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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 number of interactions suggests an extensive structural variation within HS. 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 specific motifs that make HS highly versatile, protein-

binding cell regulators (3–5). HS proteoglycans (HSPGs) are predominantly present on cell surfaces and in the extracellular matrix.

Little is known about the roles proteoglycans and GAGs play in the lung. However, their distribution over various lung components (6), their strategic ultrastructural location (7), and their changes during developmental stages (8) suggest that they are of crucial importance to the architecture and functioning of the lung. Due to their location in basement membranes and on cell surfaces, HSPGs deserve special attention.

The importance of specific HS modifications for lung functioning has recently been demonstrated in mice lacking *N*-deacetylase/*N*-sulfotransferase-1 (NDST-1), an enzyme involved in the deacetylation and sulfation of glucosamine residues in HS. NDST-1^{-/-} mice develop respiratory distress syndrome and die shortly after birth of respiratory failure (9, 10). This effect has been attributed to immature type II pneumocytes, resulting in shortage of lung surfactant. Sulfation is a major determinant of the response of alveolar type II cells to growth factors (11). Growth factors like fibroblast growth factors are retained by HS. Electron microscopical studies using cationic probes indicate that HS in the basement membranes of alveolar type I and type II cells and of alveolar endothelial cells are differentially sulfated (12, 13). Sulfated proteoglycans play a central role in the modulation of the extracellular matrix of pulmonary fibroblasts, and HSPGs are involved in neutrophil trafficking to the alveolar space (14).

Studies of HSPGs have mainly been focused on the protein core, whereas research on HS in the lung has been limited to the estimation of total HS content. Detailed structural analysis of HS domains have not been performed, simply because appropriate tools were lacking. Only a few antibodies that recognize HS epitopes have been generated, primarily because of the nonimmunogenic nature of HS. To circumvent this, we adapted the phage display technology to obtain specific antibodies against HS (15, 16).

In this study, we report on the isolation, characterization, and application of single chain antibodies selected against HS isolated from human lung. We provide evidence for the existence of several, differentially distributed HS epitopes in human lung, and show that binding of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) to the alveolar matrix of human lung is mediated via HS. The antibodies may be very instrumental in elucidation of the role of HS domains in health and disease.

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

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Abbreviations: basic fibroblast growth factor, bFGF; bovine serum albumin, BSA; enzyme-linked immunosorbent assay, ELISA; glycosaminoglycans, GAGs; heparan sulfates, HS; HS proteoglycans, HSPGs; *N*-deacetylase/*N*-sulfotransferase-1, NDST-1; phosphate-buffered saline, PBS; vascular endothelial growth factor, VEGF-1.

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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 V_H 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 10^8 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); Microton 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 P5D4) 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 AA1), 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 (ab1316) 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 \pm 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 ($16,000 \times g$ 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 ($16,000 \times g$ 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 centrifuga-

tion ($16,000 \times g$ 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 V_H gene DNA segments, antibody expressing clones were sequenced using PelB-seq (5'-CCGCTGGATTGTTATTACTC-3') (located within the PelB leader sequence), and For Link-seq (5'-GCCACCTCCGCTGAACC-3') (located in the linker region between the V_H and the V_L 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 (OD₆₀₀) of 0.5 was reached. Induction was effectuated by the addition of IPTG. After

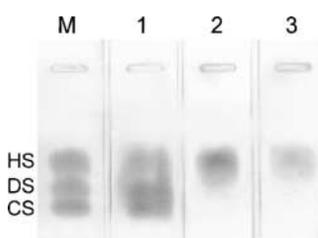


Figure 1. Agarose gel electrophoresis of human lung HS used for biopanning. The gel was run in 50 mM Ba(Ac)₂ pH 5.0 and stained by a combined azure A-silver procedure. M, marker; lane 1: total lung GAG; lanes 2 and 3: human lung HS after DEAE sepharose column chromatography

(eluting at 0.5 M NaCl [2], and 1.0 M NaCl [3] followed by chondroitinase ABC digestion). HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate.

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 $5,000 \times g$ 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 $\mu\text{g}/\text{ml}$ 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 MgCl_2 , 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, V_{H3} 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 that were desulfated and N-sulfated, desulfated and N-acetylated, N-desulfated and N-acetylated, and various HS preparations. Periplasmic fractions containing antibodies were incubated for 90 min in 96-well microtiter plates previously coated with modified heparin/HS preparations. The plates were rinsed with PBST and ELISA was performed as described above.

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 $\text{Ca}(\text{Ac})_2$, 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 stubs the antibody 3G10 was used. For chondroitin sulfate stubs 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] NaN_3). 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 $\mu\text{g}/\text{ml}$ 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 V_{H3} 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 [a], EV4D12 [b], EV3D6 [c], EV3A1 [d], and anti-stub antibody 3G10 [e]). 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, bronchioles, 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 $22 \pm 2\%$, EV3B2 by $14 \pm 4\%$, and EV4F8 by $19 \pm 2\%$ (values are mean \pm SD, $n = 4$). In case of heparin, only antibody EV3A1 was blocked by $39 \pm 3\%$. For VEGF, only binding of antibody EV3A1 to heparin could be slightly inhibited ($11 \pm 1\%$). 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

TABLE 1
Characteristics of anti-HS antibodies

Antibody	CDR3 sequence	V _H family	Germline segment
EV3A1	GKRRRQ	V _H -3	DP-42
EV3B2*	GKMMLNR	V _H -3	DP-38
EV3C3*	GYRPRF	V _H -3	DP-42
EV3D6	WMHLRVRH	V _H -1	DP-5
EV4D6	GARPRAN	V _H -3	DP-38
EV4D12	HAPLRNTRTNT	V _H -3	DP-38
EV4F8	GMRPRL	V _H -3	DP-38

Given are the antibody code, amino acid sequence of the V_H complementarity-determining region 3 (CDR3), V_H family and the germline segment (DP numbering). CDR3 sequences are shown in single-letter amino acid code. V_H families and DP segments were deduced from the V BASE using DNAPLOT alignment (<http://www.mrc-cpe.cam.ac.uk/mt-doc/restricted/DNAPLOT.html>) by applying the full-length V_H sequences of the anti-HS antibody clones (nomenclature according to Tomlinson and coworkers [40]).

* Antibodies EV3B2 and EV3C3 have been partially described (15).

TABLE 2
Reactivity of anti-HS antibodies with modified HS and heparin molecules

Test Substance	Antibody Code						
	EV3A1	EV3B2	EV3C3	EV3D6	EV4D6	EV4D12	EV4F8
Heparin, porcine intestinal mucosa	+	++	+	++	++	+	++
Heparin, <i>N</i> -desulfated and <i>N</i> -acetylated	-	-	-	-	+/-	-	-
Heparin, completely desulfated and <i>N</i> -sulfated	-	-	-	-	-	+/-	-
Heparin, completely desulfated and <i>N</i> -acetylated	-	-	-	-	-	-	-
HS, bovine kidney	-	+	+	+	+	+	+
HS, intestinal mucosa	-	+	+/-	+/-	+	+	+
HS, <i>N</i> -desulfated and <i>N</i> -acetylated	-	-	-	-	-	-	-
K5, capsular polysaccharide from <i>E. coli</i> *	-	-	-	-	-	-	-

Periplasmic fractions of the antibodies were applied to various GAG preparations immobilized on microtiter plates. Bound antibodies were detected using anti-c-Myc mouse monoclonal antibody 9E10, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG, after which enzymatic activity was measured using p-nitrophenyl phosphate as a substrate. Substrate affinity: ++, very strong; +, strong; +/-, moderate; -, absent ($n = 3$).

* Similar to the HS precursor polysaccharide.

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., $XBBXBX$ (B, basic amino acid residue; X, any amino acid residue [20]). Three out of seven antibodies bear the sequence GX_1RPRX_2 (X_1 : any amino acid; X_2 : 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-resulfation 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 3
Immunostaining for HS epitopes defined by anti-HS antibodies

Structure	Antibody Code						
	EV3A1	EV3B2	EV3C3	EV3D6	EV4D6	EV4D12	EV4F8
Alveoli							
Basement membrane epithelium	-	+/-	+	-	+/-	+/-	+/-
Basement membrane capillaries	-	+	++	-	+	++	+
Bronchioli							
Epithelial cells	-	-	+	-	-	-	-
Basement membrane epithelial cells	+	+	++	+	+	++	+
Basement membrane smooth muscle cells	-	+	+	-	-	+/-	-
Blood vessels							
Basement membrane endothelium	+	+	++	+	+	++	++
Basement membrane smooth muscle cells	-	+/-	+	-	-	+/-	-
Mast cells	-	+	+	-	-	-	+
Macrophages	++	+	+	-	-	+	+/-

Periplasmic fractions of bacteria expressing anti-HS antibodies were applied to cryosections of human lung. Bound antibodies were visualized by incubation with anti-c-Myc mouse monoclonal antibody 9E10, followed by biotinylated anti-mouse IgG. Sections were counterstained with Mayers' hematoxylin. Staining: ++, strong; +, moderate; +/-, weak; -, absent ($n = 3$).

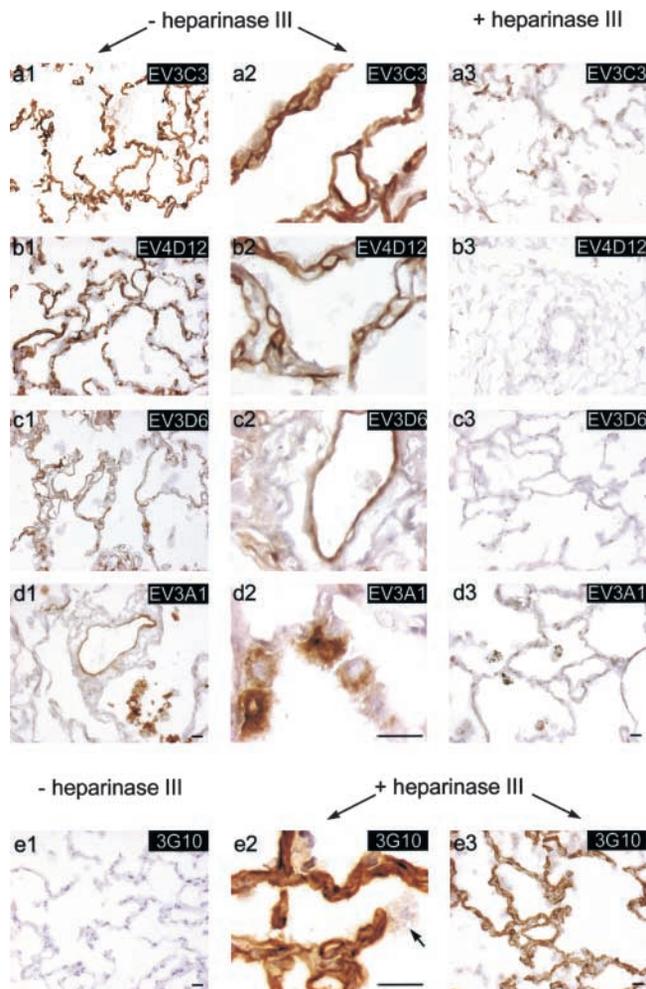


Figure 2. Immunostaining for HS epitopes defined by anti-HS antibodies. Nontreated and heparinase III–treated cryosections of human lung were incubated with periplasmic fractions containing antibody EV3C3 (a), EV4D12 (b), EV3D6 (c), EV3A1 (d), or anti-heparan sulfate stub antibody (3G10) (e). Bound antibodies were visualized by incubation with anti-*c-Myc* mouse monoclonal antibody 9E10, followed by biotinylated anti-mouse IgG. Sections were counterstained with Mayer's hematoxylin. Untreated tissue (a1–d1, a2–d2) showed HS epitopes differentially distributed throughout parenchymal tissue (for details *see text*). Staining was lost or strongly decreased after heparinase III treatment (a3–d3), indicating the HS-nature of the epitopes. Staining of heparan sulfate stubs in heparinase III–treated tissue showed alveolar HS to be present in alveolar and capillary basement membranes (e2–e3). Alveolar macrophages are negative (arrow in e2). Scale bars: 20 μm .

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.

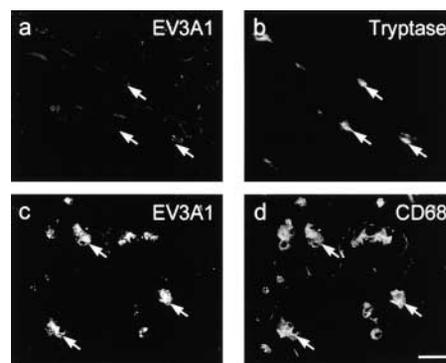


Figure 3. Staining of macrophages by antibody EV3A1. Cryosections of human lung were incubated with periplasmic fractions of antibody EV3A1 (a, c). Bound antibodies were visualized by incubation with rabbit polyclonal anti-*c-Myc* IgG, followed by Alexa 488–conjugated goat anti-rabbit IgG. Tryptase, identifying in mast cells, was visualized using mouse anti-human mast cell tryptase, followed by Alexa 594–conjugated goat anti-mouse IgG (b). Macrophages were visualized using mouse anti-human CD68, followed by Alexa 594–conjugated goat anti-mouse IgG (d). Note that EV3A1 reacted strongly with macrophages, but not mast cells. Scale bar: 50 μm .

For antibody EV3C3, heparinase III abolished basement membrane staining but not staining at some regions in the alveolar wall. Heparinase I, however, abolished all staining. Heparinase III cleaves primarily at unsulfated hexuronic acid residues, especially if there are relatively few sulfate groups on the adjacent residues. In contrast, heparinase I prefers highly sulfated regions including sulfated iduronic acid residues. Therefore, we suggest that EV3C3 recognizes highly sulfated regions in HS. Possibly, although speculative, free EV3C3-positive HS, not bound to a core protein, stick to positively charged areas in the alveolar wall and can only be removed by more extensive digestion by heparinase I.

Our results implicate that in human lung at least seven different HS epitopes are present. Already in the early seventies it was recognized that pulmonary HS are exceptionally

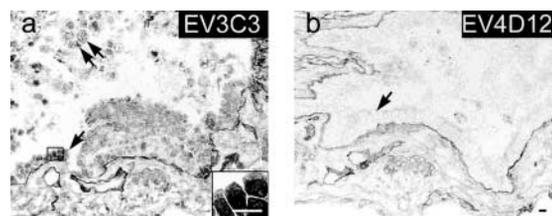


Figure 4. Staining of bronchiolar epithelium by antibody EV3C3. Cryosections were incubated with antibody EV3C3 (a) and EV4D12 (b). Bound antibodies were visualized by incubation with anti-*c-Myc* mouse monoclonal antibody 9E10, followed by biotinylated anti-mouse IgG. Sections were counterstained with Mayer's hematoxylin. Note that EV3C3, but not EV4D12, shows a distinct intracellular staining in the bronchiolar epithelium, nuclei appearing to be positive (insert). Single arrows, epithelial cells of bronchioli; double arrows in a, detached epithelial cells. Scale bars: 10 μm .

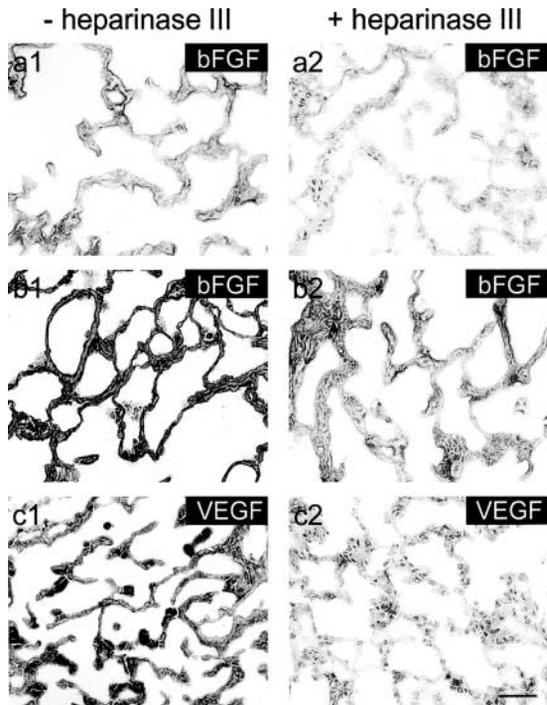


Figure 5. Localization of bFGF and VEGF in human lung. Non-treated (*a1–c1*) and heparinase III–treated (*a2–c2*) cryosections of human lung were incubated with an antibody to bFGF (*a, b*) or VEGF (*c*). Sections *b* and *c* were pretreated with 20 $\mu\text{g/ml}$ bFGF or VEGF before incubation with 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 .

diverse with respect to sulfation (22–24). The biosynthesis of HS allows for a large number of different epitopes. First a precursor polysaccharide is formed, which is subsequently subjected to a number of modifications (25). Regions where the precursor molecule is not modified consist of D-glucuronic acid-N-acetyl-glucosamine (GlcA-GlcNAc) repeats and are known as N-acetylated (NA) domains. These act as spacers between the highly modified and sulfated domains (S-domains). In these S-domains, extensive modifications occur, especially additions of sulfate groups and epimerisation of GlcA to IdoA. The bifunctional enzyme NDST catalyzes the first modification. Mice deficient in NDST isoform 1, but not 2, die shortly after birth due to respiratory failure caused by immaturity of type II pneumocytes, which results in insufficient production of surfactant (9, 10). This indicates that NDST-1, but not -2, is essential for the maturation of type II pneumocytes, and implies the importance of specific sulfate patterns in HS. NDST-1–deficient mice have under-sulfated HS in which N-sulfation and 2-O sulfation are reduced, but in which 6-O sulfation is normal (9, 26). The antibodies identified in this study recognize specific sulfation patterns and may be valuable tools to study HS saccharides in health and disease.

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 DL_{CO} 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 for 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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