Emerging Views of Heparan Sulfate Glycosaminoglycan Structure/Activity Relationships Modulating Dynamic Biological Functions

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Heparan sulfate glycosaminoglycans (HSGAGs) are an important subset of complex polysaccharides that represent the third major class of biopolymer, along with polynucleic acids and polypeptides. However, the importance of HSGAGs in biological processes is underappreciated because of a lack of effective molecular tools to correlate specific structures with functions. Only recently have significant strides been made in understanding the steps of HSGAG biosynthesis that lead to the formation of unique structures of functional importance. Such advances now create possibilities for intervening in numerous clinical situations, creating much-needed novel therapies for a variety of pathophysiological conditions including atherosclerosis, thromboembolic disorders, and unstable angina. (Trends Cardiovasc Med 2002;12:71–77). © 2002, Elsevier Science Inc.

Heparan sulfate-like glycosaminoglycans (HSGAGs) are complex acidic polysaccharides present both within the extracellular matrix (ECM) that surrounds cells and at the cell surface as proteoglycans (termed proteoglycans) (Figure 1). HSGAG proteoglycans are found at the surface of virtually every cell type, where they act as important biological mediators of various cell-related events such as proliferation, morphogenesis, adhesion, migration, and cell death (apoptosis) (Sasisekharan and Venkataraman 2000; Tumova et al. 2000). Given their role in fundamental cellular events, HSGAGs have been found to be important regulators of biological processes ranging from embryogenesis (Perrimon and Bernfield 2000) to hemostasis (Rosenberg et al. 1997) as well as in the pathophysiology of disease states, such as atherosclerosis and thromboembolic disorders (Kolset and Salmivirta 1999). This review focuses on recent scientific advances that have furthered our understanding of how HSGAG structure impinges on function. Specific emphasis will be placed upon how these advances have increased our appreciation of the importance of complex polysaccharides in vascular biology, and how this understanding offers new potential for the development of novel classes of therapeutics.

That HSGAGs can regulate such a variety of biological processes is a function of the chemical diversity inherent in the HSGAG polymer. A disaccharide repeat unit characterizes all HSGAGs; however, HSGAGs from various sources differ both in terms of their length (that is, the number of disaccharide repeat units) and differential chemical modifications within the HSGAG chain (Figure 1) (Salmivirta et al. 1996; Turnbull et al. 2001).

Each disaccharide unit of an HSGAG chain contains a β-D-glucosamine linked to an uronic acid, which can be one of two C5 epimers, either α-L-iduronic or β-D-glucuronic acid. Other than the C5 position of the uronic acid, HLGAGs vary in their chain length, that is, the number of disaccharide repeat units, and degree of sulfation and acetylation of each disaccharide unit. O-sulfation of the disaccharide repeat can occur at the 2-O position of the uronic acid and the 6-O and 3-O positions of the glucosamine. Thus, for a given disaccharide unit within an HSGAG structure, each site is either sulfated or unsubstituted, creating eight possible combinations. In addition, as mentioned above, there are two possibilities for the uronic acid component of each disaccharide unit, that is, either iduronic acid or glucuronic acid, giving rise to a total of 16 different possible disaccharide combinations. Finally, the N-position of the glucosamine can be sulfated, acetylated, or unsubstituted (three possible states). Taking this final factor into account results in 48 possible disaccharide building blocks. Thus, HSGAGs have the potential to carry more information than any other biopolymer, including either DNA (made up of four bases) or proteins (made up of 20 amino acids). Consider, for instance, a simple polymer of each kind made of six units. For DNA, there are 46 or 4096 possible sequences for this hexamer. In contrast, for a hexapeptide, there are many more possibilities, 206 or 64 million possible permutations. However, for HSGAGs, a polymer made of six disaccharide units could have a total of over 12 billion possible sequences, a staggering degree of variation, over 100 times as much as for polypeptides and two million times that of DNA!

- HSGAGs in Cardiovascular Hemostasis

Given the information density of HSGAGs, each HSGAG chain contains multiple sequences that specifically regulate multiple components of the cardiovascular system (Figure 2a). In some cases, it has been shown that certain HSGAG structures provide protective effects against disease processes such as atherosclerosis and unstable angina (Kanabrocki et al. 1992). For instance, HSGAGs lining the luminal surface of endothelial cells effectively provide electronegativity, and

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the atherogenic process. The pharmacological administration of highly sulfated HSGAGs restores the electronegativity of the damaged endothelium lining, thereby limiting further damage and preventing thrombosis. In addition, cell surface HSGAGs, through interactions with lipoprotein lipase and apolipoproteins B and E, are involved in the metabolism of lipoproteins including HDL by regulating their uptake and metabolism (Figure 2a) (Kolset and Salmivirta 1999, Rosenberg et al. 1997). In this manner, HSGAGs mediate the “lipid clearing effect” of plasma. Finally, cell surface HSGAGs, depending on structure and concentration, can either inhibit or promote growth factor signaling, thus regulating SMC proliferation during vascular stenosis and atherosclerosis. Importantly, many of these biological observations have been successfully translated to the clinic; specific examples will be illustrated below.

The protective effects of highly sulfated HSGAGs have been extensively demonstrated in coronary diseases such as myocardial infarction and unstable angina. Thus, HSGAG structures impinge on vascular biology in multiple ways. To differentiate roles for specific HSGAG sequences requires the application of newly developed technologies for the isolation and characterization of biologically important HSGAG sequences. Below, we focus on two specific examples where such studies have offered a clear understanding of how specific HSGAG structures impinge on function, resulting in significant advances both scientifically and clinically.

- **HSGAGs in Coagulation**

Of the many functions regulated by HSGAGs, one of the best studied is their role in the maintenance of hemostasis, or regulation of blood volume and composition. In vivo, under normal, non-pathologic conditions, HSGAGs at the surface of endothelial cells actively modulate the activity of the proteases involved in the coagulation cascade, including tissue factor, factor Xa, and factor IIa (thrombin), among others (Figure 2b) (Rosenberg 2001). As might be expected from such an information dense molecule, HSGAGs impinge on almost every step of the coagulation cascade, meaning that these complex polysaccharides play multifaceted roles in maintaining a hydration sphere to the vascular wall that is important for the normal tone and fluidity of the circulating media (Figure 2a). The disruption of this lining by direct injury to the blood vessels is one of the primary initiating factors of the atherogenic process.
hemostasis, thus providing a potent basis for their pharmacological use (see below). Predominantly, HSGAGs serve as potent negative regulators of coagulation, preventing hypercoagulable states and maintaining hemostasis (Figure 2b). One of the best recognized roles for HSGAGs in the coagulation cascade is the interaction of HSGAGs with antithrombin III (ATIII) (Jin et al. 1997). The entire coagulation process is under the control of a group of glycoprotein serine protease inhibitors (also known as serpins), the most important of which is ATIII. Many of the coagulation proteases, most notably factor Xa and IIa, are inhibited by ATIII, which forms an equimolar inhibitory complex with these enzymes. Much attention has focused on the binding of ATIII to HSGAGs; indeed, this interaction has become the prototypic example of a HSGAG–protein interaction. ATIII’s anticoagulant activity is mediated by binding to specific HSGAG sequences, viz., a pentasaccharide sequence within the HSGAG chain with the sequence HNAc/8S,6S GN3S,3S,6S I2S HNS,6S (Figure 3a) (Desai et al. 1998; Shriver et al. 2000). Binding of ATIII to this HSGAG pentasaccharide sequence at the cell surface of endothelial cells induces a conformational change in the protein, increasing by over three orders of magnitude its ability to inhibit selected proteases of the coagulation cascade, most notably factors Xa and IIa.

Importantly, the interaction of ATIII with factor Xa occurs by a different mechanism than its interaction with factor IIa (Nugent 2000, Petitou et al. 1999). In each case, the first event temporally is the binding of ATIII to the pentasaccharide sequence followed by ATIII’s conformational change and concomitant activation. For the inhibition of factor Xa, this conformational change is sufficient for the formation of a high-affinity protein–protein inhibitory complex (Jin et al. 1997). The Xa:ATIII complex irreversibly traps the protease as a reaction intermediate covalently bound to the serpin. Conversely, ATIII’s inhibition of thrombin requires a HLGAG chain containing at least 16 saccharide units (Figure 3b) (Petitou et al. 1999). Thrombin is thought to bind, through nonspecific ionic interactions, to the nonreducing end of the chain. Upon thrombin binding, activated ATIII interacts with thrombin via the formation of an ATIII:IIa:HSGAG ternary complex. Thus, in this case, the HSGAG chain acts as a bridge or platform, promoting the formation of an ATIII:IIa high affinity complex (Figure 3b).

In addition to the role of HSGAGs in binding to ATIII and activating it, HS-
GAGs are known to play other important roles in maintaining hemostasis. Recently, it has been found that HSGAGs actively facilitate the release of tissue factor pathway inhibitor (TFPI, also known as extrinsic pathway inhibitor) (Fareed et al. 2000, Sandset et al. 2000). TFPI is a polymodal inhibitor that inhibits tissue factor, factor VIIIa, factor Xa, and proteases released from macrophages and leukocytes. Other important components of the coagulation system that are modulated by HSGAGs include heparin co-factor II (HCII) (Ramp et al. 2001, Nader et al. 2001) and von Willebrand Factor (vWF) (Antman and Handin 1998). In light of the fact that a given HSGAG chain contains multiple distinct sequences, it seems highly likely that these molecules play other roles in preventing blood coagulation and maintaining the hemostatic balance.

Given the multidimensional role of HSGAGs, these molecules have been used as highly effective antithrombotic agents. Highly sulfated HSGAGs isolated from mast cells have been used therapeutically for over 60 years as anticoagulants. This highly sulfated population of HSGAGs, termed “heparin,” is a potent anticoagulant, used in a wide variety of thromboembolic disorders, including deep vein thrombosis, pulmonary embolism, arterial thromboses, and acute coronary syndromes like myocardial infarction and unstable angina (Antman 2001). Of note is the fact that the multitude of clinical effects initiated by heparin have not been recapitulated with synthetic agents to date.

- **Creation of Low Molecular Weight Heparins**

Unfractionated heparin (UFH) has proven to be a highly effective anticoagulant and antithrombotic agent for a variety of clinical indications. However, the side effects associated with heparin therapy are many and varied. Side effects such as heparin-induced thrombocytopenia are primarily associated with the long, polysulfated chain of pharmaceutical heparin, which provides binding domains for various proteins beyond what is therapeutically required (Baglin 2001). To circumvent the deleterious effects associated with heparin therapy while maintaining its potent anticoagulant activity, numerous strategies have been designed to create low molecular weight heparins (LMWHs), molecules with reduced chain length and, accordingly, reduced side effects. A number of strategies have been employed to create LMWHs, including controlled chemical cleavage (for instance, nitrous acid degradation or radical-mediated chain depolymerization), enzymatic digestion, and size fractionation of heparin; each has met with a varying degree of clinical success (Casu and Torri 1999; Gunay and Linhardt 1999).

LMWHs are currently defined as having molecular masses of less than 6 kDa (compared to 9–12 kDa for UFH) and an anti-Xa: anti-IIa ratio of 1.5:1 (again compared to an anti-Xa to anti-IIa ratio of approximately 1:1 for UFH) (Fareed et al. 2000). Of note is the fact that one of the consequences of the production of various LMWHs is a reduction in the amount of anti-IIa activity associated with the polysaccharide chain. This is because the length of LMWHs, as well as the placement within the polysaccharide chain of the ATIII binding site, prevents the efficient formation of an ATIII, thrombin, polysaccharide complex required for the thrombin inhibition. As such, current LMWHs are appropriate heparin substitutes in some clinical situations, but not others. For instance, LMWHs, with their pronounced ability to inhibit factor Xa, have proven to be ideal agents for the treatment and prevention of deep vein thrombosis but not for arterial thrombosis, where antithrombin activity is required. Recently, it has been noted that LMWHs differ from one another in terms of their anti-Xa activity, bioavailability, and overall clinical efficacy (Jeske and Fareed 1999). However, owing to the complex chemical nature of LMWHs, differences in function cannot currently be attributed to differences in structure, a key link that would enable the production of a new generation of LMWHs with improved clinical profiles.
Several novel technologies have been brought to bear on this important problem. Recently, a high throughput sequencing strategy was developed for the structural analysis of biologically and clinically important HSGAGs (Turnbull, Powell, and Guimond 2001; Venkataraman et al. 1999). This technology combines state-of-the-art analytical methodologies with a bioinformatics framework to quickly determine unknown sequences. With the use of this technology, it was found that certain means of degrading the heparin chain to create LMWHs resulted in cleavage of the ATIII binding site (Shriver et al. 2000). Furthermore, as predicted from the structural analysis, it was found that such strategies resulted in the generation of LMWHs with lower activity in vitro and in vivo compared to UFH (Shriver, et al. 2000). Using such a strategy, it should prove possible to generate LMWHs with increased activity and decreased side effects, thus creating a more potent, more chemically defined second-generation LMWH.

**HSGAGs as Modulators of Angiogenesis**

In addition to the role of HSGAGs in modulating the coagulation process, these complex polysaccharides are known to be potent mediators of angiogenesis (Folkman and Shing 1992, Sasisekharan et al. 1994). As in hemostasis, HSGAGs play a multifaceted role in maintaining the angiogenic balance. However, unlike in the case of coagulation, where HSGAGs largely prevent hypercoagulation, different HSGAG sequences can play distinct, even opposite, roles in the angiogenic process.

Fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are two of the primary angiogenic factors that have been extensively characterized (Nugent and Iozzo 2000; Robinson and Stringer 2001). HSGAGs have been shown to play essential roles in the angiogenic activities of both FGF and VEGF, particularly for FGF2 signaling, where specific regulatory roles for HSGAGs are known. Currently, both FGF2 and VEGF are being tested for their ability to promote therapeutic angiogenesis in cases of ischemic cardiovascular disease. Promising results have been obtained in animal models where administration of either FGF2 or VEGF increased angiogenesis and the development of new collateral vessels resulting in improved perfusion and histology (Freedman and Isner 2001). However, there is a need to improve formulation, stability, pharmacokinetics, and potency of these angiogenic factors. A clear understanding of the modulatory role of cell surface HSGAGs in FGF and VEGF signaling will provide a basis for designing more stable and potent pro-angiogenic factors.

Recent evidence has demonstrated that specific HSGAG sequences modulate the activity of FGF. Developmental studies in Drosophila have demonstrated that loss of specific HSGAG sequences results in morphogenic defects associated with inactivation of FGF-mediated signaling pathways (Lin et al. 1999). Other studies have shown that HSGAGs at the cell surface are necessary for high-affinity binding of FGF, presentation of FGF to its transmembrane receptor, and concomitant activation of intracellular signaling pathways.

**Mechanism of HSGAG Action on FGF Activity**

HSGAGs interact with growth factors like FGF at every known step of the growth factor’s trajectory starting from its release into the ECM until its binding at the cell surface of a recipient cell (Figure 4). Several mechanisms seem to be important in vivo.

- **HSGAG Sequences in the ECM Sequester FGF.** HSGAG proteoglycans in the ECM, particularly in the basement membrane, can bind FGF and store it in an inactive form (Chang et al. 2000). Degradation of these “storage” forms of HSGAGs results in the facile release of active FGF, enabling a rapid response to external stimuli without the need for new protein synthesis.

- **HSGAGs Form a Gradient Regulating FGF Diffusion from the ECM to the Cell Surface.** Developmental studies in Drosophila have demonstrated that HSGAGs are one of the primary mechanisms enabling the formation of a concentration gradient for growth factors, cytokines, and other signaling molecules (Perrimon and Bernfield 2000). Thus, by differential binding of FGF to HSGAGs in the ECM and at the cell surface, the diffusion of FGF becomes a highly regulated process thus controlling the rate and strength of FGF signaling.

- **HSGAGs Mediate the Formation of an Active Signaling Complex at the Cell Surface.** FGF oligomerizes in the pres-

![Figure 4. HSGAGs regulate growth factor signaling in multiple ways in the molecule's trajectory from the originator cell to the recipient cell.](image-url)
ence of specific HSGAG sequences (Kwan et al. 2001). Binding of FGF oligomers, mediated by cell surface HSGAGs, to tyrosine kinase receptors results in increased receptor oligomerization, increased transphosphorylation, and accordingly, increased activation of intracellular signaling pathways. In addition to mediating FGF oligomerization, HSGAGs can either promote or inhibit binding of particular FGF family members to a given receptor isofrom (Berry et al. 2001). As such, HSGAG sequences can act as molecular switches, modulating the affinity of a given FGF for various receptor isoforms.

Thus, HSGAGs, depending on sequence and the biological context, can serve multiple regulatory roles in FGF signaling. Importantly, FGF is used here as an illustrative example (albeit an important one); other growth factors and cytokines that are currently used clinically bind to HSGAGs, including VEGF (as mentioned above), interferon gamma, tissue plasminogen activator, and others. Each is regulated by HSGAGs of differing sequence and via similar, but subtly different, mechanisms. Given the complexity of the system the question then arises of how to translate this wealth of biological information into therapeutically viable means of intervening in growth factors’ signaling pathways. To this complex problem, several novel technologies can be applied. First, as was the case with the structural analysis of anticoagulant heparin, sequencing approaches promise to shed some light on inhibitory and activatory HSGAG sequences (Venkataraman et al. 1999). Structural analysis of these sequences, in conjunction with crystal structure analysis, should make possible the development of a completely novel set of growth factor agonists and antagonists. In addition, given our understanding of certain HSGAG–protein systems (for instance, the FGF:HSGAG system), protein engineering approaches have been applied towards the development of clinical preparations of growth factors like FGF with higher activity and less variable pharmacokinetics. Three distinct methodologies have been employed. The first is to create a covalent FGF–HSGAG complex using a defined HSGAG sequence that is known to promote FGF signaling activity (Pye and Gallagher 1999). A related approach is to dimerize peptides with high affinity to an isoform of the FGF receptor; dramatically increasing activity (Ballinger et al. 1999). An additional approach that has met with a higher degree of success is the creation, via recombinant protein technology, of an oligomeric FGF construct, mimicking the role of HSGAGs in the formation of an FGF complex that binds with high affinity to cell surface receptors. Recently, it has been shown that such a construct has a markedly increased ability to induce angiogenesis in vivo (Kwan et al. 2001). The improved pharmacodynamics and pharmacokinetics of engineered FGFs should prove useful in the treatment of cardiovascular diseases; for instance, a potent FGF may expedite the process of angiogenesis to improve the perfusion of ischemic myocardium.

• Future Directions

The structure–function paradigm outlined in this review opens new approaches and strategies for therapeutic intervention at the cell–tissue–organ level. For example, an understanding of how HSGAGs, in a dynamic fashion, impinge on the topological and functional organization of cells like smooth muscle cells and endothelial cells can lead to important breakthroughs in heart and blood vessel regeneration. A sophisticated understanding of ECM–cell interactions will propel forward the field of organ repair and/or tissue engineering by permitting the targeting of a whole organ instead of a single cell. Also, identification of specific sequences that impinge on a particular biological process will allow for the development of novel molecular therapeutics based on polysaccharide structure. Synthetic molecular mimics of HSGAG sequences may provide new agents to combat diseases such as atherosclerosis, thromboembolic disorders, and many others.

Three important advances, in addition to the newly devised sequencing approaches outlined above, make such a vision realizable. First, the recent cloning of many of the genes involved in the biosynthesis of HSGAGs and HSGAG proteoglycans promises to provide a basis for the dramatic increase in our appreciation of the role of HSGAGs in modulating fundamental biological processes, including atherosclerosis and tumorigenesis (Sugahara and Kitagawa 2000). Presently, transgenic animals are becoming available with defined genetic changes in HSGAG biosynthesis, including the absence of specific sulfotransferase genes (for instance, 2-O sulfotransferase or N-deactylase/N-sulfotransferase) or specific protein core sequences of proteoglycans (for instance, glypican, syndecan, or perlecan isoforms). Together, these transgenic animals should provide a solid foundation to further investigate the biological roles of specific HSGAG structural motifs in many biological processes. Second, recent advances in the development of array technologies will enable the rapid identification and sequencing of in vivo HSGAG structures that bind with high affinity to given proteins of therapeutic and/or biological importance (Keiser et al. 2001). Similar array technologies in proteomics have provided a key technology for the identification of proteins important in a biological process. Finally, significant advances have been made in the synthesis of HSGAG sequences and HSGAG mimetics, including the development of combinatorial libraries for the discovery and optimization of lead compounds (Petitou et al. 1999, Plante et al. 2001). Similar to the solid-phase synthesis of DNA and polypeptides that has enabled the biotechnology revolution, so should the development of rapid synthetic approaches to oligosaccharide generation enable the creation of new oligosaccharide-based therapeutics.

In short, a better understanding of the role of HSGAGs is likely to become very important for the rapid development and expansion of the field of glycomics, or the study of how proteins and saccharides interact with one another, thus impinging on cell physiology. Especially with the maturation of the field of genomics and the sequencing of the human genome, the next stage in obtaining a complete understanding of how genotype translates into phenotype is through the fields of proteomics and glycomics.

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