

Theme Issue Article

Heparin, heparan sulfate and heparanase in inflammatory reactions

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Summary

Heparan sulfate (HS) proteoglycans at the cell surface and in the extracellular matrix of most animal tissues are essential in development and homeostasis, and are implicated in disease processes. Emerging evidence demonstrates the important roles of HS in inflammatory reactions, particularly in the regulation of leukocyte extravasation. Heparin, a classical anticoagulant, exhibits anti-inflammatory effects in animal models and in the clinic, presumably through interference with the functions of HS,

as both polysaccharides share a high similarity in molecular structure. Apart of regulation during biosynthesis, the structures of HS and heparin are significantly modulated by heparanase, an endoglycosidase that is upregulated in a number of inflammatory conditions. Exploring the physiological roles of HS and heparin and the mode of heparanase action in modulating their functions during inflammation responses is of importance for future studies.

Keywords

Polysaccharide, proteoglycans, structure/function, chemokine, vascular endothelium, transmigration

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Heparin and heparan sulfate

Molecular properties and biosynthesis

Heparin and heparan sulfate (HS) are linear carbohydrate polymers that are expressed in the form of proteoglycans (PGs). A PG molecule is characterised as a glycoconjugate composed of a core protein to which the carbohydrate polymers are covalently attached. Whereas heparin and HS share great similarity in molecular structure, e.g. the polymer chains are composed of repeating disaccharide units of glucosamine (GlcN) and hexuronic acids (glucuronic or iduronic acid) that are sulfated at various positions (Fig. 1), the core proteins are not related and show distinct expression patterns.

Heparin is almost exclusively produced by connective tissue type mast cells as serglycin proteoglycan to which typically 10–15 sugar polymer chains are attached. The nascent heparin chain is averagely 60–100 kDa (1), together with the core protein, a heparin-PG molecule is approximately 750–1,000 kDa. The heparin product prepared from animal tissues generally does not contain serglycin, but is a mixture of saccharide fragments of

M_r 5–30 kDa, indicating degradation of the macromolecule heparin within mast cells.

In contrast to heparin, HS is ubiquitously found in essentially all tissues in the form of different types of proteoglycans (HSPG) present on the cell surface and in the extracellular matrix (ECM) and basement membranes. Two main types of cell-surface bound HSPG core proteins have been identified: the transmembrane syndecan with four isoforms, carrying HS near their extracellular tips and occasionally also chondroitin sulfate chains near the cell surface (2), and the glycosylphosphatidyl inositol-linked (GPI) glypican with six isoforms, carrying several HS side chains near the plasma membrane and often an additional chain near the tip of its ectodomain (3). Two major types of ECM bound HSPG are found: agrin, abundant in most basement membranes, primarily in the synaptic region (4); and perlecan, another dominant class of pericellular HSPG, with a widespread tissue distribution and a very complex modular structure as an important ECM component (5).

Heparin and HS share a common biosynthesis pathway occurring in the Golgi system. The process is initiated by formation

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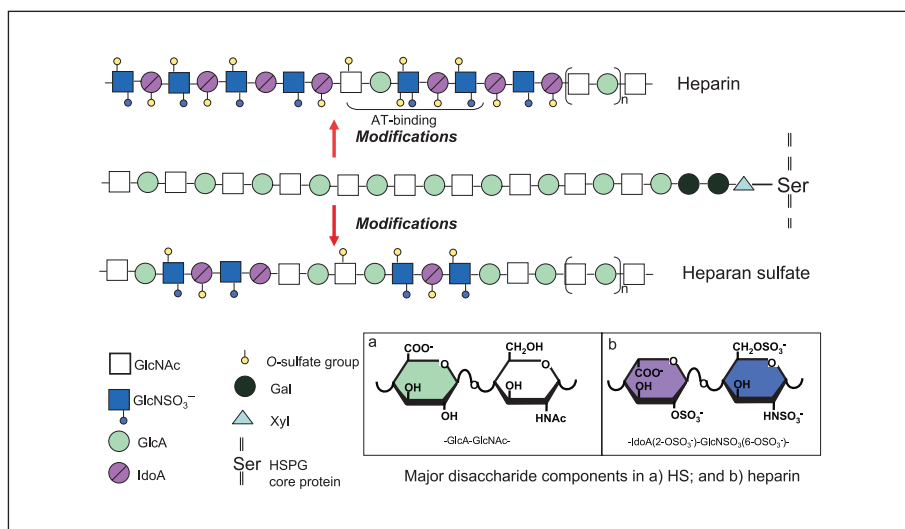


Figure 1: Simplified illustration of the biosynthesis of HS and heparin. The polymerisation of GlcNAc and GlcA is accompanied by modifications including *N*-deacetylation/*N*-sulfation of the GlcNAc, C5-epimerisation of GlcA to IdoA, O-sulfation of the IdoA at C2, and of GlcNS at C6 and C3. The pentasaccharide that specifically binds to antithrombin (AT) is indicated.

of a polysaccharide-protein linkage region, attaching four sugar units (glucuronyl-galactosyl-galactosyl-xylosyl) to a serine residue in the core protein (Fig. 1) (6). This tetrasaccharide sequence is extended by alternating addition of *D*-acetylglucosamine (GlcNAc) and *D*-glucuronic acid (GlcA) residues, forming repeating disaccharides of (GlcA β 1,4-GlcNAc α 1,4)_n. During this polymerisation process, the repeating disaccharide units are modified by a series of reactions in a sequential mode (7). The first modification is *N*-deacetylation/*N*-sulfation of GlcNAc units, followed by C5-epimerisation of GlcA to *L*-iduronic acid (IdoA) residues, and *O*-sulfation at C2 of IdoA and C6 of GlcNS residues. Rarely the sulfation occurs at C3 of GlcNS units (7), a hallmark modification for formation of the antithrombin (AT)-binding pentasaccharide sequence as outlined in Figure 1. The modification reactions in heparin biosynthesis are more 'extensive' or 'complete', resulting in a complex structure that is relatively 'homogenous'; while the modifications in HS biosynthesis tend to be more 'limited' or 'selective', resulting in a pattern that is far more heterogeneous. The tissue/cell specific structure of HS is believed to be a result of a strict regulation during biosynthesis (8, 9). It is currently assumed that these regulations are established to enable subtle and selective modulation of interactions between HS and various proteins (10, 11).

Functions of heparin and HS

Heparin, as a potent anticoagulant, has been used in the clinic for more than half a century and remains the major medicine for prevention and treatment of venous thromboembolism (12). The mechanism of the anticoagulation activity of heparin resides in the unique pentasaccharide sequence (indicated in Fig. 1) in the polymer, discovered almost 30 years ago (13–15). This pentasaccharide specifically binds antithrombin, a protease inhibitor that subsequently inhibits coagulation proteases. However, the fact that heparin is basically non-detectable in the blood raises a question regarding its physiological function as an anticoagulant. Although the exact biological functions of heparin are not fully uncovered, it is recognised that heparin is vital for storage of proteases in mast cells (16) and may regulate release of proteases that participate in inflammatory reactions (17).

HS was initially characterised as a by-product of heparin (18) and is known today as an indispensable molecule in development and homeostasis (2, 19, 20). Considerable biochemical and cellular studies demonstrate that HS, as a ubiquitous molecule, is implicated in a spectrum of biological activities, including affecting protein conformation (21), enhancing protein-protein interactions, acting as co-receptors for growth factors, and sequestration of protein ligands on cell surfaces and in the ECM (20). HS can also protect proteins from degradation (22), regulate protein transport through basement membranes (23) and mediate internalisation of proteins (24). The most direct evidences for the essential roles of HS are obtained through mutational studies of genes involved in the biosynthesis of HS in animal models. Complete elimination of HS production by disrupting one of the glycosyltransferase genes, *EXT1*, in mice ceased embryo development before gastrulation (25). By contrast, targeted disruption of genes encoding enzymes involved in the later stages of HS biosynthesis, for example hexuronic acid 2-*O*-sulfotransferase (*Hs2st*^{-/-}), glucosyl *N*-deacetylase/*N*-sulfotransferase-1 (*Ndst-1*^{-/-}) and GlcA C5-epimerase (*Hsepi*^{-/-}), resulted in strikingly varied phenotypes, albeit that all the mutant animals were neonatal lethal (26–28). Current research is aimed at increasing our understanding of the functional specificity of HS. Recently, an array of new approaches has begun to illuminate HS structure-function relationships, especially in relation to growth factor mediated signalling (29–31).

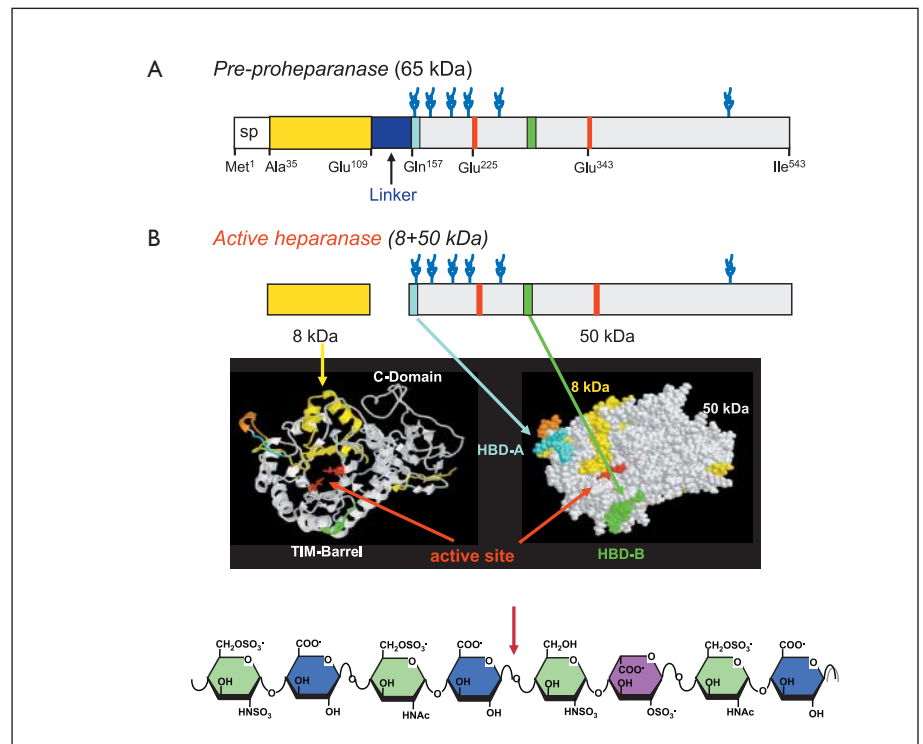
Heparanase

Activity and functional properties

The existence of mammalian endo-glycosidase was first evidenced in a mastocytoma system where the enzymatic activity was found to cleave heparin (32). The enzyme was characterised as an endo-glucuronidase that specifically cleaves the glyco-bond between GlcA and GlcN. It was believed that isoforms of heparanase might exist, however, simultaneous cloning of the same gene by several groups indicated that mammalian cells express primarily a single dominant functional heparanase

Figure 2: Mode of heparanase action.

A) Processing and activation of heparanase. The signal peptide (sp) and the linker segment are cleaved by proteases during processing; B) Predicted model of the 50 + 8 kDa heparanase heterodimer. The active site (E225 & E343; red), Tim-barrel, C-terminus domain (C-domain), and heparin/HS binding domains (HBD) site A (blue) and site B (green), are indicated; C) Cleavage of heparin/HS by heparanase (arrow).



(33–35). Although a protein that shares ~40% similarity with heparanase was reported as heparanase 2 (36), expression of the gene failed to yield endo-glycosidase activity (our unpublished data). Moreover, our recent success in knockout of the heparanase gene in mice has proved that there is only one gene encoding for heparanase with endo-glycuronidase activity (37).

Heparanase is produced as a latent protein that is processed at the N-terminus into a highly active enzyme composed of two polypeptides, 8 kDa and 50 kDa, in a form of heterodimer (Fig. 2) (38–40). The proteolytic processing mechanism, primarily by cathepsin L (41), may have critical regulatory effects on heparanase activity. Heparanase gene expression can be regulated by early growth response transcription factor (42), p53 (43) and inflammatory cytokines (44), as well as by hypoxia (45). The active form of heparanase has been found to be secreted from late endosomes/lysosomes after processing, and secretion was stimulated by extracellular cues activating the protein kinase A (PKA) and protein kinase C (PKC) signalling pathways (46). Soluble heparanase exhibits maximal endo-glycuronidase activity between pH 5.0 and 6.0 and is inactivated at pH higher than 8.5. Apart of its well-characterised enzymatic activity, heparanase was noted to exert also enzymatic independent functions (47).

Because of the universal presence and multifaceted roles of HS, its cleavage by heparanase directly affects essentially all the functions of HS. Notably, as a major component of the ECM, modulation of HSPG by heparanase is critical to the integrity and functional state of the ECM and basement membrane of blood vessel walls (48). Enzymatic degradation of HS is, therefore, involved in fundamental biological phenomena, ranging from morphogenesis, development and homeostasis to pathological conditions such as inflammation, amyloidosis, diabetic nephropathy, cancer metastasis and angiogenesis.

Transgenic animals

To explore the physiopathological implications of heparanase, we have established transgenic mice that either overexpress the human heparanase gene (49) or are null in expression of the endogenous gene (37). Interestingly, neither overexpression nor elimination of the enzyme in mice resulted in drastic phenotypes. Nevertheless, these mice have provided excellent models for investigating various HS-related biological/pathological processes. It was found that overexpression of heparanase can accelerate wound angiogenesis (50) and murine hair growth (51), and regulate the retention and proliferation of haematopoietic progenitor cells (52). In a systemic amyloidosis model, organs overexpressing high levels of the enzyme escaped amyloidosis upon induction (53), presumably due to extensive degradation of HS. The newly generated heparanase knockout mice produce non-degraded HS chains and display upregulated expression of certain matrix metalloproteinases (MMPs) (37). These properties may potentially affect physiological and pathological activities of the mice in response to specific challenges. Our ongoing research applying these animal models to study inflammatory related diseases is expected to shed light on the mode of action and involvement of heparanase in inflammatory reactions.

Implications in inflammation

HS in inflammation reactions

Inflammation is a reactive response of the body to harmful stimuli. This protective action of the body is primarily achieved by the increased movement of leukocytes from the blood into the injured tissues, followed by a cascade of biochemical reactions that propagate and mature the inflammatory response, involving the local vascular system, the immune system and various cells at

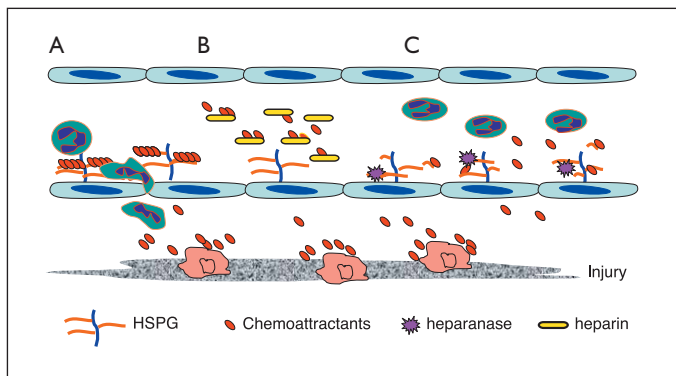


Figure 3: Implications of heparan sulfate, heparin and heparanase in inflammatory reactions. A) Functions of HS. HSPG on the endothelial surface congregates and presents chemoattractants, e.g. MIP2 and interleukin-8, to guide and promote leukocytes to crawl and transmigrate; B) Anti-inflammatory effect of heparin. Administered heparin competes with the endogenous HS on endothelial surface for interaction with chemoattractants, resulting in reduced adhesion and transmigration of leukocytes; C) Modulation effect of heparanase. Upregulated heparanase activity released by activated platelets and leukocytes results in extensive cleavage of cell surface HS that, consequently, loses its capacity to present chemoattractants to rolling leukocytes.

the injured sites. An essential step in the process is initiated by interaction of selectins on leukocytes with endothelial surface-associated HS, causing circulating cells to slow down and roll along the vessel wall (54). Then, HS on the endothelial surface activate leukocyte adhesion molecules, the integrins, thereby promoting strong adhesion to stop the rolling leukocyte and enable its transmigration out of the blood vessel (Fig. 3) (55, 56). Another important step of the process is activation of chemokines and interleukins and their presentation by HS on the endothelial surface for attracting the rolling leukocytes (Fig. 3). Recent *in vivo* studies, applying real-time and time lapse intravital video-microscopy to monitor inflamed microvessels (57), revealed that the adhered leukocytes often crawl transversely or even backwards towards the chemoattractant, indicating that the 'oriented' extravasation of leukocytes is chemoattractant dependent, that maybe modulated by the endothelial surface HS (our unpublished data).

A rich body of evidence shows that HS coordinates the inflammatory response at a number of levels in an inflammatory reaction. Biochemical experiments revealed interactions of HS with an array of inflammatory chemokines and cytokines, including microphage inflammatory protein (MIP)-1 α (58), RANTES (59) as well as interleukin (IL)-2 (60), IL-8 (61) and IL-10 (62). Moreover, HS also interacts with molecules expressed on cell surfaces, such as selectins (63, 64). HS seems to trigger integrins on the endothelial cell surface by immobilising chemokines (65). The ability of HS to interact with different types of chemokines is largely owed to the heterogeneous nature of the polysaccharide structure, so that the chemokines selectively bind to subsets of HS structures, raising the possibility that HS participates in determining the specificity of leukocyte recruitment *in vivo* (66).

In contrast to the affluent information obtained from *in vitro* experimentation, direct *in vivo* evidence for the exact functional roles of HS is circumstantial. It was reported that biliary-pancre-

atic infusion of HS in rats resulted in local inflammatory response visualised as rapid infiltration of neutrophils and macrophages into the pancreas (67). Notably, a recent study on a mouse model where one of the HS biosynthetic enzymes, NDST1, was selectively inactivated in either leukocytes or endothelial cells, provided the first direct *in vivo* evidence for the function of HS in leukocyte transmigration. The experiments clearly showed an impaired response of endothelial cells, but not of the leukocytes to inflammation stimuli, though both cell types expressed HS chains with reduced *N*- and *O*-sulfation (68). The results nicely show the dependence of L-selectin on HS structures expressed on the endothelial cell surface. These findings indicate that endothelial cell expression of HS is essential for multiple stages of leukocyte activity, while selectin may interact with a less sulfated HS expressed on the endothelial surface. Apart of direct involvement in leukocyte recruitment, HS has been shown to improve immune cell proliferative responses and to induce Th1 cytokine responses in healthy animals (69).

Anti-inflammatory effect of heparin

Besides its anticoagulant properties, heparin has an anti-inflammatory potential, as demonstrated in animal studies and clinical trials (70–73). Heparin and related compounds have been shown to benefit patients with bronchial asthma (74), ulcerative colitis (75) and skin injuries including burns (76). These effects of heparin may be a result of interfering with the binding of L- and P-selectin to HS (54). In fact, administration of heparin into inflamed microvessels of mouse attenuated leukocyte transmigration (our unpublished data). Although these clinical and experimental data convincingly demonstrate the anti-inflammatory effect of heparin, the drug has not been approved for application as an anti-inflammatory agent, mainly due to the potential risk of bleeding. Nevertheless, the accumulated information is of importance for illustrating the mechanisms underlying the observed effects, and is useful for the development of anti-inflammatory heparin mimetics.

Modulation of heparanase in inflammatory reactions

The finding that *Ndst1* deficient endothelial cells bearing low sulfated HS are defected in their response to inflammatory stimuli provides evidence that structural alterations (i.e. sulfation) in HS are critical for communication with leukocytes and chemokines (68). Conceivably, mice that are genetically deficient in other HS modifying enzymes and hence in HS structure (26–28) should be subjected to similar studies. Concomitantly, apart of HS synthesis enzymes, an important universal modulator of HS structure and function is heparanase, hypothesised to participate in inflammation through degradation of HS. First, upregulation of heparanase was reported in different inflammatory conditions. We have found that heparanase is expressed by the vascular endothelium at the site of inflammation, in degradation of the subendothelial basement membrane and subsequent vascular leakage – a hallmark of delayed type hypersensitivity (DTH) skin reactions (77). It was demonstrated that heparanase gene promoter activation occurs in the inflammation site upon the onset of a DTH response. Upregulation of heparanase has also been found in colonic epithelium of inflammatory bowel disease (IBD) (78). We found that heparanase expression was non-de-

tectable in specimens derived from normal colon tissues, whereas strong heparanase staining was observed in Crohn's disease and ulcerative colitis. Heparanase staining was primarily detected in epithelial rather than immune cells, indicating that heparanase levels are elevated under chronic inflammatory conditions. The stimulation of heparanase expression in the course of IBD and the resulting heparanase-mediated shedding of syndecan 1 (79) and degradation of its HS side chains represent an important mechanism underlying chronic inflammation of the colon and associated tumorigenesis. Moreover, heparanase activity was recently found to be dramatically elevated in synovial fluid from rheumatoid arthritis (RA) patients (80). The findings suggest an important role for heparanase in promoting joint destruction and RA progression, indicating heparanase as a reliable prognostic factor for RA progression and an attractive target for the treatment of RA (80).

Taking into account that heparanase is responsible for degrading both heparin (81) and HS (82), it is conceivable that degradation of macromolecular heparin in mast cells may modulate release of granule proteases that are involved in inflammation (17). On the other hand, increased heparanase activity in activated inflammatory cells may accelerate the turnover of HS production and thereby induce alterations in HS structure (i.e., over sulfation), as found in the liver of transgenic mice overexpressing heparanase. The increased sulfation of HS induced by up-regulated heparanase (83) may facilitate, for example, the interactions of HS with chemokines. Second, being a major component of the ECM, degradation of HS by heparanase can affect the architecture of the ECM and thereby promote release of chemokines anchored within the ECM network. Moreover, remodeling of the ECM may facilitate transmigration of inflammatory cells towards the injury sites.

Having little knowledge about the regulation of heparanase gene expression, the increased heparanase activity often seen in

inflammatory sites may be the result of induced gene expression and/or increased enzymatic activity. Further studies are needed to address the regulation of heparanase expression during diverse inflammatory diseases.

Conclusion

Facing the multiple functional roles of HS in inflammatory responses and the fact that heparin has indeed an anti-inflammatory effect, it is of urgent importance to elucidate the mode of action and regulatory role of HS in the inflammatory process. For example, it is important to find out whether HS is involved in transcytosis of chemokines (84), whether the fine structure of HS is regulated under inflammatory conditions, and how the chemokines are presented by cell surface HS. In line with the findings observed with *Ndst1* mutant cells (68), our ongoing experiments indicate that overexpression of heparanase in mice results in increased rolling rate of leukocytes activated by MIP-2, but reduced adhesion and transmigration of the cells (our unpublished data). Moreover, the leukocytes in heparanase overexpressing mice appear to crawl randomly, rather than towards the chemoattractant. These findings suggest that HS is critical in presenting the chemoattractant to the rolling leukocytes, consequently guiding the crawling direction. It is also of interest to investigate the effect of HS on heparanase processing and activation, and the effect of heparanase on the expression and bioavailability of inflammatory chemokines and cytokines. The extensive degradation of HS on endothelial cells that overexpress heparanase may result in reduced chemoattractant storage on the endothelial surface. This finding is supported by the reduced recruitment of neutrophils observed in heparanase overexpressing mice upon lipopolysaccharide stimulation (our unpublished data).

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