Age-dependent Modulation of Heparan Sulfate Structure and Function

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Heparan sulfate interacts with growth factors, matrix components, effectors and modulators of enzymatic catalysis as well as with microbial proteins via sulfated oligosaccharide domains. Although a number of such domains have been characterized, little is known about the regulation of their formation in vivo. Here we show that the structure of human aorta heparan sulfate is gradually modulated during aging in a manner that gives rise to markedly enhanced binding to isoforms of platelet-derived growth factor A and B chains containing polybasic cell retention sequences. By contrast, the binding to fibroblast growth factor 2 is affected to a much lesser extent. The enhanced binding of aorta heparan sulfate to platelet-derived growth factor is suggested to be due to an age-dependent increase of GlcN 6-O-sulfation, resulting in increased abundance of the trisulfated L-iduronic acid (2-O-SO_3)GlcNSO_3(6-O-SO_3) disaccharide unit. Such units have been shown to hallmark the platelet-derived growth factor A chain-binding site in heparan sulfate.

Interactions of the sulfated glycosaminoglycan (GAG) heparan sulfate (HS) with various proteins affect the biological activity, tissue localization, and turnover of the protein ligands (1–3). Such interactions, generally electrostatic in nature, regularly involve specific oligosaccharide domains generated by an elaborate biosynthetic machinery in the Golgi apparatus. HS formation starts by assembly of an initial (GlcA-GlcNAc)_n polymer. Parts of the nascent polymer are subsequently modified by N-deacylation/N-sulfation of GlcNAc residues, and further modifications, including C-5 epimerization of GlcA residues into IdcA residues as well as O-sulfation at various positions, occur mainly in the vicinity of the previously incorporated N-sulfate groups (3, 4). The O-sulfate groups are predominantly found at the C-2 position of IdcA residues and the C-6 position of GlcN residues. The protein-binding HS domains typically reside within the N-sulfated regions, their functional specificity being determined by the pattern of modification, particularly the positioning of sulfate groups. Although information regarding the structures of the recognition sites for individual proteins (5–11) and the general features of HS biosynthesis (3) is accumulating, the biological control of HS structure and function remains poorly understood. Nevertheless, HS species from various cells and tissues clearly differ in their structure and in some studies such differences have been correlated to differential protein-binding properties (12–15). These and other findings (discussed in Refs. 1 and 3) suggest that the biosynthesis of HS is subject to regulation during development or aging. Control of the appropriate expression of functional HS domains in given organs or at particular developmental stages would appear essential, whereas, conversely, perturbed regulation could contribute to various pathologies. In the present study we have explored the aging aortic wall as a model to gain insight into the control of HS structure and function in humans. Aging is a strong predisposing factor to atherosclerosis, characterized by endothelial damage, lipid accumulation, and cell proliferation in arterial wall plaques (16). HS has been attributed multiple roles in these processes by interacting with lipoprotein lipase (17) and with growth factors such as basic fibroblast growth factor (FGF-2) and platelet-derived growth factors (PDGFs) (18–21). HS binds to and enhances the mitogenic activity of FGF-2 (22) and regulates the tissue localization of PDGFs (20). PDGFs are homo- or heterodimers of two closely related, A and B, polypeptide chains. The PDGF-A chain exists as two variants due to alternative mRNA splicing. The longer variant (PDGF-Aα) contains a C-terminal polybasic sequence that serves as a cell retention signal and is associated with the localization of PDGF to the surface of the producer cell or to the extracellular matrix (21), presumably due to interactions with HS (20). A similar but not identical retention signal is found at the C terminus of the PDGF-B propeptide chain (PDGF-Bβ) (21). This propeptide may undergo various N- and C-terminal proteolytic processing events that give rise to multiple forms of cell-associated PDGF-B species (23).

MATERIALS AND METHODS

Isolation and Radiolabeling of Heparan Sulfate—Tissue samples from human abdominal aorta were obtained at autopsy and stored at 70 °C until processed for HS isolation. The surrounding connective tissue was removed, and the sample, encompassing the entire thickness of the vessel wall, was cut into fine pieces with a scalpel. The samples were defatted essentially as described (15) with the exception that ethyl ether was replaced by ethanol. Defatted samples (dry weight, 0.15–2.0 g) were subjected to protease digestion with papain (Sigma; 5 mg/g of defatted tissue) in 25 ml of 0.05 M Tris, pH 5.5, 0.01 M EDTA, 2 M NaCl, 0.01 M cysteine/HCl at 60 °C for 18 h and centrifuged (10 min at 2000 × g), and the supernatants were applied to columns of DEAE-Sephacel (1.5 × 5 cm; Amersham Pharmacia Biotech) equilibrated with 0.05 M Tris, pH 7.2. The column was washed with 0.05 M sodium acetate, pH 4.0, followed by elution of the DEAE-bound material (containing sulfated GAGs) by a linear gradient of LiCl (0.15–2.0 M) in the acetate buffer (15). 2-ml fractions were collected and analyzed for uronic acid content by the carbazole reaction (24). Fractions containing GAGs were pooled, dialyzed against water, lyophilized, and digested with 1 unit of chondroitinase ABC (Seikagaku Corporation) and 125 units of endonuclease (Benzonase, Benzon Pharms A/S) in 0.05 M Tris-HCl, pH 8.0, 1 mM MgCl_2, 0.05 M sodium acetate at 37 °C overnight (25). The digest was heated for 2 min and applied to a DEAE-Sephacel column (1.5 × 5 cm) equilibrated with 0.2 M NH_4HCO_3 and eluted by a linear gradient of NH_4HCO_3, 0.2–2.0 M. Fractions containing HS were identified by the carbazole reaction, pooled, and freeze-dried, and the material was stored at −20 °C until further use. Treatment of the purified HS preparations with HNO_3 at pH 1.5 resulted in quantitative degradation of the material into lower molecular weight species as demonstrated by
chromatography of intact and HNO₂-treated samples on a column of Superose 12 (data not shown), indicating that the purification procedure yielded pure HS.

For radiolabeling, HS samples (80–150 µg) were N-deacetylated in hydrazine hydrate (Fluka Chemie AG) containing 30% water and 1% hydrazine sulfamide (Merck) for 2–4 h at 100 °C (26), desalted, and reacetylated using [3H]acetic anhydride (Amersham Pharmacia Bio- tech) as described (15). The specific activity was calculated after measurement of the uronic acid content of the [3H]HS samples by the carbazole reaction.

Assay of Heparan Sulfate-Protein Interaction—The binding of [3H]HS preparations to recombinant PDGF-AA₉ (27), PDGF-BB₉ (33 pmol/incubation), and FGF-2 (29 pmol/incubation) (Pepro Tech EC) was studied by a nitro- carbazole reaction.

PDGF Affinity Chromatography—5 mg of recombinant PDGF-AA₉ was mixed with an equimolar amount of heparin and immobilized to 3 ml of CH-Sepharose CL4B (Amersham Pharmacia Biotech) in 0.5 M NH₄HCO₃. The column was equilibrated with Tris-buffered saline, and the bound material was eluted using a linear NaCl gradient (0.15–2.0M in 50 m M Tris-HCl, pH 7.4). Fractions of 1 ml were collected and analyzed for radioactivity in a liquid scintillation counter.

Structural Analysis of Heparan Sulfate—Samples of HS (~30 µg) were subjected to cleavage with nitrous acid at pH 1.5 (28). The cleavage products were end-labeled by reduction with 250 µg of diethylamine (Laboratories) and 20 µg of chloramine T (Eastman Kodak) in 5 ml of 0.2 M NH₄HCO₃, pH 7.4, and subjected to electrophoresis on Whatman number 3MM paper in 6.5% HCOOH, pH 1.7.

RESULTS

We first examined the binding of radiolabeled human aorta HS from a young and an old individual to FGF-2 and to dimers of the long isoforms of PDGF A or B chains (designated PDGF-AA₉ and PDGF-BB₉ below) (30). A filter trapping procedure was employed, involving interaction of labeled HS and the protein ligand in solution followed by rapid passage of the mixture through a nitrocellulose filter. Protein and protein-bound HS chains (but not free HS chains) were retained on the filter (8). We found that the binding capacity for PDGF-AA₉ and PDGF-BB₉ of HS from a 76-year-old individual was 4–5 times higher than that of HS from a 21-year-old individual (Fig. 1A), whereas the binding to FGF-2 differed only marginally between the two HS samples. To assess the inter-individual variation in these interactions, we next compared the binding of six HS preparations, from three young and three old subjects, to PDGF and FGF-2. The results of this experiment (Fig. 1B) indicated a virtually invariable level of binding of HS to a given protein ligand within each of the two age groups but marked differences in binding characteristics between these groups. The effect of age thus was selectively expressed for different proteins, in accord with the data shown in Fig. 1A. These findings clearly point to the occurrence of age-dependent differences in HS structure that affect the binding of HS to PDGF and FGF-2 in distinct ways. The effect of this structural transition with respect to PDGF binding was further examined by affinity chromatography of HS from young and old subjects on a column of immobilized PDGF-AA₉ (Fig. 1C). Both types of HS contained material that remained bound to the growth factor at physiological pH and ionic strength, as well as a fraction of unbound material. The PDGF-binding chains amounted to 50.0±2.4% versus 81.8±1.5% (mean ± S.E. from three and two samples, respectively) of the total HS from young subjects aged 20 (21 years) and 22 years) and three old (70, 76, and 81 years) subjects were tested for binding to PDGF-AA₉, PDGF-BB₉, and FGF-2 (same amounts as in A). The binding of HS from old subjects is set as 100%. The means ± S.D. are shown. C, samples of [3H]HS from three subjects aged 20 (solid line), 21 (dashed line), and 22 (dotted line) years (Young subjects) and two subjects aged 76 (dashed line) and 80 years (solid line) (Old subjects) were subjected to chromatography on a column of PDGF-AA₉-Sepharose as described under "Materials and Methods." The arrow indicates the start of the gradient.
and old subjects, respectively. These results thus confirm not only the correlation between individual age and PDGF binding ability of aortic HS but also the striking inter-individual similarity within each age group.

Binding of HS to PDGF-AAL and FGF-2 involves structurally distinct oligosaccharide domains. The former domain is comprised by N-sulfated ~8-mer sequences with at least one trisulfated \( \text{IdceA}(2-\text{OSO}_3)\text{GlcNSO}_3(6-\text{OSO}_3) \) disaccharide unit (8), whereas the minimal FGF-2-binding site contains an essential \( \text{IdceA}(2-\text{OSO}_3) \) residue but no 6-O-sulfate groups (5, 7, 31). Given these data, we wanted to examine whether the differential protein binding of aorta HS from young and old subjects would correlate with the O-sulfate substitution pattern of the N-sulfated regions of HS. We therefore determined the disaccharide composition of these regions in a total of 15 HS preparations from subjects aged 20–84 years (including the six preparations used in the binding experiments). The results of this analysis (Table I) demonstrated an age-dependent increase in the proportion of the \( \text{IdceA}(2-\text{OSO}_3)\text{GlcNSO}_3(6-\text{OSO}_3) \) unit (Fig. 2). Calculation of the overall extent of 2-O- and 6-O-sulfation indicated that this increase was due to an approximate doubling of the level of 6-O-sulfate substitution of GlcNSO3 residues in the old subjects (Fig. 2), whereas the IdceA 2-O-sulfation remained high and essentially unchanged (Fig. 2). The 6-O-sulfation of GlcNSO3 residues showed an almost linear increase between the age of 20 and 40 years; in the still older subjects the levels of 6-O-sulfation were somewhat scattered but consistently higher than in the young individuals (Fig. 2).

We further determined the degree of N-sulfate substitution of GlcN residues from the pattern of HS depolymerization following cleavage with HNO2 (at pH 1.5; see “Materials and Methods”). Five of the HS samples (subject aged 20, 21, 74, 76, and 80 years) were analyzed (Fig. 3) and found to contain essentially similar proportions of GlcNSO3 units (means ± S.D., 39 ± 2% of total GlcN units).

Finally, we assessed the degree of sulfation of the sequences comprised of alternating N-sulfated and N-acetylated disaccharide units. These domains typically harbor GlcN 6-O-sulfate groups but few or no IdceA 2-O-sulfate groups (4) and are recovered as tetrasaccharides after cleavage of HS with HNO2 at pH 1.5. Analysis of tetrasaccharides from four HS samples (subject aged 20, 30, 50, and 80 years) indicated essentially similar proportions of nonsulfated, monosulfated, and disulfated species (76 ± 3, 20 ± 3, and 4 ± 1% of all tetrasaccharides, respectively, expressed as means ± S.D.). The increase in 6-O-sulfate substitution of GlcN units in the old individuals thus is essentially confined to the contiguous N-sulfated domains of HS.

The major alteration in human aorta HS in association with

| Table I | Analysis of O-sulfated disaccharide species derived from the contiguous N-sulfated regions of human aorta heparan sulfate |
|---------|---------------------------------------------------------------------------------------------------------------|
| Samples of HS were treated with HNO2 at pH 1.5, resulting in deaminative cleavage at GlcNSO3 residues. The cleavage products were radiolabeled by reduction with NaB3H4. Disaccharides were recovered by gel chromatography and analyzed by anion exchange HPLC as described. |

| Subject characteristics | Disaccharide composition | % of O-sulfated disaccharides |
|-------------------------|--------------------------|-------------------------------|
| Subject | Age | Sex | GlcA/2-OSO3, aManR | GlcA-aManR (6-OSO3), | IdeA-aManR | GlcA/2-OSO3, aManR (6-OSO3), | IdceA(2-OSO3)-aManR | % of O-sulfated disaccharides |
| 1 | 20 | M | 0.6 | 3.3 | 2.4 | 86.7 | 7.0 |
| 2 | 21 | M | 0.8 | 2.4 | 1.1 | 82.4 | 7.5 |
| 3 | 22 | M | 1.0 | 2.6 | 1.5 | 86.4 | 8.5 |
| 4 | 31 | M | 1.8 | 4.6 | 1.4 | 83.3 | 8.0 |
| 5 | 31 | M | 1.6 | 3.7 | 1.7 | 82.6 | 10.5 |
| 6 | 37 | M | 1.1 | 3.8 | 2.0 | 83.0 | 10.1 |
| 7 | 43 | M | 1.0 | 4.4 | 1.8 | 81.4 | 11.5 |
| 8 | 47 | M | 1.3 | 4.1 | 2.6 | 78.1 | 14.0 |
| 9 | 50 | M | 1.4 | 3.6 | 2.0 | 82.9 | 10.0 |
| 10 | 70 | F | 0.8 | 6.4 | 1.8 | 74.0 | 16.9 |
| 11 | 75 | M | 2.0 | 4.2 | 3.2 | 78.7 | 12.0 |
| 12 | 76 | F | 0.7 | 3.8 | 1.8 | 81.9 | 11.9 |
| 13 | 80 | M | 0.6 | 3.8 | 2.2 | 80.5 | 12.9 |
| 14 | 81 | F | 1.2 | 4.2 | 2.5 | 78.3 | 13.8 |
| 15 | 84 | M | 1.8 | 5.5 | 4.0 | 74.3 | 14.4 |

*a In samples 12 and 13, macroscopic atherosclerotic lesions were present.*

![Fig. 2. Age-dependent changes in O-sulfate substitution of human aorta HS.](image-url)
Atherosclerotic lesions are frequently encountered in human aorta, and HS-binding growth factors such as FGFs and PDGFs are thought to contribute to the pathological smooth muscle cell migration and proliferation characterizing the disease (16). The expression level of the PDGF-A chain is high in human arterial smooth muscle cells and increases markedly during the conversion of monocytes into macrophages (32). It has been shown that PDGF isoforms containing the Aα or B chains are retained at cell surfaces or in the extracellular matrix by HS (20, 21). In the arterial wall, increased binding of PDGF to HS could thus result in extracellular accumulation of PDGF. Such early changes might facilitate later pathophysiological processes such as aberrant smooth muscle cell migration and growth in individuals prone to develop atherosclerotic disease. Moreover, we note that the trisulfated Idecα(2-OSO3)-GlcNSO3(6-OSO3) disaccharide units found to promote binding of PDGF to HS has also been implicated in binding of lipoprotein lipase (6), a cell surface-bound enzyme that catalyzes the breakdown of triglycerides and affects the cellular uptake of lipids (17). The observed change in HS structure thus is likely to have more widespread functional implications than those emphasized in this study.

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