis [12, 22, 23]. Secondly, increased HA synthesis and turnover represents an early response during lung inflammation in general and mesenchymal cell activation in particular [8, 12]. HA regulates cell migration, differentiation and proliferation *via* interaction with specific cell-surface receptors (CD44 or RHAMM) [12, 22], but also *via* interaction with toll-like receptors 2 and 4 [14]. The interaction of HA with CD44 regulates leukocyte rolling and activation, as well as tumour metastasis. Furthermore, CD44-dependent clearance of HA fragments is crucial in resolving lung inflammation in the bleomycin model of lung injury [26], demonstrating an essential role for CD44 in the resolution phase of inflammation.

It is of interest to note that the biological effects of HA vary depending on its average molecular mass [12, 23]. Under physiological conditions, HA is a polymer of high average molecular mass (>1,000 kDa). In contrast, HA fragments of lower molecular mass accumulate following tissue injury, which are cleared *via* binding to the cell surface receptor CD44. HA with lower molecular mass (300–500 kDa) has been reported to prolong the survival of eosinophils *in vitro* [33]. HA fragments (<200 kDa) induce the expression of cytokines, chemokines or inducible nitric oxide synthase by macrophages [34], affects ECM turnover in murine alveolar macrophages [34] and stimulates TGF- β 1 synthesis [33]. It has been shown that fragmented HA with an average molecular mass of

250 kDa induces the expression of inflammatory genes [35], while HA of higher molecular weight exhibited the opposite effect and suppressed chemokine expression [23]. Therefore, it would be of interest to elucidate in future studies whether HA expressed in the vascular system of control donor lung specimen is of different average molecular weight than in IPAH specimens. In this context, dysregulation of *Has*/*Hyal* expression and/or activity may lead to the generation of HA of different molecular masses, thereby exhibiting distinct biological effects, *e.g.* facilitating PASC migration and proliferation, which potentially contribute to the pathogenesis of IPAH.

The aforementioned *in vivo* experiments were supported by the current observations in primary cultures of PASC. It was found that TGF- β 1, but not BMP-2, significantly stimulated total GAG synthesis and HA secretion, an effect which coincided with an increased *Has*1 gene expression. The TGF- β 1-dependent increase in GAG synthesis was mediated *via* the Smad and p38 MAPK pathways. The current results are in agreement with previous studies in human fibroblast-like synoviocytes, which have revealed that TGF- β 1 is a potent stimulus for *Has*1 expression. Furthermore, blocking p38 MAPK inhibited TGF- β 1-induced *Has*1 expression by 90% [36], which underlines the importance of the p38 signalling pathway for TGF- β 1-regulated expression of *Has* isoforms. Since TGF- β 1 is a potent stimulus for PASC migration, *Has*1-mediated HA synthesis may represent a necessary step for PASC activation and migration. The aforementioned changes in *Has*/*Hyal* expression, along with the change in HA synthesis and content in IPAH, may also potentially result from the tissue hypoxia observed in IPAH. Notably, it has previously been reported that hypoxia potentiates GAG synthesis by primary lung fibroblasts induced by TGF- β or PDGF-BB [16, 37], suggesting that hypoxia is a synergistic regulator of the increased GAG deposition observed in IPAH.

In conclusion, the results of the present study demonstrate an increased hyaluronic acid content in lungs from idiopathic pulmonary arterial hypertension patients, which was associated with increased hyaluronan synthase 1 and decreased hyaluronoglucosaminidase 1 gene expression. Synergistic regulation of glycosaminoglycan-metabolising enzymes in favour of accumulation may, thus, regulate pathological remodelling in idiopathic pulmonary arterial hypertension by favouring the activation state of pulmonary arterial smooth muscle cells.

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