Heterogeneity of Heparan Sulfates in Human Lung

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Heparan sulfates (HS), a class of glycosaminoglycans, are long linear complex polysaccharides covalently attached to a protein core. The HS molecules are made up of repeating disaccharides onto which modification patterns are superimposed. This results in a large structural heterogeneity and forms the basis of specific interactions of HS toward a vast array of proteins, including growth factors and proteases. To study HS heterogeneity in the lung, we used phage display technology to select seven antibodies against human lung HS. Antibodies reacted with HS/heparin, but not with other glycosaminoglycans or polyanions. Sulfate groups were essential for antibody binding. The amino acid sequence of the antibodies was established, the complementarity determining region 3 of the heavy chain containing basic amino acids. The antibodies defined HS epitopes with a characteristic tissue distribution. Antibody EV3A1 primarily stained macrophages. Other antibodies primarily stained basement membranes, but with different preference toward type of basement membrane. Antibody EV3C3 was the only antibody which clearly reacted with bronchiolar epithelial cells. In human lung parenchyma, basic fibroblast growth factor and vascular endothelial growth factor were largely bound by HS. Some antibodies blocked a basic fibroblast growth factor–binding site of HS, and one antibody blocked a vascular endothelial growth factor–binding site of heparin. Taken together, these data suggest a specific role for HS epitopes in human lung. The antibodies obtained may be valuable tools to study HS in pulmonary diseases.

Heparan sulfates (HS) are members of the glycosaminoglycan (GAG) family, consisting of repeating disaccharide units onto which modification patterns are superimposed. HS bind and modulate a myriad of molecules, including growth factors, cytokines, proteases, antiproteases, matrix molecules, and viral and bacterial proteins (1, 2). This large structural heterogeneity is based on the synthesis of HS, including deacetylation, sulfation, and epimerization. The structural diversity of HS is brought about by specific chain modifications during the biosynthesis of HS, including deacetylation, sulfation, and epimerization. The addition of, e.g., sulfate groups leads to the generation of HS, including deacetylation, sulfation, and epimerization. The amino acid sequence of the antibodies was established, the complementarity determining region 3 of the heavy chain containing basic amino acids. The antibodies defined HS epitopes with a characteristic tissue distribution. Antibody EV3A1 primarily stained macrophages. Other antibodies primarily stained basement membranes, but with different preference toward type of basement membrane. Antibody EV3C3 was the only antibody which clearly reacted with bronchiolar epithelial cells. In human lung parenchyma, basic fibroblast growth factor and vascular endothelial growth factor were largely bound by HS. Some antibodies blocked a basic fibroblast growth factor–binding site of HS, and one antibody blocked a vascular endothelial growth factor–binding site of heparin. Taken together, these data suggest a specific role for HS epitopes in human lung. The antibodies obtained may be valuable tools to study HS in pulmonary diseases.

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Materials and Methods

Lung specimens were obtained from patients undergoing lobectomy or pneumonectomy for a localized malignant pulmonary process, at the University Lung Centre Nijmegen or the Rijnstate...
Hospital Arnhem, the Netherlands. A human semi-synthetic antibody phage display library (17) (now officially named synthetic scFv Library No.1) was generously provided by Dr. G. Winter, Cambridge University (Cambridge, UK). This library contains 50 different VH genes with a synthetic random complementarity determining region 3 (CDR3) segments, which are 4–12 amino acid residues in length. The heavy chains are combined with a single light chain gene (DPL16). The library contains over 10^6 different clones and all antibodies contain a c-Myc tag.

All chemicals used were purchased from Merck (Darmstadt, Germany) unless stated otherwise. Bacterial medium (2xTY) was from Gibco BRL (Paisley, Scotland); chemically modified heparan sulfate kit, chemically modified heparin kit, anti-chondroitin sulfate (CS)/dermatan sulfate (DS) “stub” antibody (2B6), anti-HS “stub” antibody (3G10) and chondroitin 4,6-disulfate from squid cartilage were from Seikagaku Kogyo (Tokyo, Japan). Heparin from porcine intestinal mucosa, HS from bovine kidney and from porcine intestinal mucosa, chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, hyaluronate from human umbilical cord, DNA from calf thymus, dextran sulfate, sodium azide, bovine serum albumin (fraction V), chondroitinase ABC (from Flavobacterium heparinum), and rabbit anti-rat bFGF were from Sigma (St Louis, MO); Microlon 96-well microtiter plates were from Greiner (Frickenhausen, Germany); polystyrene Maxisorp Immunotubes were from Nunc (Roskilde, Denmark); mouse anti-c-Myc monoclonal IgG (clone 9E10) and mouse anti-VSV monoclonal IgG (clone PS4D) were from Boehringer Mannheim (Mannheim, Germany); rabbit anti-c-Myc polyclonal IgG (A-14) was from Santa Cruz Biotechnology (Santa Cruz, CA); alkaline phosphatase–conjugated rabbit anti-mouse IgG, mouse anti-human mast cell tryptase (clone A1A1), and mouse anti-human CD68 (clone KP1), were from Dakopatts (Glostrup, Denmark); Alexa 488–conjugated goat anti-mouse IgG and Alexa 594–conjugated goat anti-mouse IgG were from Molecular Probes (Eugene, OR); Mowiol (4–88) was from Calbiochem (La Jolla, CA); Plasmid DNA isolation kit was from Qiagen (Hilden, Germany); and ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from PE Applied Biosystems (Norwalk, CT). Hematoxylin was from Fluka Biochemika (Buchs, Switzerland). Mouse anti-human VEGF (ab3116) clone VG1 was from Abcam (Cambridge, UK). Human recombinant (hr) VEGF-165 and rat recombinant (rr) bFGF, cloned in prokaryotic vector pQE16, were a gift from the Department of Pathology, University Hospital Nijmegen (Nijmegen, The Netherlands). All experiments were performed at ambient temperature (22°C), unless stated otherwise.

Isolation of Heparan Sulfate from Human Lung Tissue

Lung tissue was collected from 8 individuals, 5 were male, and 3 were female. Small lung specimens were taken from resected lung lobes, not showing any sign of the underlying disease for which the patient underwent surgery (mostly lung cancer) or obstruction pneumonia. Subjects (54 ± 7; mean age ± SD) had spirometric values in the normal range. Per gram (wet weight) of human lung tissue, 4 ml 50 mM sodium phosphate buffer, pH 6.5, containing 2 mM EDTA, 2 mM cysteine, and 10 U papain were added and digestion was performed for 16 h at 65°C. The digest was centrifuged (16,000 × g for 20 min at 4°C) and the supernatant containing the GAGs was subjected to mild alkaline borohydride digestion (0.5 M NaOH/0.1 M NaBH₄ at 4°C) to remove residual peptides from the GAGs. After overnight digestion, the mixture was neutralized by addition of 6 M HCl. Proteins were precipitated for 30 min at 0°C by addition of 100% (wt/vol) trichloroacetic acid to a final concentration of 15%. Precipitated proteins were removed by centrifugation (16,000 × g for 20 min at 4°C), and GAGs were precipitated by addition of 5 volumes of 100% ethanol to the supernatant and incubation overnight at −20°C. After centrifugation (16,000 × g for 30 min at 4°C), the pelleted GAGs were washed with 70% ethanol, dried, and dissolved in MilliQ. To obtain GAG preparations which contained only HS, chondroitinase ABC, which digests chondroitin sulfate and dermatan sulfate, was added (1 IU/100 mg of GAG in 25 mM Tris-HCl, pH 8.0) and incubation was performed for 16 h at 37°C. The efficacy of chondroitinase ABC treatment was evaluated by agarose gel electrophoresis (18). HS were further purified using DEAE Sepharose column chromatography (18), using 0.2 M, 0.5 M, 1.0 M and 2.0 M NaCl in 10 mM Tris-HCl elution steps, pH 6.8. The 0.5 and 1.0 HS fractions were pooled, ethanol-precipitated, and residual salt removed by a 70% (vol/vol) ethanol wash. HS preparations were dissolved in MilliQ, checked for purity (Figure 1) and stored at 4°C.

Selection of Anti-GAG Antibodies

Phage display-derived antibodies were obtained as described (15) using four rounds of panning against HS (0.5 M and 1.0 M fraction). Briefly, antibody-expressing phages were added to HS-coated tubes, and bound phages were eluted at high pH to allow for the infection of Escherichia coli TG1 cells. After overnight amplification, phages were rescued by the addition of helper phage and used for further rounds of selections.

Screening for Bacteria Expressing Antibodies against Glycosaminoglycans

Screening for bacteria expressing anti-HS antibodies was as described (15). Briefly, single colonies picked from the last two rounds of selection were grown in 96-well polystyrene plates until bacterial growth was visible. Antibody production was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration 1 mM). Plates were centrifuged, and the supernatant containing soluble antibodies was applied to wells of polystyrene microtiter plates previously coated with HS. Bound antibodies were detected using mouse anti-c-Myc, followed by incubation with alkaline phosphatase–conjugated rabbit anti-mouse IgG. Alkaline phosphatase activity was measured using p-nitrophenyl phosphate as a substrate. Absorbance was measured at 405 nm. To establish the CDR3 and Vh gene DNA segments, antibody expressing clones were sequenced using Pebl-seq (5'-CCCGTGTAGTGTATAC-3') (located within the PeBl leader sequence), and For Link-seq (5'-GCCACCT-CCGCTTAGAACC-3') (located in the linker region between the Vh and the Vl genes). For this purpose, double-stranded DNA was isolated using standard procedures.

Large Scale Preparation of Antibodies

To obtain large amounts of soluble antibodies, periplasmic fractions from infected bacteria were isolated (15). Briefly, bacteria were grown at 37°C until an optical density (OD₅₅₀) of 0.5 was reached. Induction was effected by the addition of IPTG. After
incubation at 30°C for 3 h, the culture was centrifuged, and the pellet was resuspended in 200 mM sodium borate buffer (pH 8.0) containing 160 mM NaCl, and an ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail (1 mM). After centrifugation at 5,000 x g for 30 min at 4°C, the supernatant (representing the periplasmic fraction containing the antibodies) was filtered through a 0.45 µm filter, dialyzed overnight at 4°C versus phosphate-buffered saline (PBS), and stored at −20°C.

Characterization of Antibodies by Enzyme-Linked Immunosorbent Assay

Affinity of the antibodies to various molecules was evaluated by enzyme-linked immunosorbent assay (ELISA) as described (15). Briefly, wells were coated with the molecules concerned by incubation with 100 µl of a 10 µg/ml solution in wells of a 96-well microtiter plate for 16 h at 4°C. The wells were rinsed with PBS containing 0.1% (vol/vol) Tween-20 (PBST) and blocked with 2% bovine serum albumin (BSA) in PBS containing 0.05% (vol/vol) Tween-20. Antibodies were added and allowed to bind for 90 min. Bound antibodies were detected by incubation with 10-fold diluted mouse anti-c-Myc monoclonal antibody 9E10, followed by incubation with 1:1,000 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG. The plates were rinsed six times with PBST following each incubation. Enzyme activity was detected using 100 µl 1 mg p-nitrophenyl phosphate/ml 1 M diethanolamine/0.5 mM MgCl₂, pH 9.8, as a substrate. Absorbance was read at 405 nm. All assays were performed at least three times and representative results are shown. As a control, wells were incubated with an irrelevant antibody TSC01 (CDR3 sequence LGFHS, Vι3 family, germline segment DP40).

To evaluate which chemical groups are important for recognition of the antibodies, an ELISA with modified HS/heparin preparations (from porcine intestine) was performed, including heparins from various HS preparations in 96-well microtiter plates previously coated with modified heparin/HS preparations. Antibodies were incubated for 90 min with 2-fold diluted antibodies containing 3% normal horse serum for 45 min. Bound antibodies were detected as described.

Characterization of Antibodies and Localization of HS Epitopes by Immunohistochemistry

Human lung cryosections (5 µm) were fixed in 4% paraformaldehyde. After rinsing in PBS for 10 min, cryosections were incubated in hydrogen peroxide solution (0.3% in PBS, pH 7.3) to quench endogenous peroxidase activity. Subsequently, cryosections were washed for 10 min in PBS, blocked with PBS containing 0.05% (vol/vol) Tween-20 and 2% (wt/vol) BSA for 10 min, and incubated with 2-fold diluted antibodies containing 3% normal horse serum for 45 min. Bound antibodies were detected by incubation with 1:10 diluted mouse anti-c-Myc monoclonal antibody 9E10 containing 3% normal horse serum for 45 min. After washing with PBST (2 × 10 min), cryosections were incubated with biotinylated anti-mouse IgG containing 3% normal horse serum, for 45 min, washed with PBST (2 × 10 min), and incubated with Vectastain Elite ABC-kit (Vector, Burlingame, CA) for 45 min. Sections were rinsed in PBST and incubated with 3,3'-diaminobenzidine (DAB) solution to identify bound antibody. After a final wash in PBS, sections were counterstained with Mayer's hematoxylin and mounted with Entellan (Merck, Darmstadt, Germany). As a control, cryosections were incubated with an irrelevant antibody TSC01.

To evaluate the specificity of the antibodies, cryosections were digested with the glycosidases heparinase III, heparinase I (both digest HS) 0.04 IU/ml in 50 mM NaAc/50 mM Ca(Ac)₂, pH 7.0 or chondroitinase ABC (digests chondroitin sulfate and dermatan sulfate) 0.02 U/ml in 25 mM Tris-HCl, pH 8.0, (2 h at 37°C, refreshing the enzyme after 1 h). As a control, cryosections were incubated in reaction buffer without enzyme. After washing three times with PBS and blocking for 30 min with PBS containing 0.05% (vol/vol) Tween-20 and 2% (wt/vol) BSA, cryosections were incubated with antibodies and processed for immunohistochemistry as described above. The efficiency of heparinase III and chondroitinase ABC treatment was evaluated by incubation of cryosections with antibodies against GAG-“stubs,” generated by the glycosidases. For HS the antibody 3G10 was used. For chondroitin sulfate the antibody 2B6 was used. All tests were performed at least three times.

To evaluate whether the antibodies react with heparin or mast cells in situ, human lung cryosections were rehydrated, blocked with PBS containing 0.05% (vol/vol) Tween-20 and 2% (wt/vol) BSA for 30 min, and incubated with 2-fold diluted antibodies for 90 min. Bound antibodies were detected using 1:100 diluted anti-c-Myc rabbit polyclonal antibody A-14 and goat anti-rabbit IgG Alexa 488, each for 60 min. For detection of mast cell tryptase, 1:500 diluted mouse anti-human mast cell tryptase and goat anti-mouse IgG Alexa 594 were included in the incubations. Macrophages were detected by 1:500 diluted CD68, and goat anti-mouse IgG Alexa 594. After each incubation, cryosections were washed, fixed in 100% methanol, air-dried, and embedded in Mowiol (10% [wt/vol] in 0.1 M Tris-HCl, pH 8.5/25% [vol/vol] glycerol/2.5% [wt/vol] NaN₃). As a control, cryosections were incubated with an irrelevant antibody TSC01.

Inhibition of Antibody Binding to Heparin and HS by bFGF and VEGF

To study whether the HS epitope defined by the antibodies is involved in the binding of bFGF and/or VEGF, polystyrene microtiter plates were coated with HS from bovine kidney or heparin from porcine intestinal mucosa. Subsequently, 100 µl of a solution containing bFGF- (19) or VEGF-165 (0.3 µg/ml PBS, containing 0.05% [vol/vol] Tween-20 and 2% [wt/vol] BSA) was applied for 60 min to wells of the polystyrene microtiter plate. At these amounts, a maximum number of bFGF- or VEGF-165-binding sites on HS and heparin are occupied, as determined using anti-growth factor antibodies. After washing, antibodies were applied. The amount of antibodies was chosen such that, in an ELISA without growth factors, 50% staining was obtained, 100% being the value obtained using saturating amounts of antibody. This set-up was chosen to sensitively detect a reduction of HS-bound antibodies by growth factors. Bound antibodies were detected as described.

Results

Selection of Anti-HS Antibodies

A human synthetic phage library containing phages expressing antibodies was biopanned against HS isolated from human lung. After four rounds of panning, seven antibodies were selected (Table 1). The antibodies were different with respect to their amino acid sequence of the complementarity-determining region 3 and/or VH gene.

Characterization of Anti-HS Antibodies

Using ELISA, antibodies were shown to be reactive for the lung HS preparation as well as for heparin, a highly sulfated form of HS. HS from other sources was also recognized,
except for EV3A1, which only reacted with heparin (Table 2). None of the antibodies was reactive with other glycosaminoglycans such as dermatan sulfate and chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, keratan sulfate and K5 (similar to the HS precursor polysaccharide), nor with other polyanionic molecules such as dextran sulfate and DNA.

**Analysis of HS Epitopes Recognized by the Antibodies**

To determine which chemical groups are important for recognition, we tested all seven antibodies for reactivity with chemically modified heparin and HS preparations (Table 2).

None of the antibodies reacted with K5 capsular polysaccharide from *E. coli* (which is similar to the HS precursor polysaccharide), indicating that additional modifications are essential for binding. Except for antibodies EV4D12 and EV4D4, none of the antibodies reacted with heparin that was completely desulfated/N-acetylated, desulfated but N-sulfated heparin, or N-desulfated/N-acetylated, indicating that N- and O-sulfate groups are essential. Only antibody EV4D12 reacted, although weakly, with heparin that was completely desulfated and N-sulfated, suggesting that N-sulfation is of major importance for the binding of this antibody. Antibody EV4D6 was the only antibody which partially recognized N-desulfated and N-acetylated heparin (but not HS), indicating that the presence of N-sulfate groups is not absolutely essential for binding.

**Localization of HS Epitopes in Human Lung**

To study the location of the HS saccharides defined by the antibodies, we performed immunohistochemistry using cryosections of human lung. Each antibody showed a defined pattern of reactivity (Table 3, Figure 2: shown are the antibodies EV3C3 [α], EV4D12 [β], EV3D6 [γ], EV3A1 [δ], and anti-stub antibody 3G10 [ε]). All antibodies, except for EV3A1 and EV3D6, primarily stained basement membranes of alveoli, bronchioli, and blood vessels. The antibodies differed in preference toward different types of basement membranes. EV3D6 was the only antibody which was only reactive with basement membranes of bronchioli and blood vessels (Figure 2, c1–c2). Basement membranes of alveoli were not recognized by EV3D6. For the other antibodies, staining intensity of basement membranes of blood vessel endothelium, bronchioli, and capillaries was identical, or stronger compared with alveolar basement membranes. Basement membranes of smooth muscle cells of blood vessels and bronchioli were recognized by the antibodies EV3C3, EV4D12 (Figure 2, a1–a2, b1–b2), and antibody EV3B2 (not shown), whereas the other antibodies were completely negative. Antibody EV3A1 was primarily reactive with macrophages (Figures 3c and 3d). It stained granules of uneven size, likely lysosomes. It was not reactive with mast cells (Figures 3a and 3b). EV3C3 was the only antibody which clearly stained the epithelial cells of bronchioli (Figure 4a); other antibodies (e.g., EV4D12, Figure 4b) were negative in this respect. Note that EV3C3, but not EV4D12, shows a distinct intracellular staining in bronchiolar epithelium. Nuclei of bronchiolar cells appeared to be reactive with EV3C3 (Figure 4a, insert). Macrophages were recognized by all antibodies, except for EV3D6 and EV4D6 (data not shown). Mast cells were recognized by the antibodies EV3B2, EV3C3, and EV3F8 (data not shown). Thus, the staining patterns of anti-HS antibodies show a unique distribution of HS epitopes in human lung.

To ascertain HS specificity of the antibodies, cryosections of human lung tissue were treated with heparinase III, heparinase I, or chondroitinase ABC, before incubation with the antibody. Staining was absent or strongly decreased after treatment with heparinase III (Figure 2, a3–d3), whereas treatment with chondroitinase ABC had no effect (data not shown). For antibody EV3A1 staining of macrophages was not abolished by treatment with heparinase III, nor with heparinase I. For antibody EV3C3 some staining at discrete, but unidentified, places in the alveolar wall still remained after treatment with heparinase III (Figure 2, a3). After treatment with heparinase I, however, staining was completely lost.

**Inhibition of Antibody Binding by bFGF and VEGF**

To study if antibody-defined HS saccharides were involved in growth factor binding, the inhibition of antibody binding to HS/heparin by bFGF and VEGF was studied. Using bFGF, binding of antibody EV3C3 to HS was blocked by 22 ± 2%, EV3B2 by 14 ± 4%, and EV4F8 by 19 ± 2% (values are mean ± SD, n = 4). In case of heparin, only antibody EV3A1 was blocked by 39 ± 3%. For VEGF, only binding of antibody EV3A1 to heparin could be slightly inhibited (11 ± 1%). No effect of VEGF was observed for the other antibodies. In tissue, endogenous bFGF could be detected at sites of basement membranes (Figure 5, a1), and staining was removed after heparinase III treatment (Figure 5, a2). After applying recombinant bFGF, most basement membranes were stained (Figure 5, b1), and staining was decreased after heparinase III treatment (Figure 5, b2). No endogenous VEGF could be detected. Recombinant VEGF-165 added to the sections was located in basement membranes of alveoli, blood vessels, bronchioli, and macrophages (Figure 5, c1). Heparinase III digestion greatly abolished VEGF binding to sections (Figure 5, c2).

**Discussion**

In this study we describe the selection of seven antibodies against human lung HS from a semi-synthetic phage display
library. Phage display has proven to be a very useful technique to select antibodies against poorly immunogenic molecules, such as HS. All antibodies selected in this study recognize different HS epitopes, as indicated by their staining patterns and reactivity toward various HS preparations. For all antibodies, the CDR3 region of the heavy chain, which is of prime importance for the specificity and affinity of the antibodies, contained two or more basic amino acid residues likely involved in binding to negatively charged HS. HS-binding consensus sites contain basic amino acids, e.g., XBBXB(B, basic amino acid residue; X, any amino acid residue [20]). Three out of seven antibodies bear the sequence GXRPRX; (X1: any amino acid; X2: hydrophobic amino acid). We suggest that this sequence forms a potential GAG-binding site.

Of the seven antibodies selected against lung HS, two (EV4D12 and EV4F8) are identical to antibodies selected against HS from bovine kidney and human skeletal muscle (15, 16). Their CDR3 sequences are HAPLRNTRTNT and GMRPRL, and it indicates that common HS saccharides are present in these organs. The position of sulfate groups is of major importance for the binding of the antibodies. The requirement of both N- and O-sulfate groups for epitope recognition was indicated by chemically modified heparins. Overall desulfation completely abolished recognition by all antibodies. N-desulfation could not restore the heparin-antibody interaction, except (partly) for EV4D12. N-desulfation abolished reactivity with all antibodies, except EV4D6. Because CS as well as DS were not bound by any of the antibodies, sulfation patterns specific for HS are likely to be important in the structure of the epitopes involved in binding.

For two antibodies (EV3A1 and EV3C3), staining could not be completely abolished using heparinase III treatment. For EV3A1, treatment with heparinase III abolished staining of basement membranes, but not of macrophages (Figure 2, d3). Treatment with heparinase I also did not remove staining. Staining for HS/heparin stubs, generated by heparinases, was positive after treatment with heparinase I, but not III, indicating that the HS/heparin in macrophages is not a substrate for heparinase III and therefore probably not bound to a core protein (heparinase III cleaves HS in a region near the core protein, and HS is then washed away.

### Table 2

Reactivity of anti-HS antibodies with modified HS and heparin molecules

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>EV3A1</th>
<th>EV3B2</th>
<th>EV3C3</th>
<th>EV3D6</th>
<th>EV4D6</th>
<th>EV4D12</th>
<th>EV4F8</th>
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<tbody>
<tr>
<td>Heparin, porcine intestinal mucosa</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>+</td>
</tr>
<tr>
<td>Heparin, N-desulfated and N-acetylated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Heparin, completely desulfated and N-sulfated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Heparin, completely desulfated and N-acetylated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HS, bovine kidney</td>
<td>-</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>HS, intestinal mucosa</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HS, N-desulfated and N-acetylated</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>K5, capsular polysaccharide from E. coli*</td>
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* Similar to the HS precursor polysaccharide.

### Table 3

Immunostaining for HS epitopes defined by anti-HS antibodies

<table>
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<tr>
<th>Antibody Code</th>
<th>EV3A1</th>
<th>EV3B2</th>
<th>EV3C3</th>
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<th>EV4D6</th>
<th>EV4D12</th>
<th>EV4F8</th>
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<td>Alveoli</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>−</td>
<td>+/−</td>
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<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
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<tr>
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</tr>
<tr>
<td>Basement membrane endothelium</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Basement membrane smooth muscle cells</td>
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<td>+/−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−/−</td>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
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Periplasmic fractions of bacteria expressing anti-HS antibodies were applied to cryosections of human lung. Bound antibodies were visualized by incubation with anti-ε-Myc mouse monoclonal antibody 9E10, followed by biotinylated anti-mouse IgG. Sections were counterstained with Mayers’ hematoxylin.

Staining: +, ++, very strong; +, strong; +/−, moderate; −, absent (n = 3).
from the tissue section). This situation may be analogous to that of heparin in mast cells. In mast cells heparin is not bound to a core protein (it is cleaved off by an endogenous endoglucuronidase) and also cannot be washed out after treatment with heparinase I, probably because of a tight binding of heparin (fragments) to positively charged molecules (histamine, proteases). Also, 3-O sulfation of glucosamine residues inhibits cleavage of heparin at that site (21), and heparin with a high degree of 3-O sulfation may be more resistant to heparinase I digestion compared with heparin, which is less 3-O sulfated.
Figure 5. Localization of bFGF and VEGF in human lung. Non-treated (a1–c1) and heparinase III-treated (a2–c2) cryosections of human lung were incubated with an antibody to bFGF (a, b) or VEGF (c). Sections b and c were pretreated with 20 μg/ml bFGF or VEGF before incubation with the antibody to bFGF or VEGF. Heparinase III treatment removed endogenous bFGF (a2), and reduced binding of recombinant bFGF and recombinant VEGF to sections (b2, c2). After digestion with heparinase III, endogenous bFGF could not be detected (a2). Staining of sections treated with heparinase III before incubation with bFGF or VEGF was markedly decreased (b2 and c2). Scale bar: 50 μm.

A well recognized feature of HS/heparin is the binding of growth factors. bFGF could (partially) prevent three (out of seven) antibodies to bind to HS. This suggests that a number of antibodies are not recognizing domains in HS/heparin involved in binding bFGF/VEGF. The three antibodies that were blocked cover the sites to which bFGF binds in sections. Interestingly, these three antibodies are the only ones reactive with mast cells (see Table 3). In the case of VEGF, only antibody EV3A1 could be partially inhibited by VEGF from binding to heparin, and this antibody did not completely cover the structures reactive with exogenously applied VEGF. The binding of growth factors to immobilized HS in ELISA may be quite weak. It should be noticed that in tissue, HS chains are clustered on a core protein in a way that is probably not possible in microtiter plates. In tissue, therefore, a multivalency effect may be expected which results in a stronger binding of growth factors compared with that seen in ELISA. In this respect it is notable that for example for glypican-1 (a HS-proteoglycan) a $K_D$ of 0.12 nM has been found for VEGF-165 which is considerably lower than what is generally found for HS (27).

The staining patterns of the antibodies raise curiosity about the function of the distinct HS epitopes involved in human lung. Antibody EV3A1 showed a quite different staining pattern compared with the other antibodies, as it reacted strongly with macrophages. Frevert and coworkers (28) showed positive staining for HS on the cell surface of alveolar macrophages. Macrophages are distributed throughout connective tissues and participate in both defense- and injury-related processes (29). They are thought to play a central role in the fibroproliferative response, and studies indicate that they produce bFGF (30) and generate an abundant amount of VEGF (31). Interestingly, EV3A1 was the only antibody that could compete with VEGF for HS. In lung the role of VEGF, which is bound by HS, has recently attracted much attention. In rats, a blockade of the VEGF receptor results in lung alveolar cell apoptosis and emphysema (32, 33). In humans, lung tissue from patients with emphysema contains less VEGF compared with controls. Also, lower levels of VEGF in sputum correlate well with lower FEV1 and DLCO levels (34).

Proteoglycans and GAGs may have a specific role in the pathogenesis of pulmonary diseases. Next to binding and modulation of growth factors/cytokines, GAGs (especially HS) can function as strong inhibitors of neutrophil elastase (35–37), and may thus influence the protease/antiprotease balance. Alterations in HS would have consequences for the protective HSPG barrier of the alveolus (7). In the urine of patients with emphysema, a decreased content of the HS epitope JM403 was found together with a normal content of HS (38), suggesting a structural alteration in or an altered processing of HS molecules in the lungs of emphysematous patients. Studies on chemically and enzymatically modified HS indicate that the JM403 epitope contains one or more N-unsubstituted glucosamine and D-glucuronic acid units, and is located in a region of the HS chain composed of mixed N-sulfated and N-acetylated disaccharide units (39). This is an example of a structural alteration in HS associated with a pulmonary disease. The availability of seven HS epitope-specific antibodies further allows to identify changes in the fine structure of HS associated with pulmonary conditions.
In conclusion, using phage display technology seven antibodies were selected against HS from human lung. Antibodies recognize different epitopes and some of them compete with growth factor binding to HS. The binding of bFGF and VEGF to the alveolar matrix of human lung is likely to be mediated via HS. The availability of anti-lung HS antibodies and their encoding DNAs may provide valuable tools to study more accurately alterations in HS in health and disease.

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References