Original Article

Antiproliferative Heparan Sulfate Inhibiting Hyaluronan and Transforming Growth Factor-β Expression in Human Lung Fibroblast Cells

A Possible Antifibrotic Therapy?

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Abstract

The objective of this study was to examine the effects of heparan sulfate (HS) on factors involved in the remodeling of connective tissue observed in patients with fibrotic respiratory disorders such as asthma. A suitable working model is to stimulate human fetal lung fibroblasts in vitro with structurally different forms of HS. Highly sulfated and iduronic acid (IdoUA)-rich HS specifically decreased cell proliferation, production of hyaluronan (HA), transforming growth factor (TGF)-β_1, and TGF-β-induced α-smooth muscle actin but did not affect the overall proteoglycan production in the cells. These repressed factors are suggested to play a critical role in the early stages of remodeling and myofibroblast activation. Low sulfated and IdoUA-poor HS did not display any effects on these factors. Furthermore, analysis of the protein expression pattern by two-dimensional gel electrophoresis revealed a 70% increased expression of annexin II, which has previously been shown to have a high affinity for both heparin and HS. Heat-shock protein 27 and arsenite translocating factor, both involved in actin organization and polymerization, were also increased in the HS-stimulated cells. Thus, the reduced expression of HA and TGF-β_1, both important in the development of fibrosis, seems to be mediated by specific changes in protein expression of the fibroblast. The observed inhibition of cell proliferation, HA, and TGF-β_1 allows speculation of highly sulfated HS as an antifibrotic candidate in the early stage of remodeling.

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Introduction

Heparin and the closely related heparan sulfate (HS) are extracellular matrix (ECM) molecules and members of a family of polysaccharides termed glycosaminoglycans *(1)*. For many years, heparin has been explored as a suitable anticoagulative agent, but it also posses anti-inflammatory characteristics and has therefore been used as a therapeutic agent for many inflammatory diseases such as asthma *(2,3)*. Several studies have suggested antiasthmatic effects of heparin in both asthmatic subjects and animal models by inhibiting the recruitment of inflammatory cells and inactivation of chemokines *(4,5)*. Therefore, a modified heparin such as HS has the potential to become a basic asthmatic treatment in the future if separating its anticoagulative effects *(6,7)*. Although successful treatments are available for the inflammatory process in asthma, such as corticosteroids, a suitable therapeutic agent is yet to be discovered for the remodeling process, which is considered an important factor in the progression of asthma and other fibrotic respiratory disorders *(8)*.

The subepithelial fibrotic process observed in patients with asthma has previously been described as an abnormal wound-healing response leading to a deposition of ECM components in the lamina reticularis and hyperplasia of alveolar smooth muscle cells *(9)*. Because fibroblasts regulate the normal turnover of the ECM, they are important target cells when studying fibrotic disorders. When fibroblasts are stimulated with transforming growth factor (TGF)-β, the cells differentiate into an activated myofibroblasts phenotype *(10)*. These cells are characterized by elevated levels of α-smooth muscle actin (α-SMA) and increased production of ECM components, such as collagens and proteoglycans.

The ECM has an important role in influencing cell behavior through several different pathways and has recently been shown to be heterogeneously expressed in the lung of asthmatic patients *(11,12)*. Components of the ECM such as HS-proteoglycans are considered to affect wound closure, re-epithelialization, and granulation tissue formation by storage and activation of cytokines and growth factors *(13)*. The prerequisite for this is a defined structure of the disaccharide glucuronic acid (GlcUA) organized into block regions of repeats of *N*-acetyl- and *N*-sulfated glucosamine (for review, see ref. *14*). The *N*-sulfated regions are often exposed to further modifications: epimerization of GlcUA into iduronic acid (IdoUA) followed by 2-*O*-sulfation and 6-*O*-sulfation on the *N*-sulfated glucosamine. HS chains are attached to different core proteins and are most abundant in cell membranes such as glypican-6 and syndecan-4, but are nevertheless also present both intracellularly and in the ECM.

In this study, we used two structurally distinct forms of HS: a heparin-similar form termed HS6 that is rich in IdoUA and sulfate, and HS2 that is poor in IdoUA and sulfate. The effects of these two HS forms on factors important for the remodeling process, such as hyaluronan (HA), TGF-β, and α-SMA, were studied by using human fetal lung fibroblasts (HFL-1). These cells serves as an important complement to animal models and patient-related material when studying cellular activation associated with respiratory disorders *(15,16)*. Moreover, the effects of these two HS forms on the proteome were analyzed using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization time-of-flight detector mass spectrometry (MALDI-TOF MS).

Materials and Methods

Materials

Fibroblasts from human lung embryonic tissue were from ATCC (Manassas, VA). Cell culture flasks and well plates were from Nunc Brand (Roskilde, Denmark). Glucosamine isotopes were from Amersham Biosciences (Uppsala, Sweden). DEAE-52 cellulose columns were from Whatman (Maidstone, UK) and the scintillation counter was an LKB 1214 Rackbeta (Uppsala, Sweden). The IsoDalt system, including gel cassettes and casting box, was from Hoefer (San Francisco, CA). Equipment for isoelectric focusing (IEF), Multiphor II, immoboline DryStrip Kit, Immobobiline DryStrip (18 cm, pH 3.0–10.0 non-linear [NL]; 18 cm, pH 4.5–5.5) immobilized pH gradient (IPG) buffer (3–10 NL and 4.5–5.5), and Drystrip cover fluid were purchased from Amersham Biosciences. Iodoacetamide and sodium thiosulfate were from Sigma (St. Louis, MO). Trypsin was purchased from Promega (Madison, WI*). Coomassie Brilliant Blue was from Bio-Rad (Hercules, CA). Duracryl (30%, 0.65% Bis) and all other chemicals, unless otherwise stated, were of electrophoresis grade (ESA Chelmsford, MA). Gels and 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer were from Invitrogen (Carlsbad, CA). Annexin II antibody (Santa Cruz Biotechnology Inc. Europe, Heidelberg, Germany) and α-SMA antibody (Dako A/S, Glostrup, Denmark) were used in recommended concentrations in 0.05% Tween-Tris-buffered solution (TBS) buffer according to the manufacturer. HS2 and HS6 were prepared from beef lung according to Fransson et al. *(17).* The final preparation step was separation on a Sepharose 6 column under dissociative conditions to remove cytoactive components bound to the HS. The procedure for the determination of total hexosamine, IdoUA, and sulfate content has earlier been described *(18–20).* Chemical data for the HS2 and HS6 used in the study are summarized in Table 1.

Table 1
Chemical Data for the Derivatives of Heparan
Sulfate Used in the Study

Sample	O-SO$_3$ HexN mole%	N-SO$_3$ HexN mole%	IdoUA HexUA mole%	IdoUA-O-SO$_3$ HexUA mole%	M_r (kDa)
HS2	30	26	30	10	20
HS6	91	72	65	60	20

Chemical data for HS2 and HS6 used in the study. Relative molecular mass (M_r) was determined by gel chromatography on Superose 6 calibrated with HS species of known molecular mass (determined by light scattering). HexN, hexosamine; HexUA, hexuronic acid; O-SO$_3$, ester sulfate; N-SO$_3$, *N*-sulfamate, IdoUA, iduronic acid; mole%, mole (HexN/HexUA)/mole polysaccharides.

The HS2 preparation had approx 1/3 IdoUA units, most of which were nonsulfated. In contrast, the HS6 preparation had approx 2/3 IdoUA units that were mostly 2-O sulfated.

Cell Cultures

Cells were grown in 25 cm^2 flasks in medium supplemented with 10% fetal calf serum (FCS) and 1% glutamine, at 37°C in a humidified incubator with 5% CO$_2$. Cells were regularly checked for mycoplasma with a GEN-PROBE Rapid Detection System (Gen Probe, San Diego). HS2 and HS6 were added in a concentration of 100 μg/mL. TGF-β$_1$ was used in a concentration of 10 ng/mL in medium containing 0.4% serum.

Cell Proliferation Assay

Cells were stimulated in 96-well plates with HS2 or HS6 in 10% FCS supplemented medium for 24, 48, and 96 h as described previously and fixed at different intervals with 1% glutaraldehyde in Hanks balanced salt solution for 15 min at room temperature. After removal of fixation solution, the amount of cells was determined with a previously described crystal violet method *(21).*

HA and Proteoglycan Assays

HFL-1 cells were cultured for 24 h +/− HS2, HS6, TGF-β_1, and HS6/TGF-β_1 in ^{35}S or ^{3}H-glucosamine supplemented sulfate-poor medium (0.11 mM SO$_4^{2-}$) for 24 h. The HA and proteoglycans with incorporated ^{3}H-glucosamine or ^{35}S were isolated on a DE-52 ion-exchange chromatography column and quantified according to a method described by Westergren-Thorsson et al. *(22)* as radioactivity in a scintillation counter using a dpm-correctional program.

Western Blotting

HFL-1 cells were grown to confluence and stimulated for 24 h +/− HS2, HS6, TGF-β_1, and HS6/TGF-β_1. The cells were harvested in homogenization buffer (20 mM HEPES, 2 mM MgCl$_2$, 1 mM EDTA, pH 8.0, with 2 mM orto-vanadate and 10 µM leupeptin) and centrifuged for 5 min at 1500g at 4°C. Aliquots were taken from the supernatant to compare protein content by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Equal amounts of total protein were applied on SDS-PAGE followed by Western blotting using monoclonal mouse antibodies for human annexin II and α-SMA. The membrane was stained with 28 mM diaminobenzidine in dimethylformamide diluted 1:10 in a citrate-phosphate buffer (35 mM trisodiumcitrate, 67 mM Na$_2$HPO$_4$, pH 5.0) supplemented with H$_2$O$_2$. The intensity of the bands was quantified using Gel Pro Analyzer™ software (Media Cybernetics, Silver Spring, MD).

TGF-β Measured With ELISA

Cells were cultured in 24-well plates and stimulated for 24 h with 1, 10, and 100 µg/mL HS2 or HS6. The quantitative determination of TGF-β_1 and TGF-β_2 in culture medium from the cells was performed using the human TGF-β immunoassay Quantikine kit (R&D Systems, Abingdon, UK).

Protein Expression Analysis

Cells were cultured in six-well plates and stimulated +/− HS2 or HS6 for 24, 48, and 96 h. The cells were harvested in 7 M urea, 2 M thiourea, and 2% CHAPS. An appropriate amount of sample was diluted in rehydration buffer (7 M Urea, 2% CHAPS, fresh IPG buffer v/v 0.5% 3–10 NL, dithiothreitol [DTT] w/v 0.3%) to a final amount of up to 100 µg for analytical gels. For narrow zoom gels, an IPG buffer v/v 0.5% 4.5–5.5 was used in the rehydration buffer. The strips were rehydrated overnight and covered with Drystrip cover fluid to minimize evaporation. The IEF was preformed in a Multiphor II unit using the Immobiline DryStrip Kit according to the instructions from the manufacturer. The IEF was carried out in 500 V for 1 h, 1000 V for 1 h, and 4000 V for 12 h up to at least 50 kVh.

The gel strips were equilibrated in a two-step procedure in an equilibration solution containing 6 M urea, 2% SDS (w/v), 30% glycerol (w/v), 50 mM Tris/HCl, and 65 mM DTT for 10 min. The equilibration buffer was then switched to a solution containing 259 mM iodoacetamide for an additional 10 min.

Prior to second dimension, 14% gels (sized 300 × 300 mm) were cast. The electrophoresis tank was filled with running buffer containing 24 mM Tris Base, 0.2 M glycine, and 0.1% SDS. The equilibrated strips were dipped in the running buffer and placed on top of the SDS PAGE gels. The second dimension was carried out at 100 V for 18 h until the tracking dye reached the end of the gels. The silver staining procedure was a modified protocol from Shevchenko et al. and has previously been described *(12,23)*.

Image Analysis

Gels were scanned using a Fluor-S™ Multi-Imager (Bio-Rad Laboratories, Sundbyberg, Sweden) and Quantity One (version 4.0.3, Bio-Rad Laboratories). Spot analysis was

performed using the PDQuest (version 6.2.0) 2-D gel analysis system (Bio-Rad discovery series, Bio-Rad Laboratories). Gels from single experiments were analyzed in the same match set, using the same parameters for spot detection and editing the gels. After correcting for the background, the amount of protein was expressed as integrated optical density (IOD). The intensity of an individual spot was expressed as parts per million IOD of the total IOD.

Trypsin Digestion and Mass Spectrometry Identification

Gel pieces were washed with 50 mM ammonium bicarbonate buffer followed by three rounds of acetonitrile. The excised spots were treated overnight with 10 ng/mL trypsin (Promega) and acidified with 0.5% trifluoroacetic acid (TFA), and the peptides were applied on an Anchor-chip^{TM} plate (Bruker Daltonics, Bremen, Germany) with 2 mg/mL 2.5 dihydroxy benzoic acid (Bruker-Daltonics) as matrix. The MALDI-TOF MS instrument used was a Bruker Reflex (Bruker-Daltonics) mass spectrometer. The instrument, equipped with a delayed extraction ion source, uses a nitrogen laser at 337 nm and was operated in reflector mode at an accelerating voltage of 20 kV.

Statistical Method

Mean ± standard errors of the mean were calculated. Student's *t*-test was used to evaluate the differences of the means between groups.

Results

HS6 Reduces Cell Proliferation in HFL-1 Cells

Fibroblast cultures obtained from HFL-1 cells were stimulated and incubated for up to 96 h with HS2 and HS6 (Fig. 1A). A significant reduction of 38% in cell proliferation was observed in cultures stimulated with

HS6 ($p < 0.01$; Fig. 1B). This inhibitory effect of HS6 on cell proliferation has previously been shown to be independent of cytotoxic effects, measured as release of lactate dehydrogenase (LDH) *(24)*. No change in cell growth was observed in cell cultures stimulated with HS2, suggesting that the fine structure of HS6 is involved in cell proliferation *(17,20,21)*.

HS Decreases the Production of TGF-β_1 in HFL-1 Cells

In fibrotic tissue, fibroblasts proliferate and differentiate into activated myofibroblasts by the influence of TGF-β. By analyzing the effects of HS on the production of TGF-β, the importance of HS in the early phase of fibrosis can be established. When the cells were stimulated with 100 μg/mL of HS6, the production of TGF-β_1 significantly decreased by 50% compared with the control levels ($p < 0.05$; Fig. 2). No effect could be seen for any of the HS forms at concentrations below 100 μg/mL. The TGF-β_1 production in the HS2-stimulated cells was unaffected when compared with the control, showing that HS2 is less effective in inhibiting the TGF-β_1 production in HFL-1 cells. Thus, HS6 specifically decreases TGF-β_1 production in HFL-1 cell cultures to a much higher extent than HS2, but only at levels of 100 μg/mL. The production of another isoform, TGF-β_2, was also measured under similar conditions to determine the specificity of the response. Neither HS6 nor HS2 affected the levels of produced TGF-β_2 (data not shown).

HS Affects HA and α-SMA But Not Proteoglycan Production in HFL-1 Cells

TGF-β_1 has a strong stimulatory effect on HA, proteoglycans, and α-SMA, all of which have been used as fibrotic markers. The next set of experiments aimed at quantifying the levels of these markers after stimulation by HS and/or TGF-β_1 to see whether HS had any inhibitory effects on these markers
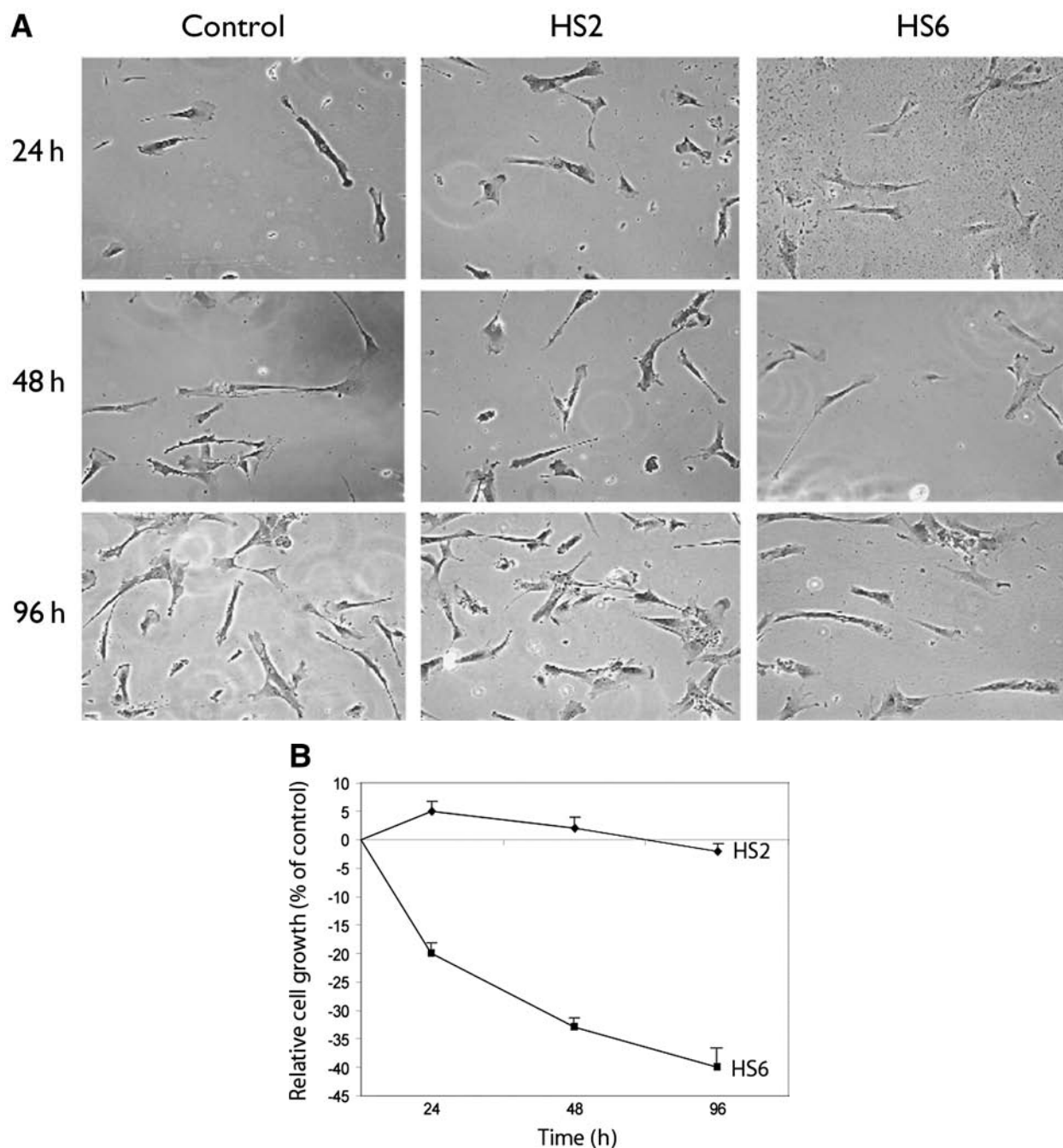
A

B

Fig. 1. HS6 reduces proliferation of human fetal lung (HFL)-1 cells in vitro. This figure displays the effects of heparan sulfate (HS) derivatives on cell proliferation. HFL-1 cells were stimulated with 100 μg/mL of HS6 or HS2 for 24, 48, and 96 h **(A)**. The cells were grown in 10% fetal calf serum supplemented medium in 96 well plates at a density of approx 5000 cells/well. The number of cells was estimated by fixing the cells with 1% glutaraldehyde in Hanks balanced salt solution for 15 min at room temperature. After removal of fixation solution, the amount of cells was determined with a previously described crystal violet method *(21)* at different intervals and by measuring the amount of crystal violet absorbed at 595 nm. The data is presented as relative growth rate **(B)**.
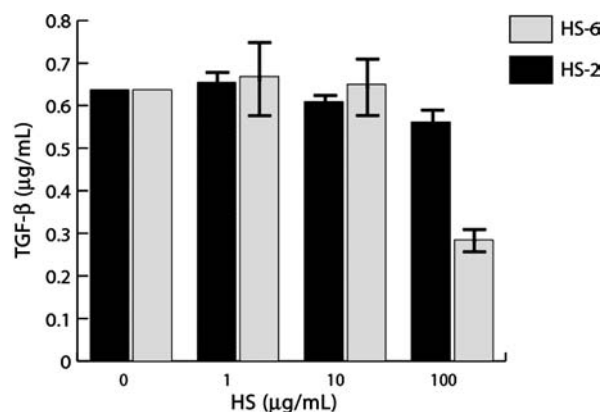
Fig. 2. HS6 inhibits transforming growth factor (TGF)-β_1 production in vitro. This figure shows the effect of heparan sulfate (HS) derivatives on TGF-β_1 production. Human fetal lung (HFL)-1 cells were cultured in 0.4% fetal calf serum supplemented medium in 96 well plates and stimulated with 1, 10, or 100 µg/mL HS2 or HS6 for 24 h. The quantitative amount of TGF-β_1 produced from the HFL-1 cells was determined by ELISA and is presented as µg/mL.

(Fig. 3A). The HS6-stimulated cells showed a 2.5-fold decrease ($p < 0.05$) in HA production as compared with the HS2-stimulated cells. The HA production decreased by 35% when stimulated with HS2. Furthermore, the previously stimulatory effect of TGF-β_1 was decreased to control levels by the addition of HS6.

The total production of proteoglycans as a result of HS2 and HS6 stimulation were also studied in HFL-1 cells (Fig. 3B). Although a decrease in proteoglycan synthesis was observed in the HS6-stimulated cells, this was not significant ($p = 0.21$). Therefore, HS does not seem to have a conclusive effect on the total proteoglycan production.

The expression of the myofibroblast marker α-SMA was analyzed by Western blotting (Fig. 3C). In cells costimulated with TGF-β_1 and HS6, the α-SMA was reduced to control levels, suggesting that HS6 reduces TGF-β-induced α-SMA expression in HFL-1 cells.

Protein Expression by Gel-Based Proteomics in HS-Stimulated Fibroblasts

To further analyze the molecular mechanism of HS on HFL-1 cells, we determined the differential protein expression pattern by using 2-DE and MS. Series of triplicate 2-D gels were run from both the HS2- and HS6-stimulated HFL-1 cells and are confined in the master image (Fig. 4A) which comprises 1438 spot annotations from a total gel number of 40. In total, 75 proteins were regulated in the HS2-stimulated cells and 87 in the HS6-stimulated cultures compared with the controls. Of these regulated proteins, 34 were unique for the HS6 cultures. Eleven proteins were identified of those that showed a quantitative difference in their expression pattern, where eight of these were significant (Fig. 4B). After 24 h of stimulation, the following increases were observed in the HS6-stimulated cells: annexin II (70%), peptidyl-prolyl *cis-trans* isomerase (PPI; 40%), ubiquitin carboxyl-termini (50%), and two isoforms of heat shock protein 27 (50 and 30% [not significant], respectively). Identified proteins that did not show any significant changes in expression patterns were thioredoxin peroxidase, antioxidant protein II, and disulfide isomerase. The increased expression in the 2-DE assay of annexin II by HS2 and HS6 was confirmed using Western blotting (Fig. 4C,D).

To further analyze the expression profile from the stimulated HFL-1 cells, we used specific narrow zoom gels to study the proteins in the p*I* range of 4.5–5.5. This region is very difficult to study in fibroblasts using a global p*I* 3–10 NL expression profile, mainly because of the presence of highly expressed actin isoforms. In this narrow p*I* 4.5–5.5 region, 11 different regulated proteins could be revealed where apolipoprotein A1 (Apo A1); (two-fold decrease), phosphoserine phosphatase (three-fold increase), arsenite
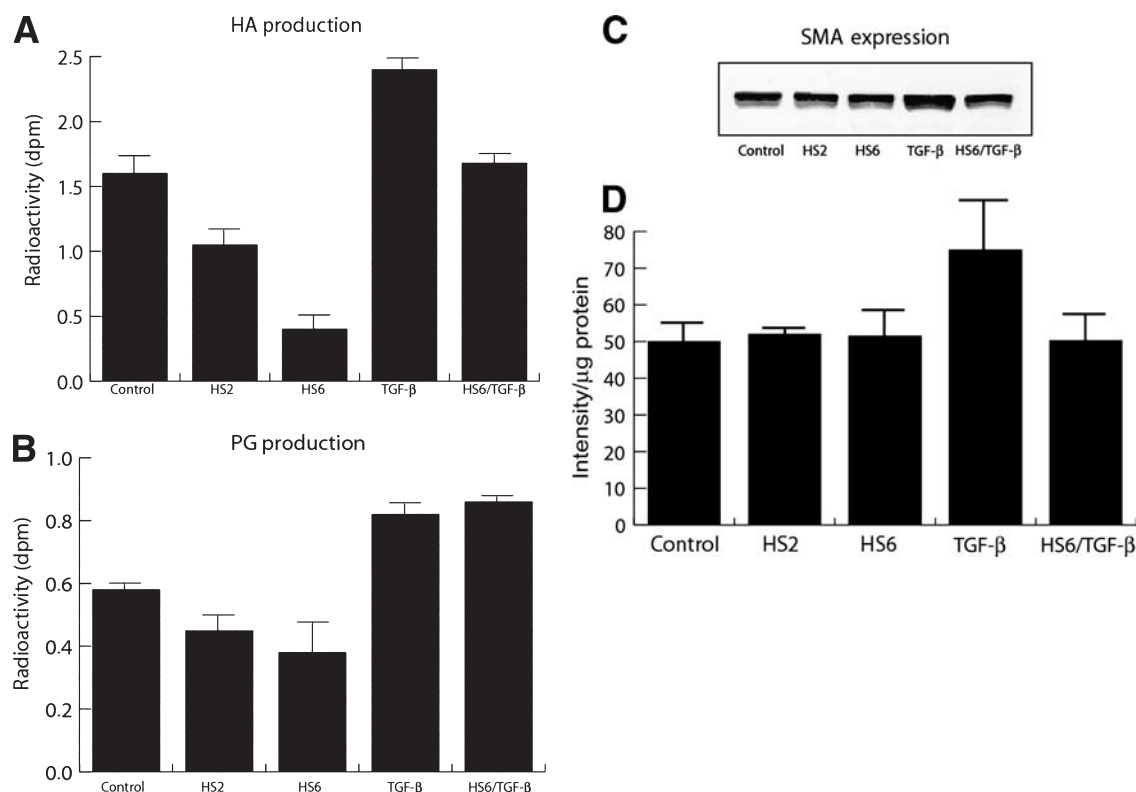
Fig. 3. HS6 reduces hyaluronan (HA) and transforming growth factor (TGF)-β-induced α-smooth muscle actin (α-SMA) expression, but does not affect total production of proteoglycans in vitro. These figures display the effects of heparan sulfate (HS) derivatives on HA, proteoglycan (PG), and α-SMA expression. The cells were stimulated for 24 h with 100 μg/mL of HS2 or HS6, or 10 ng/mL of TGF-β_1 or HS6/TGF-β_1 respectively, and grown in ^{3}H-glucosamine or [^{35}S]-supplemented sulfate-poor 10% fetal calf serum (FCS) medium. DEAE-52 columns were used to separate the proteoglycans from the cell medium. The quantification of HA (as incorporated ^{3}H-glucosamine) **(A)** and total PG production (as incorporated ^{35}S) **(B)** was determined using a scintillation counter and is presented as dpm. To study α-SMA expression **(C)**, cells were stimulated as described above in 10% FCS supplemented medium. The expression was quantified by Western blotting **(D)**.

translocating factor (five- and seven-fold increase, respectively), and FK506 binding-protein 65 kDa (20-fold increase) displayed significant regulations by HS2, HS6, or both. Histograms of triplicate gel quantified expressions are displayed from these selected proteins (Fig. 5).

Discussion

In this study, we have observed a reduced production of HA and TGF-β_1 as well as a reduction of TGF-β_1-induced α-SMA expression in fibroblasts stimulated with HS6.

Although the production of proteoglycans was unaffected, these findings suggest a novel function for HS6 in regulating early fibrotic markers where its fine structure plays an important role in mediating its effects. In the early phase of fibrosis, TGF-β has been shown to increase ECM molecules such as HA and biglycan, which are considered important markers for recruitment and differentiation of myofibroblasts both in vitro and in vivo (22,26). Because the stimulation of TGF-β occurs by self-activation (27) and TGF-β binds to HS, it may be possible that this TGF-β/HS6
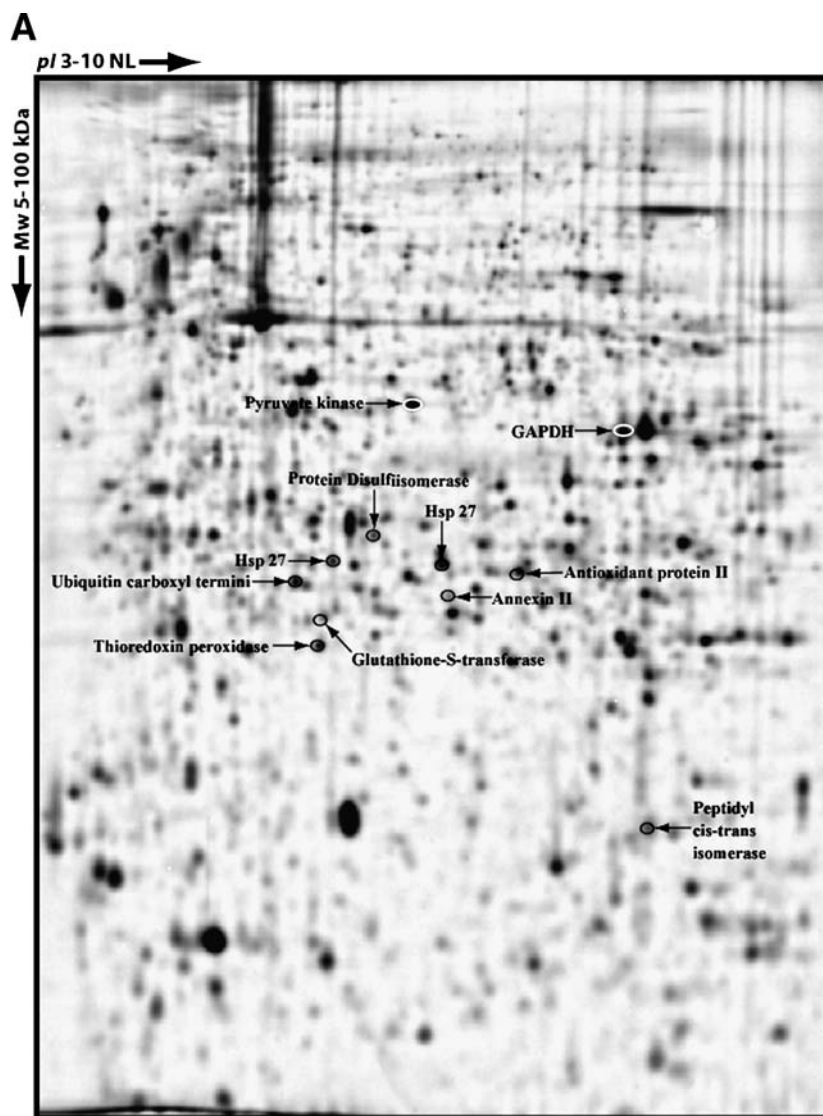
Fig. 4. Global protein expression profile using two-dimensional gel electrophoresis (2-DE) on heparan sulfate (HS)-stimulated human fetal lung (HFL)-1 cells. These figures illustrate the differential protein expression pattern for the HS-stimulated cells. The cells were cultured in six-well plates for 24, 48, and 96 h +/− HS2 or HS6, and harvested in solubilization solution (7 *M* urea, 2 *M* thiourea, 2% CHAPS). The lysed cells were separated by 2-DE in the nonlinear pH range of 3.0–10.0. The protein expression pattern for the master gel is presented and the arrows indicate identified proteins that showed a differential expression pattern between HS2 and HS6 stimulation **(A)**. The quantitative expression patterns for the identified proteins are presented as a function of time **(B)**, where the data are presented as relative change and 100% indicates the expression level in the control. Arrows indicate a significant difference when compared with control. To study annexin II expression **(C)**, we stimulated cells for 24 h with 100 µg/mL of HS2 or HS6, or with 10 ng/mL of transforming growth factor (TGF)-β_1 or HS6/TGF-β_1, respectively in 0.4% fetal calf serum supplemented medium. The expression was quantified by Western blotting **(D)**. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
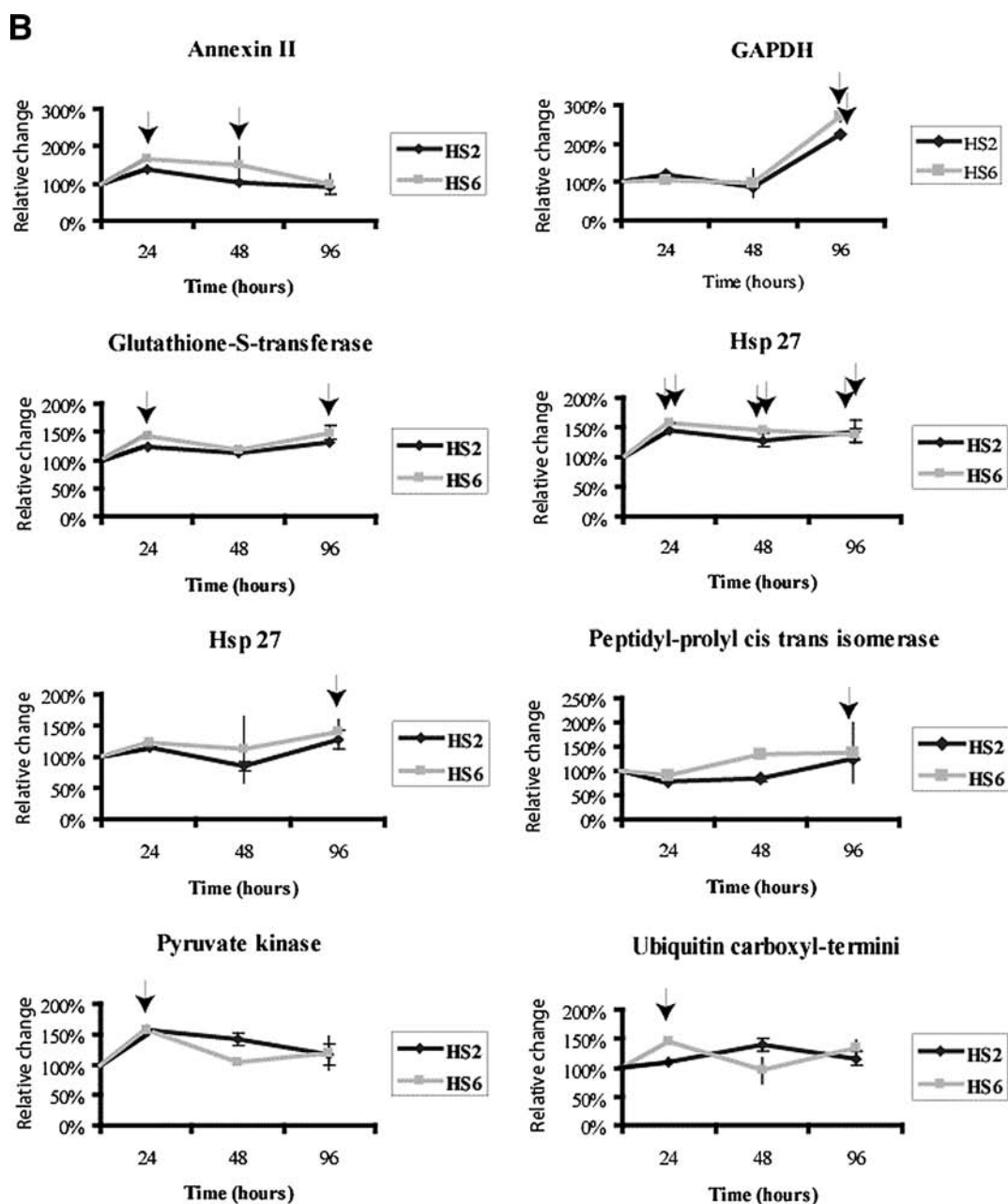
B

Fig. 4. (*continued*)

complex could reduce the activity of TGF-β, thus regulating the early stage of fibrosis by decreasing the myofibroblast differentiation. A similar suggestion has been reported earlier where the binding of TGF-β to the ECM molecule decorin resulted in its inactivation by preventing the TGF-β-induced biglycan production but not the production of the PG-100 *(28)*. More recently, decorin has also been suggested to have a therapeutic value by reducing TGF-β-induced lung fibrosis in vivo *(29)*. In combination with our present findings, this suggests that TGF-β and proteoglycans are regulated through different pathways.
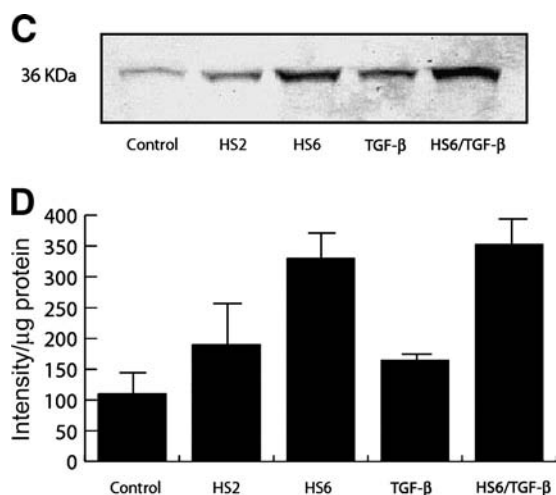
Fig. 4. (*continued*)

Furthermore, our findings with HS6 on fibroblast proliferation and early fibrotic markers emphasize its potential in reducing the deposition of ECM components observed in the fibrotic lung. However, neither of the HS forms had any effect on the basal expression level of α-SMA, suggesting that the inhibition of TGF-β-stimulated α-SMA is upstream of the transcription level for α-SMA.

HS6 affected the fibroblast proteome by specifically inducing an increase in annexin II. An altered expression of annexin II has previously been shown in other cell types on stimulation with TGF-β_1 *(30)*. Several in vitro roles have been suggested for annexin II, such as a docking protein associated with endosomes and exocytic vesicles influencing membrane-membrane or membrane-cytoskeletal interactions related to vesicular trafficking *(31)*. Furthermore, annexin II has also been shown to bind both heparin and HS, thereby undergoing a larger conformational change when binding to heparin than HS *(32)*. We have previously shown that HS6, but not HS2, is internalized and further translocated into the nucleus *(24)*. Though the intracellular HS-mediated effects are still under speculation, HS has the ability to affect transcription factors such as NF-κB *(33)*. The internalization

of HS6 may therefore explain the observed differences in cell proliferation, TGF-β_1 expression, and enzymes involved in HA production.

Both HS2 and HS6 increased the expression of two hsp27 isoforms, which are proteins involved in stress resistance and actin organization. In smooth muscle cells, hsp27 appears to be the link between muscle contraction and protein kinase C, therefore taking an important part in the signal transduction pathway *(34)*. This emphasizes the link between HS and the reorganization of the cytoskeletal proteins, though the structural differences between the two HS forms seem to be of less importance in this case. The other identified proteins, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, glutathione *S*-transferase, and ubiquitin carboxyl-termini, are involved in stress resistance and different metabolic, scavenger, and folding processes *(35–37)*. PPI is a protein thought to play an active role in folding and trafficking events *(38)*. Another group of proteins that share the characteristics of PPIs are the FK-binding proteins, of which we were able to characterize the 65-kDa isoform by using narrow p*I* 4.5–5.5 interval gels. This protein showed an increased expression when stimulated with both HS forms and has been associated with ECM proteins such as tropoelastin during its transportation through the cell *(39)*, further emphasizing the role of HS in intracellular trafficking. An interesting finding is the major increase of the ATPase arsenite translocating factor compared with the control. Arsenite and annexin II have previously been shown to be induced and involved in stabilization and reorganization of the actin cytoskeleton *(40)*. To summarize our proteomic data from the cells stimulated with the distinct forms of HS, we observed an increase in proteins involved in intracellular trafficking events and cytoskeletal interactions, which yields new insights on a molecular level into the biological mechanism of HS.
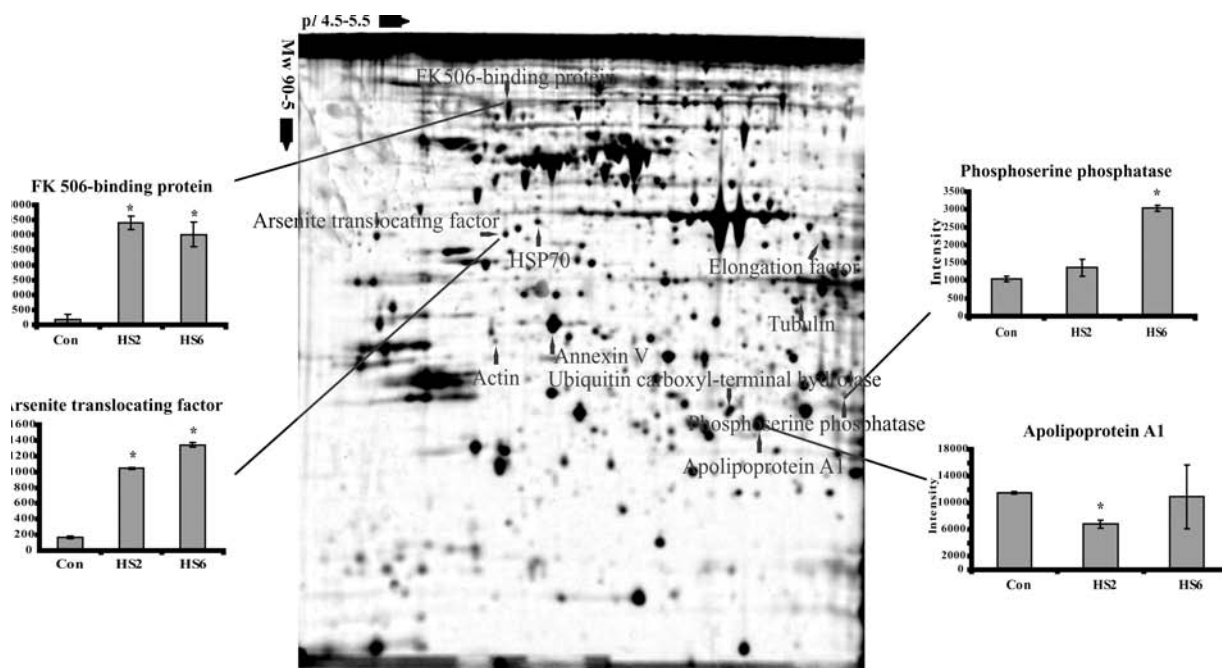
Fig. 5. Narrow protein expression pattern in heparan sulfate (HS)-stimulated human fetal lung (HFL)-1 cells. This figure shows the master gel in the pH range of 4.5–5.5 for the heparan sulfate (HS)-stimulated cells. The cells were grown in six-well plates for 24, 48, and 96 h +/− HS2 or HS6 and harvested in solubilization solution (7 *M* urea, 2 *M* thiourea, 2% CHAPS). The lysed cells were separated by two-dimensional gel electrophoresis in the narrow pH range of 4.5–5.5. The master gel is presented with the arrows indicating the differentially expressed proteins. The graphs illustrate the proteins that showed a statistical significance in the quantitative expression pattern when compared with the control. * Indicates a significant difference when compared with control.

In conclusion, we report a reduction in fibrotic markers such as HA, TGF-β_1, and TGF-β_1-induced α-SMA by a specific form of HS6 in HFL-1 cells. Moreover, we observed an increased expression of cytoskeletal and trafficking-associated proteins in the HS-stimulated cells such as annexin II, hsp27, and arsenite translocating factor. These findings open up new possible ways of treating various fibrotic diseases and further emphasizing the importance of heparin/HS derivatives as future antifibrotic therapeutic agents.

Acknowledgments

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