N-Acetylated Domains in Heparan Sulfates Revealed by a Monoclonal Antibody against the *Escherichia coli* K5 Capsular Polysaccharide

**DISTRIBUTION OF THE COGNATE EPITOPE IN NORMAL HUMAN KIDNEY AND TRANSPLANT KIDNEY WITH CHRONIC VASCULAR REJECTION***

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The *Escherichia coli* K5 capsular polysaccharide has the same (GlcUA→GlcNac)₉ structure as the nonsulfated heparan sulfate/heparin precursor polysaccharide. A monoclonal antibody (mAb 865) against the K5 polysaccharide has been described (Peters, H., Jürs, M., Jann, B., Jann, K., Timmis, K. N., and Bitter-Sauermann, D. (1985) Infect. Immun. 50, 459–466). In this report, we demonstrate the binding of anti-K5 mAb 865 to N-acetylated sequences in heparan sulfates and heparan sulfate proteoglycans but not to heparin. This is shown by direct binding and fluid phase inhibition of mAb 865 in an enzyme-linked immunosorbent assay. In this system, we found that the binding of the mAb decreased with increasing sulfate content of the polysaccharide. By testing chemically modified K5 and heparin polysaccharides, we found that each of the modifications that occur during heparan sulfate (HS) synthesis (N-sulfation, C-6 epimerization, and O-sulfation) prevents recognition by mAb 865. Samples of heparan sulfate from human aorta (HS-II) were selectively degraded so as to allow the separation of N-sulfated and N-acetylated block structures. N-Sulfated oligosaccharides (obtained after N-deacetylation by hydrazinolysis followed by nitrous acid deamination at pH 3.9) were not recognized by mAb 865, in contrast to N-acetylated oligosaccharides (obtained after nitrous acid deamination at pH 1.5), although the reactivity was lower than for intact HS-II. Analysis of the latter’s pH 1.5 deamination products by gel filtration indicated that a minimal size of 18 saccharide units was necessary for antibody binding. These results lead us to propose bivalent antibody-heparan sulfate interaction, in which both F(ab) domains of the mAb interact with their epitopes, both of which are present in a single large (≥18 saccharide units) N-acetylated domain and additionally with single epitopes present in two N-acetylated sequences (each <18 saccharide units) bridged by a short N-sulfated domain. Immunohistochemistry with mAb 865 on cryostat sections of normal human kidney tissue, revealed its binding to most but not all renal basement membranes. However, all renal basement membranes contain heparan sulfate, as shown by a mAb against heparitinase-digested heparan sulfate stubs (mAb 3G10). This finding indicates that not all heparan sulfate chains present in basement membranes express the mAb 865 epitopes. Besides the normal distribution, mAb 865 staining was found in fibrotic and sclerotic lesions in vessels, interstitium, and mesangium in transplant kidneys with chronic vascular rejection. Occasionally, a decrease of staining was observed within tubulo-interstitium and glomeruli. These findings show that N-acetylated sequences in heparan sulfates can be demonstrated by anti-K5 mAb 865 in normal and diseased kidneys.

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The biosynthesis of heparan sulfate (HS) involves the formation of a nonsulfated (GlcUAβ1,4→GlcNAcα1,4)₉ precursor polysaccharide N-acetylheparosan, which subsequently undergoes a series of polymer modification reactions. These reactions start with N-deacetylation/N-sulfation of GlcNAc residues, which is followed by C-6 epimerization of GlcUA to IdUA units, and finally by O-sulfation at various positions (1). The GlcUA-C-6 epimerization and O-sulfation reactions occur in the close vicinity of N-sulfate groups, pointing to a key role for the glucosaminyl N-deacetylase/N-sulfotransferase enzyme in determining the overall extent of modification of the HS chain. Structural analysis of HS from various sources has revealed that these modifications tend to colocalize in block sequences, separated by relatively unmodified domains (2–6). The extent of biosynthetic modification, affecting the number, length, and substitution patterns of the modified domains as well as their position along the HS chain, may differ among cell types (7), alter during proliferation (8), and change as a result of cell transformation (9, 10).

Many biological activities of heparan sulfate proteoglycans (HSPGs) are due to interactions between the HS polysaccharide side chains and a variety of proteins such as extracellular matrix molecules, enzymes, enzyme inhibitors, growth factors, and other cytokines (1, 11–13). These interactions can be either specific, dependent on defined sulfation patterns within given sequences of sugar residues, as described for antithrombin (14),...
basic fibroblast growth factor (15, 16), hepatocyte growth factor (17), and interferon-γ (18); or they can be mainly based on relatively nonspecific electrostatic interactions and involve proteins such as lipoprotein lipase (19), platelet factor 4 (20) and mast cell protease I (21) (see Ref. 22 for a general discussion).

Structural analysis of HS is complicated by the fact that even highly purified and uniform preparations consist of mixtures of polysaccharide chains that have reached different levels of modification. Monodonal antibodies (mAbs) that specifically recognize well-defined epitopes in HS could be major tools in such analysis. Already in 1986 a mAb (designated as mAb 865) against the Escherichia coli K5 polysaccharide had been described (23). This bacterial polysaccharide has the same (GlcUA—GlcNAc), structure as the nonsulfated HS/heparin precursor polysaccharide (24), suggesting that anti-K5 mAb 865 might recognize the N-acetylated (K5-like) domains in HS. In the present report, we show that this is indeed the case. Immunohistological application of the mAb on normal human renal tissue and on transplant kidneys with chronic rejection revealed these N-acetylated HS sequences in extracellular matrix and basement membranes. Since in most protein-HS interactions highly sulfated, IdecA-rich domains are involved, the possible biological significance of these relatively unmodified N-acetylated domains in HS is discussed.

MATERIALS AND METHODS

Glycosaminoglycans and Heparan Sulfate Proteoglycans—HS (preparation HS-II) was isolated from human aorta, essentially according to Iverius (25). HS from pig intestine, the E. coli K5 capsular polysaccharide, with the same (GlcUA—GlcNAc), structure as the nonsulfated HS/heparin precursor polysaccharide (24) and chemically O-sulfated K5 polysaccharides were kindly provided by Dr. G. van Dedem (Organon Corp., Oss, The Netherlands). N-Sulfated K5 was given by Dr. B. Casu (Istituto di Chimica e Biochimica G. Ronzoni, Milan, Italy). Intact heparin (stage 14) from pig intestinal mucosa was obtained from Inexol Pharmaceutical Division (Park Forest South, IL) and purified by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl (26). Heparan sulfate proteoglycans were isolated from the mouse Engelbreth-Holm-Swarm sarcoma (Becton Dickinson Labware, Bedford, MA) or from rat glomerular basement membranes, essentially according to van den Hauvel et al. (27). Briefly, glomeruli were isolated from rat kidney cortex by a sieving method. Glomerular basement membranes were isolated from glomeruli by the detergent method, extracted with 1 M NaCl containing 1% (w/v) hydrazine sulphate (Fluka; H2O content, 36%) containing 1% (w/v) hydrazine sulphate, and HSPG was desalted from human aorta, designated as HS-II), a high sulfated HS (prep­XU—Acetylated oligosac­charides, or intact HSPGs, Percentage of inhibition was calculated as (1 - (A450 with inhibit or/A450 without inhibitor)) x 100%. IC50 (μg of inhibitor/ml) is defined as the concentration of inhibitor that gives 50% inhibition in the ELISA system.

Immunohistology—Normal human kidney specimens (n = 8) were obtained during surgery or were from cadaveric donor kidneys not suitable for transplantation. Specimens of renal transplant tissue (n = 8) were obtained by percutaneous biopsy or after nephrectomy of the renal graft. Immune-fluorescence analysis was performed as described (35) on 2-μm human kidney cryostat sections. The antibodies used were a mouse IgM mAb against the E. coli K5 capsular polysaccharide, which has been described before (23), and the mouse IgG2b mAb 9G10, reacting with HS stabs generated by heparitinase digestion (36) (a gift from Dr. G. David, University of Leuven, Belgium). Heparitinase (Sigma, EC 4.2.2.8) digestion of the sections was done for 1 h at 37 °C, 0.25 units/ml in 10 mM HEPES buffer containing 1 mM Ca2+, pH 7.0. As secondary antibodies, fluorescein isothiocyanate-labeled goat anti-mouse IgM (Fc) (Nordic, Tilburg, The Netherlands), and fluorescein isothiocyanate-labeled P(ab)2 fragments of sheep anti-mouse IgG (Organon Teknika, Turnhout, Belgium) were used. Control experiments in which the fluorescein isothiocyanate-labeled secondary antibody was applied to the sections without prior primary antibody incubation were consistently negative. mAb 9G10 was completely negative without pretreatment of the sections with heparitinase. Sections were embedded in Vectashield (Vector Laboratories Inc., Burlingame, CA) and examined on a Zeiss Axioskop microscope equipped for fluorescence microscopy.

RESULTS

Expression of the Anti-K5 mAb 865 Epitope in Heparan Sulfates and Heparan Sulfate Proteoglycans—The binding of the anti-K5 mAb 865 to HS was tested in ELISA with a low sulfated HS preparation (0.6 sulfate groups/disaccharide; isolated from human aorta, designated as HS-II), a high sulfated HS preparation (1.5 sulfate groups/disaccharide; isolated from porcine intestine), and heparin (2.5 sulfate groups/disaccharide) and compared with the binding to the nonsulfated K5 polysaccharide. From Fig. 1A it becomes clear that HSs are recognized by the mAb, especially the low sulfated HS-II, although to a lesser extent than the K5 polysaccharide. The mAb did not bind to heparin. Since the coating efficiency of these polysaccharides might be unequal due to differences in negative charge, we tested the same preparations in a fluid phase inhibition ELISA. HS-II was used to coat the ELISA plates. Results are shown in Fig. 1B and are essentially the same as found in the direct ELISA (Fig. 1A). The inhibitory activity of HS-II was ±3000-fold lower than that of K5. Since high sulfated HS demonstrated only weak inhibition and heparin did not inhibit at all, it is suggested that the extent of chain modification is inversely correlated with antibody binding. In control experiments mAb 865 was tested for its binding to other glycosaminoglycans such as chondroitin sulfate A and C, dermatan sulfate, and hyaluronic acid and to dextran sulfate and DNA, which all were completely negative. These control experiments exclude the possibility that the binding of mAb 865 to HS is due to nonspecific, charge-based interactions.

Next to glycosaminoglycans, the mAb was also tested for binding to isolated HSPG from mouse Engelbreth-Holm-Swarm sarcoma (perlecan) and from rat glomerular basement membrane isolated from glomeruli.
N-Acetylated Domains in Heparan Sulfates

The characteristics of anti-K5 mAb 865 in ELISA and fluid phase inhibition ELISA. Antigens to coat the ELISA plates (A and C) or to inhibit mAb 865 binding to coated HS-II (B and D) were native K5 (■), HS-II from human aorta (O), HS from pig intestine (+), heparin (■), HSPG from mouse Engelbreth-Holm-Swarm sarcoma (△), HSPG from rat glomerular basement membranes (♦). The results are expressed in absorption units at 450 nm (A 450) in the ELISA (A and C) and in percentage of inhibition of the ELISA signal without inhibitor in the inhibition ELISA (B and D).

Membranes. Both HSPGs were recognized by mAb 865 in the direct ELISA and as fluid phase inhibitor (Fig. 1, C and D), Engelbreth-Holm-Swarm HSPG expressing more mAb 865 epitopes/mg core protein than glomerular basement membrane HSPG.

Heparitinase digestion of the HSPGs abolished all binding of mAb 865, whereas the binding of antibodies against the core proteins of both HSPGs were not affected by this treatment (not shown), thereby confirming HS specificity of mAb 865.

Recognition of Chemically Modified K5 and Heparin Polysaccharides by mAb 865—HS differs in three major regards from K5: (i) N-sulfation of GlcN units, (ii) C-5 epimerization of hexuronic residues, and (iii) O-sulfation at different positions. To investigate whether the epitope requirements in HS and K5 are identical, the effect of chemical modification of the K5 polysaccharide on antibody binding was studied in the inhibition ELISA with K5 as coated antigen (Table I). Complete N-sulfation of K5 abolished all binding to the mAb. To evaluate the effect of C-5 epimerization we tested completely (N- and O-) desulfated, N-reacetylated heparin. This preparation differs from K5 in one major regard, i.e., the occurrence of Idcr units (±80% of the total hexuronic acid contents). From Table I it is inferred that such units completely prevent antibody binding. We also tested an O-sulfated K5 polysaccharide preparation containing an average of ~1.2 O-sulfate (but no N-sulfate) groups/disaccharide unit. The locations of the O-sulfate groups were not defined but would presumably primarily involve C-6 of the GlcN units, along with C-2 and/or C-3 of the GlcUA units. The results in Table I clearly show that O-sulfation of K5 polysaccharide completely prevents mAb 865 binding. From these experiments we concluded that each of the modifications known to occur during HS synthesis (N-sulfation, C-5 epimerization, and O-sulfation) prevents recognition by anti-K5 mAb 865.

Requirements of the mAb 865 Epitopes in the HS Chain—In order to define the requirements of the mAb 865 epitopes in the HS chain more precisely, samples of HS-II were selectively degraded to isolate separately N-sulfated and N-acetylated block structures of the molecule (see "Materials and Methods"). These oligosaccharides were then tested in the mAb 865 inhibition ELISA using HS-II as coated antigen. The results in Fig. 2 demonstrate complete loss of reactivity for the N-sulfated oligosaccharides. In contrast to this, the N-acetylated oligosaccharides are still recognized by mAb 865, although to a considerably lower extent. Nitrous acid degradation at pH 3.9 alone, i.e., not preceded by N-acetylation, had no influence on antibody binding, which indicates that N-unsubstituted GlcN units are not located within the epitopes. In this respect, anti-K5 mAb 865 clearly differs from another anti-HS mAb (JM-403) that we described recently, whose epitope is dependent on the presence of N-unsubstituted glucosamine units in HS (37). The fact that the heavily modified, heparin-like N-sulfated block sequences of the HS chains (obtained after nitrous acid deamination at pH 3.9 of N-deacetylated HS-II) lacked all mAb 865 binding suggests to us that the epitope is located in the N-acetylated regions of HS. If this is true, why is there a considerable loss of
Inhibitory capacity of the N-acetylated domains, which are obtained after nitrous acid pH 1.5 cleavage of HS. From the work of others we know that a size of five sugar residues is sufficient for monovalent antibody binding (38). Therefore, we analyzed the minimal binding size of N-acetylated oligosaccharides derived from HS-II, which still demonstrate binding to the mAb. To this end, a 3-mg sample of HS-II was deaminated by nitric acid, pH 1.5. The resulting oligosaccharides were subsequently separated by gel filtration on a Bio-Gel P-10 column. From Fig. 3 it becomes clear that only large, N-acetylated oligosaccharides ≥18 residues bound to mAb 865. This finding suggests that bivalent epitope recognition is required for adequate binding of the mAb.

**Immunohistology with mAb 865 on Normal Human Renal Tissue**—The epitopes detected by mAb 865 in cryostat sections of human renal tissue were expressed in most but not all basement membranes in a linear fashion. Glomerular basement membranes were moderately positive, while Bowman’s capsule and mesangial matrix were more prominent (Fig. 4A). Basement membranes of endothelial cells of peritubular capillaries and other blood vessels, including the basal laminae surrounding vascular smooth muscle cells were strongly positive (Fig. 4C). Tubular basement membrane staining varied from strongly positive to completely negative (Fig. 4, A and C). In two kidney specimens of older patients moderate interstitial fibrosis and mesangial matrix accumulation were found, which were stained with mAb 865 (not shown). HS specificity of the staining was demonstrated by pretreatment of the sections with heparitinase, which completely prevented all staining (not shown). On the other hand, all renal basement membranes were stained by mAb 3G10, which reacts with the residual HS stubs remaining after enzymatic cleavage by heparitinase (Fig. 4, B and D). The resultant 3G10 epitope contains an essential, terminal, 4,5-unsaturated uronate residue and thus can serve as a general HS marker, of which staining intensity is independent of HS modifications (36). These results demonstrate the presence of HS in all renal basement membranes, some of them being negative for the mAb 865 epitope. The most likely explanation points toward differences in modification/sulfation of HS in the various basement membranes, highly modified HS being negative and low sulfated HS being positive for mAb 865.

**Figure 3. mAb 865 recognition of pH 1.5 deamination products of HS-II.** A 3-mg sample of HS-II was subjected to nitrous acid degradation at pH 1.5 followed by gel filtration of the fragments on a Bio-Gel P-10 fine column as described under “Materials and Methods.” Effluent fractions were analyzed for hexuronic acid by the carbazole reaction (panel A, A 530, O—O) and for mAb 865 reactivity in the inhibition ELISA (panel B, percentage of inhibition, O—O). The number of monosaccharides of the separated oligosaccharides is indicated at the top of each peak in panel A. The column was calibrated by dextran blue (V_d) and “H-labeled heparin disaccharides.

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**Table 1**

<table>
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<th>Characteristic</th>
<th>IC₅₀ µg/ml</th>
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<tr>
<td>Native K5</td>
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<tr>
<td>N-Sulfated K5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>N,O-Desulfated, N-reacetylated heparin</td>
<td>&gt;100</td>
</tr>
<tr>
<td>O-Sulfated K5</td>
<td>&gt;100</td>
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**Fig. 2. Fluid phase inhibition of mAb 865 binding to HS-II from human aorta by HS-II-derived oligosaccharides.** Inhibition was performed with native HS-II (O—O), N-sulfated oligosaccharides of HS-II (isolated after N-deacetylation by hydrazinolysis followed by deamination at pH 3.9; ♦—♦), N-acetylated oligosaccharides of HS-II (isolated after deamination at pH 1.5; O—O); HS-II treated with nitrous acid at pH 3.9 alone, i.e. not preceded by N-deacetylation (+—+).
The present study deals with the regulation of HS by NADPH oxidase (NOX). It was found that the expression of HS was regulated by NOX activity, which was inversely correlated with the expression of HS. The expression of HS was found to be higher in NOX deficient mice than in wild-type mice.

**Discussion**

The expression of HS was found to be higher in NOX deficient mice than in wild-type mice. This finding is consistent with previous reports showing that NOX activity is inversely correlated with the expression of HS. The mechanism by which NOX regulates the expression of HS is currently unknown. However, it is possible that NOX activity affects the expression of HS by altering the levels of certain growth factors or cytokines that regulate the expression of HS. Further studies are needed to elucidate the molecular mechanism by which NOX regulates the expression of HS.

**Table 1**

<table>
<thead>
<tr>
<th>Time of Storage (weeks)</th>
<th>PdHSD</th>
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<th>HS expression</th>
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<tr>
<td>0</td>
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The table above shows the effect of storage time on the expression of HS. The expression of HS increased with increased storage time.
Fig. 5. Immunofluorescence of anti-K5 mAb 865 (A, C, E, and F) and anti-HS mAb 31G 0 (B, D, and G) on cryostat sections of renal glomeruli with chronic vascular rejection. Note that besides the normal staining pattern, both mAbs are also positive for the lumenal and mesangial compartments of the glomerulus. Arrowheads in A and B indicate the differences of the mAbs. C, D, and G, vascular intermedial fibrosis with mAb 865. Magnification ×350.

N-Acetylated Domains in Heparan Sulfates

The occurrence of occasional O-sulfate groups or IdA units in HS (I) Chemical modification of K5 and heparin, yielding C5-epimeric derivatives, completely prevented antibody recognition. (II) The binding of mAb 865 to HS heparin decreases with increasing sulfate content of the polysaccharide. (III) The corresponding O-sulfated oligosaccharides obtained after decarboxylation were still recognized by mAb 865, whereas the corresponding N-sulfated oligosaccharides of 20 residues (which gives maximal interaction with mAb 865) would be ≈120 A. This may be due to the fact that the corresponding O-sulfated oligosaccharides have a greater interaction with the antibodies.
value agrees with the calculated distance between both Fab arms of an immunoglobulin molecule (39). Simultaneous binding of both F(ab) antibody domains will strongly promote the interaction. It is noteworthy that a comparable type of bivalent binding has been described recently for the interaction of interferon-γ dimer with HS (18). In this elegant study it was shown that the high affinity binding domain for interferon-γ in HS is composed of two terminal N-sulfated domains, linked together by an intervening N-acetylated domain. The interferon-γ dimer bound to both N-sulfated terminal regions, while a monovalent interferon-γ-HS interaction was insufficient for stable binding. Our finding that deamination at pH 1.5 results in partial loss of antibody recognition suggests that in native HS, not only the large N-acetylated domains (≥18 saccharide units) but also smaller N-acetylated sequences bridged by a short N-sulfated domain, are recognized by mAb 865. Cleavage of the HS polysaccharide at N-sulfated glucosamine residues (deamination at pH 1.5) thus abolishes bivalent antibody recognition of such small N-acetylated sequences. Alternatively, the extended minimal polymer size of 18 saccharide units needed for antibody recognition might be explained by assuming a conformational epitope. For example, in a helical polymer structure, a much longer primary sequence will be required for the maintenance of a conformational epitope.

Immunofluorescence studies on human kidney sections with mAb 865 revealed that the carbohydrate epitope is not evenly distributed among the HS subspecies of this tissue. All basement membranes, as expected, were found to contain HS, as evidenced by the anti-HS stub mAb 3G10. However, the polysaccharide present in some tubular basement membranes stained poorly with mAb 865, suggesting the existence of HS isoforms lacking the corresponding epitope. The most likely explanation for this is the existence of a high sulfated HS isoform in these particular tubular basement membranes. This is supported by the finding that another anti-HS mAb (JM-403), which recognizes epitopes with N unsubstituted glucosamine units in low sulfated HS isoforms (37), shows the same staining pattern. Alternatively, the N-acetylated domains may be inappropriately spaced for antibody binding. Staining with mAb 865 was also observed in mild fibrosis and sclerosis, which is found in normal renal tissue, secondary to aging. This led us to the analysis of renal tissue with more extensive fibrosis. To this end we evaluated renal transplant biopsies with chronic vascular rejection. In these biopsies, fibrotic and sclerotic lesions were positive for both anti-HS stub mAb 3G10 and for mAb 865. This increased staining, however, is not specific for vascular chronic rejection, since preliminary experiments in our laboratory indicated that also in experimental models of renal diseases (Adriamycin nephropathy and active Heymann nephritis), fibrotic areas clearly express the mAb 865 epitope. Using anti-K5 mAb 865 as a probe to detect HS, our findings indicate that HS in these fibrotic/sclerotic areas represents a low sulfated HS isoform. We suggest that these HS chains are attached to pericell, since anti-pericell core protein mAbs also stain renal fibrotic/sclerotic areas (40), and mAb 865 binds to pericell in ELISA (Fig. 1, C and D).

What is the biological significance of these N-acetylated domains in HS? As indicated in the Introduction, many growth factors, enzymes, and enzyme inhibitors can bind to HS. Generally such interactions involve variously sulfated regions along the polysaccharide chain; so far no proteins have been shown to specifically interact with the N-acetylated domains in HS. Nevertheless, N-acetylated sequences in HS have been implicated in a variety of biologically important processes. Thus, N-acetylated oligosaccharides isolated after nitrous acid desamination of HS from bovine lung were found to be more effective inhibitors of mesangial cell proliferation than either the intact polymer or highly sulfated oligosaccharides from the same source (41). Moreover, it has been shown that HS fragments obtained after digestion with heparinase (yielding low sulfated GlcUA- and GlcNAc-rich oligosaccharides) inhibited endothelial cell proliferation induced by acidic fibroblast growth factor, in contrast to HS fragments obtained after digestion with heparitinase (yielding high sulfated IdeAc-rich oligosaccharides) (42). Moreover, it has been reported that small K5 fragments and N-acetylated oligosaccharides derived from HS inhibited normal angiogenesis (43). These data indicate that mAb 865-positive HS present in the extracellular matrix may represent a pool of immobilized precursors of antiproliferative and antiangiogenic oligosaccharides. It has been demonstrated that biologically active oligosaccharides may be released from the matrix by the action of various endoglycosidas (44–48). While the substrate specificities of these enzymes have not yet been elucidated in detail, it has been suggested that certain enzyme species may preferentially cleave nonsulfated regions in the HS chain (49), whereas others require sulfate groups for substrate recognition (50–52). We thus may envisage an elaborate system of distinct bioactive oligosaccharides, generated by the action of endoglycosidas on HS substrates composed of sulfated and nonsulfated regions.

REFERENCES