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Heparan sulfate proteoglycans in extravasation: assisting leukocyte guidance

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1. ABSTRACT

Heparan sulfate proteoglycans (HSPGs) are glycoconjugates that are implicated in various biological processes including development, inflammation and repair, which is based on their capacity to bind and present several proteins via their carbohydrate side chains (glycosaminoglycans; GAGs). Well-known HSPGs include the family of syndecans and glypicans, which are expressed on the plasma membrane and perlecan, agrin and collagen type XVIII, which are present in basement membranes. In this review, we provide an overview of the current knowledge on the role and regulation of HSPGs in leukocyte extravasation. In the non-inflamed endothelial glycocalyx HSPGs are anti-adhesive, and there are several indications that active regulation of HSPG core protein expression and/or GAG modification occurs upon inflammation. We address the current evidence for the role of HSPGs in leukocyte extravasation through interaction with the leukocyte adhesion molecule L-selectin, chemokines and other binding partners. Finally, a number of possibilities to use HSPGs as therapeutics or targets in anti-inflammatory strategies are discussed.

2. INTRODUCTION

Upon inflammation, leukocytes migrate from the bloodstream through the vessel wall towards the site of inflammation. The process of leukocyte transendothelial migration involves several sequentially acting molecules. The complexity of this system prevents inappropriate inflammatory responses, and enables combinatorial specificity (1). The multistep paradigm of leukocyte transmigration initially comprised leukocyte rolling (step 1), activation (step 2), firm adhesion (step 3) and transmigration (step 4) (2,3). However, the original paradigm has been expanded and modified based on new findings (1,4,5). The involvement of the family of heparan sulfate proteoglycans (HSPGs) at multiple levels in this process is becoming increasingly clear. The vast majority of functions ascribed to HSPGs depends on their ability to bind different cytokines, chemokines, growth factors and the leukocyte adhesion molecule L-selectin. This review describes our current knowledge of the role of HSPGs in leukocyte extravasation and trafficking, regulation of their expression and binding properties, and a number of possibilities to use HSPGs as therapeutic agents or targets in anti-inflammatory strategies.

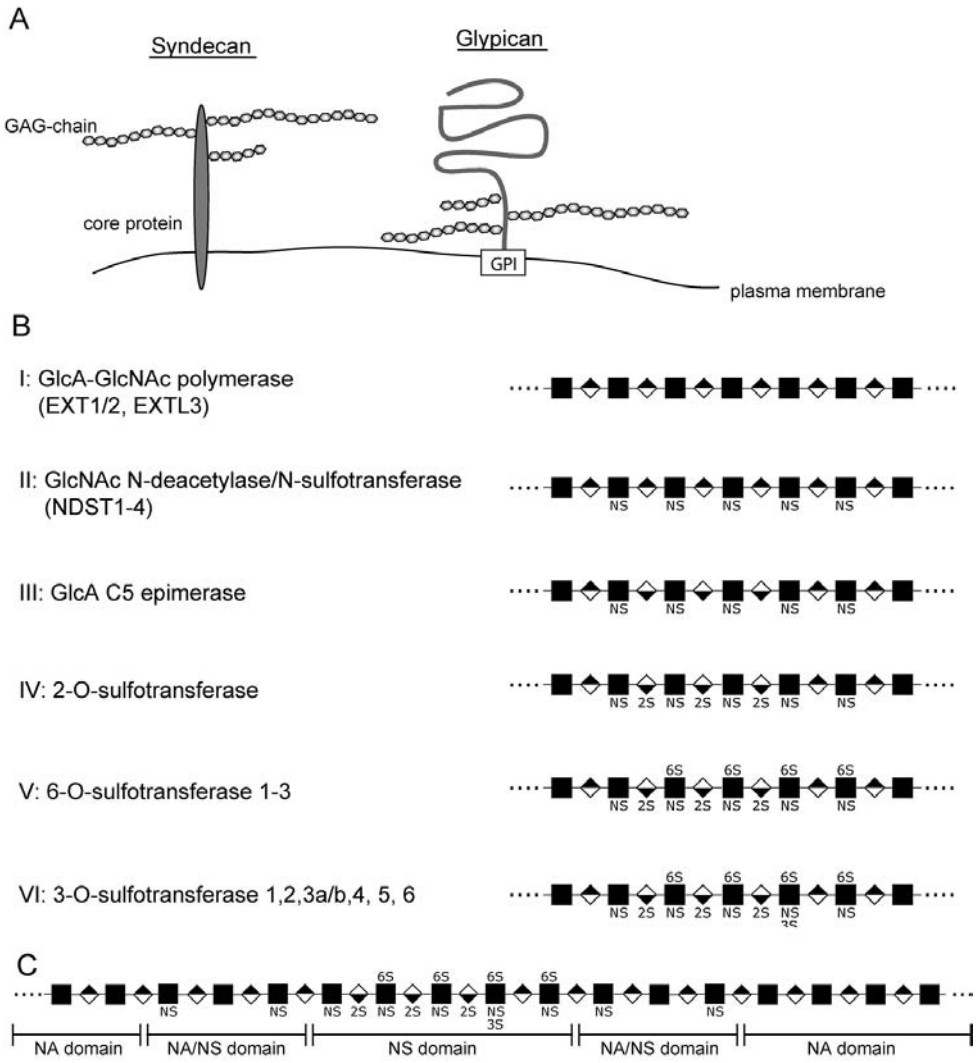

Figure 1. HSPG molecular structure and GAG-chain biosynthesis/modification. (A) HSPGs consist of a core protein with covalently linked GAG-chains. Cell surface HSPGs belong to the family of syndecans (transmembrane) or glypicans (GPI-linked). Apart from syndecans and glypicans, 'part-time' cell surface HSPGs include CD44v3 and betaglycan. (B) Schematic representation of HS synthesis/modification reactions and enzymes involved. *Filled square* = *N*-acetylglucosamine; *Top-filled diamond* = glucuronic acid; *Bottom-filled diamond* = iduronic acid; *Filled square with* 'NS' *annotation* = *N*-deacetylated, *N*-sulfated glucosamine; 2S =sulfate at C2 position, 3S= sulfate at C3 position, 6S= sulfate at C6 position (symbol nomenclature used as described at http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml). (C) The coordinated activity of these enzymes results in a typical domain organization, in which highly modified (NS) domains are flanked by moderately modified (NA/NS) domains, interspersed with stretches of unmodified (NA) domains. This sequence repeats a number of times throughout the HS-chain. Note that the enzyme reactions typically do not proceed to completion, resulting in a highly heterogeneous HS structure. Adapted from (16).

3. HSPG STRUCTURE, BIOSYNTHESIS AND PROTEIN BINDING PROPERTIES

Proteoglycans consist of a core protein to which extended linear carbohydrate chains (glycosaminoglycans; GAGs) are attached (Figure 1A). Based on the GAG-composition, proteoglycans are divided in different types, being heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), or keratan sulfate (KS) (6,7). Proteoglycans are not to be confused with N-linked glycoproteins, which bear relatively short, branched carbohydrate structures containing mannose, fucose and sialic acid residues.

HSPGs are expressed on the cell surface and in the extracellular matrix (ECM). Based on their core proteins, the majority of cell-surface HSPG belong to the family of syndecans or glypicans (Figure 1A). Four mammalian syndecans are known (syndecan-1 to -4), which are

transmembrane molecules with typically three to five HS-chains, although also CS/DS-chains can be attached to syndecan core proteins (8,9). Glypicans are linked to the cell surface by a glycosylphosphatidylinositol anchor, and six mammalian glypicans have been described (glypican-1 to -6) (10). Syndecan HS-chains are located distal from the plasma membrane, whereas glypican HS-chains are located close to the membrane (Figure 1A). Apart from syndecans and glypicans, also 'part-time' cell surface HSPGs are known, e.g. CD44 and betaglycan (11,12). ECM HSPGs include agrin, perlecan and collagen type XVIII, which are typically found in basement membranes (13,14). Another familiar molecule in this context is the pharmaceutical heparin, which is a highly sulfated free HS-chain that contains a high amount of iduronic acid. Heparin is only attached to a core protein (serglycin) inside the granules of its producing cell, the mast cell (15).

HSPGs are very heterogeneous molecules based on the large variation in their HS-chain composition. HS-chains are initially synthesized as alternating glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) residues (Figure 1B). These residues can subsequently be modified by the coordinated action of various enzymes, resulting in varying degrees of sulfation (*N*-sulfation and *O*-sulfation at various positions) and epimerization (converting GlcA to iduronic acid) (Figure 1B). Several isotypes have been identified for many HS modifying enzymes, adding to the complexity of the system. Theoretically, over 20 differently modified disaccharide units can be synthesized, and their combined diversity can result in highly heterogeneous HS-chains, which can all encode a different 'message'. Typically however, HS-chains are organized in highly modified domains with high *N*-sulfation (NS), *O*-sulfation and iduronic acid content, flanked by moderately modified (NA/NS) domains, interspersed with stretches of unmodified (NA) domains (Figure 1C). For further description of HS-biosynthesis we refer to excellent reviews on this topic (16-18).

As mentioned above, HSPGs are able to bind various cytokines, chemokines, growth factors and L-selectin through their HS-chains. Interestingly, different HSPG binding partners tend to require somewhat different GAG modification for binding (16,17,19). Important determinants for binding include degree and position of GAG-chain sulfation, epimerization, but also 3D-conformation (20,21). Therefore, differences in HS-chain fine-structure could affect the function of HSPGs and their binding partners in certain locations and under certain conditions, including inflammation.

4. HSPGS ON THE ENDOTHELIUM – STICKY OR NOT

4.1. Anti-adhesive HSPGs in the non-inflamed endothelial glycocalyx

Under non-inflamed conditions, the endothelial surface is covered with a relatively thick sheath of glycosylated molecules, referred to as the glycocalyx (22,23). HS is regarded to be the dominant GAG within the glycocalyx, although also CS/DSPGs and hyaluronic acid are present, and relative amounts can differ between different endothelial cell preparations (22). Together with sialylated glycoproteins, HSPGs are partly responsible for conferring the negative charge to the glycocalyx (Figure 2A) (22). In the absence of an inflammatory insult, the endothelial glycocalyx serves as an anti-thrombotic, anti-proliferative, and anti-inflammatory sheath. These actions are likely to depend both on masking of the underlying endothelial plasma membrane and its associated molecules, and on charge repulsion (both glycocalyx and leukocyte cell surface are negatively charged) (23). Upon inflammation, (part of) the glycocalyx can be shed, facilitating cell-cell interactions by reducing negative charge, and exposing underlying and newly synthesized molecules (Figure 2B) (22,24-27). Interest in the role of HSPGs in the endothelial glycocalyx has increased over the last few years and has been subject of several independent studies. Injection of heparitinase I (which specifically degrades HS) into mouse cremaster muscle venules increased the number of adherent leukocytes, indicating that HS in non-inflamed venules is anti-adhesive (28). Interestingly, Ox-LDL also induced degradation of the glycocalyx and increased adhesion of leukocytes (28). This effect could be inhibited by HS/heparin, which were bound to the luminal side of the endothelium, likely reflecting reconstitution into the degraded glycocalyx (28). These results suggest that changes in the glycocalyx, and its HSPGs, may play a role in atherothrombosis and -sclerosis (23,29). In addition, HSPGs were shown to function as endothelial sensors of blood flow (30,31), which may also play a role in atherosclerosis (local perturbations in blood flow) as well as other types of inflammation (e.g. reduced blood flow due to vasodilatation and endothelial swelling). In turn, recent studies show shear-dependent alterations in glycocalyx composition (23).

Although various studies show that the endothelial glycocalyx is (at least partly) shed upon inflammation, both shedding and increased exposure of one of its components, syndecan-1, has been reported in inflammatory settings (24,25,32). This apparent contradiction may be due to differences in glycocalyx composition in cultured cells compared to *in vivo*, inflammatory stimuli, and possibly vascular beds. It is however an important point, as the presence of a particular HSPG at the inflamed endothelium dictates whether it can be involved in leukocyte extravasation.

4.2. HSPGs on the inflamed endothelium: interaction with L-selectin

The first phase of transendothelial migration is defined as selectin-mediated 'rolling' of the leukocyte over activated endothelium. E- and P-selectin are expressed on activated endothelium (as well as on platelets for P-selectin), whereas L-selectin is constitutively expressed on leukocytes. All three selectins bind glycoproteins decorated with (sulfated) sialyl Lewis^x residues (33,34). In addition, binding of P- and especially L-selectin to HSPGs is now established, whereas binding of E-selectin to HSPGs remains controversial (35-40). Sulfation of HS is critical for both P- and L-selectin binding, and heparinoids inhibit inflammation by blocking P- and L-selectin activity

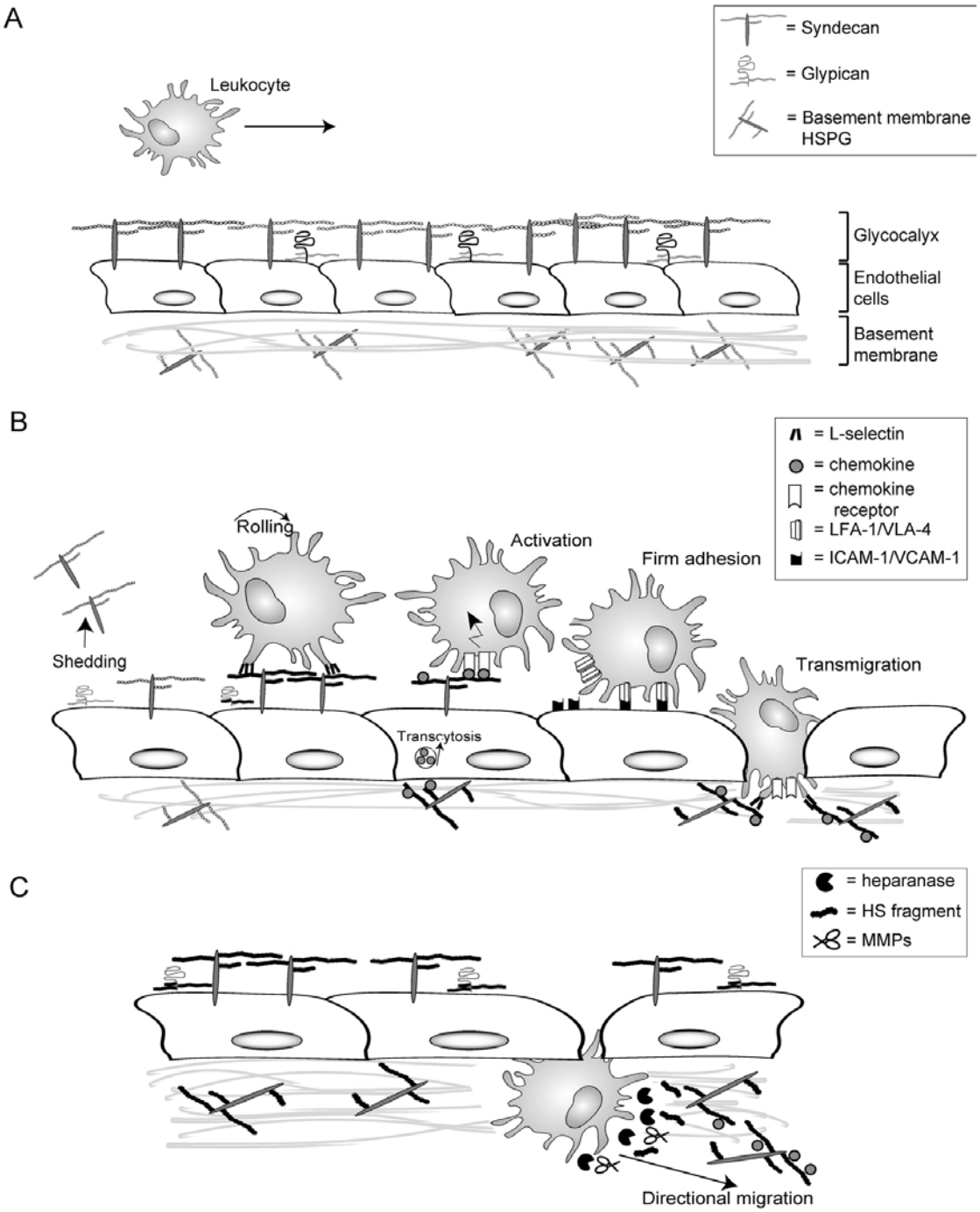

Figure 2. Inflammation can induce changes in vascular HSPGs facilitating leukocyte extravasation. (A) In the non-inflamed endothelium, HSPGs in the endothelial glycocalyx serve as anti-adhesive molecules, preventing leukocyte adhesion. (B) Upon inflammation, the anti-adhesive endothelial glycocalyx is at least partly shed, exposing underlying and newly synthesized molecules. Inflammation-induced changes in HSPGs (dark coloured GAGs) can affect leukocyte extravasation through interaction with L-selectin and chemokines. HSPGs able to bind L-selectin may be present at the endothelial cell surface, facilitating leukocyte rolling, and in the vascular basement membrane, enhancing leukocyte transmigration and possibly activation. Chemokines are transcytosed and presented by luminal HSPGs, which activates leukocytes and induces firm adhesion, and in the subendothelial matrix enhancing transmigration. (C) Leukocyte-derived enzymes can cleave of proteins (MMPs) and HS (heparanase) to facilitate extravasation. Heparanase acts at slightly lower than physiological pH and releases fragments that may still be able to bind chemokine. Local presentation of chemokines on HSPGs and/or HS fragments enhances directional migration.

(38,39,41). However, if endogenous HSPGs are involved in leukocyte transmigration *in vivo*, it should be possible to demonstrate P-selectin binding HSPGs on leukocytes (section 7) and/or L-selectin binding HSPGs on inflamed endothelium. Several groups have shown binding of recombinant L-selectin to HSPGs expressed by cultured endothelial cells intracellularly (42), associated with the cell-surface and/or ECM, or in the culture medium (43,44). Support for a functional role of the interaction between L-selectin and HSPGs is provided by Wang *et al.* who elegantly showed that reducing endothelial HS sulfation by inactivating *N*-deacetylase *N*-sulfotransferase-1 (NDST-1) impairs neutrophil extravasation (45). This could be partially explained by reduced binding of L-selectin to NDST-1-deficient endothelial cells *in vitro*, resulting in increased neutrophil rolling velocity and reduced firm adhesion. Similarly, leukocyte rolling and adhesion to endothelial cells of different origins is reduced after enzymatic removal of HS, or by antibodies that target *N*- and 6-*O*-sulfated HS domains (44,46,47). Interestingly, there are clear indications that HSPGs are not only involved in leukocyte-endothelial interactions, but also in adhesion of hematopoietic progenitor cells to bone marrow endothelial cells (48). Adhesion of progenitor cells was reduced when the bone marrow endothelial cells were cultured in the presence of chlorate, which inhibits GAG-chain sulfation, and by enzymatic removal of HS (48). Blocking studies indicated L-selectin to be one of the binding partners involved (48).

Although several studies describe a role for the interaction between L-selectin and endothelial HSPGs in leukocyte transmigration (Figure 2B), to our knowledge no single HSPG expressed on the endothelial cell surface has yet been identified as direct ligand for L-selectin. Endothelial syndecans have been proposed as likely candidates, although also glypicans, and the part-time HSPG CD44 can be considered (49-53). Examination of L-selectin ligands *in situ* using recombinant L-selectin proteins as probes has been performed, predominantly in various types of renal inflammation (54-56). In the non-inflamed kidney, L-selectin binding HSPGs are present in tubular basement membranes, but not associated with the endothelium (41,57-59). In both experimental and human renal inflammation tissues, L-selectin binding HSPGs are detected at the abluminal side of peritubular capillaries (but not at the endothelial surface), and these HSPGs are likely to be perlecan, collagen type XVIII and/or agrin (55,56). Mice deficient for perlecan-HS and collagen type XVIII showed reduced/delayed inflammation-induced monocyte influx, and the induction of perivascular L-selectin binding HSPGs in human renal biopsies correlates with increased leukocyte counts, indicating that these subendothelial HSPGs do contribute to leukocyte extravasation (55,56). Possibly, inflammation-induced endothelial damage exposes the vascular basement membrane components to leukocytes. Formally it cannot be excluded that L-selectin binding HSPGs are expressed on the luminal side of endothelial cells in these settings, as levels may be too low for detection or binding sites may be masked by plasma proteins adsorbed to HS. However, a recent study in mouse cremaster muscle venules, commonly used to study leukocyte rolling, has doubted whether L-selectin ligands are expressed at all at the luminal side of endothelial cells, as L-selectin coated beads did not significantly adhere to the endothelium but rather to adherent leukocytes (60). In this study, L-selectin mediated rolling was completely explained by secondary tethering (rolling of leukocytes over adherent leukocytes) (60). In parallel, wildtype and L-selectin knockout mice showed similar leukocyte rolling, although extravasation and extravascular locomotion was reduced in L-selectin knockout mice (61). An interesting possibility is that HSPGs do not (only) function as adhesive ligands for leukocytes via L-selectin, but may (also) activate transmigrating leukocytes via the same molecule (Figure 2B). L-selectin cross-linking was shown to activate leukocytes (62,63), and the repetitive HS domain structure could be a likely candidate to cause L-selectin cross-linking.

Summarizing, there is compelling evidence indicating that an interaction between L-selectin and vascular HSPGs enhances leukocyte extravasation, although the exact mechanism deserves additional research.

4.3. Other adhesive interactions with endothelial HSPGs

Apart from L-selectin, other leukocyte adhesion molecules have been shown to interact with HSPGs, thereby potentially affecting extravasation. Especially the interaction between HSPGs and integrins is interesting in this respect. Binding of leukocyte-expressed integrin Mac-1 (CD11b/CD18) to HS has been demonstrated years ago, and this interaction was shown to cause firm adhesion after initial rolling over P/E-selectin under flow (64,65). Also binding of HSPGs to other adhesion molecules, including CD45 and CD31 (PECAM-1) has been reported, although the functional relevance of these interactions is unclear (65-67). Interestingly, mammalian heparanase, which is mainly known to degrade HS at low pH (section 6), can in its inactive form also induce T-cell adhesion under shear flow conditions, although the exact mechanism remains to be determined (68).

5. HSPGS AND CHEMOKINES: SELECTIVE PRESENTATION

5.1. HSPGs present chemokines to facilitate directional migration

In the second step of transmigration, binding of chemokines to high-affinity receptors on leukocytes causes integrin activation and firm adhesion (69). Both cell-surface and extracellular matrix HSPGs can bind chemokines via their HS-chains (Figure 2B). In this way, chemokines are retained locally, prevented from degradation (70), and presented to leukocytes. In addition, chemokines bound to HSPGs may serve as local storage depots, or could be functionally scavenged away (69). Interestingly, both chemokines and HS show a certain amount of specificity in their interaction. Different chemokines use different protein domains for their binding to HS, which may confer HS-chain selectivity, and has even been suggested to potentially control chemokine

receptor selectivity (71,72). HS-chains in turn have different sulfation patterns favoring binding of certain chemokines over others, which may help attract different leukocyte subsets (73). This mutual selectivity can offer another level of regulation of chemokine function, which is thereby determined by cells producing the chemokine as well as their environment (74).

The notion that chemokine binding to GAGs is important for leukocyte extravasation has been elegantly demonstrated using GAG-binding mutant chemokines, which can bind and activate their high-affinity receptors, but have mutated GAG-binding domains. For example, monocyte transmigration was significantly reduced using GAG-binding mutant CCL5/RANTES compared to the wildtype chemokine (75). Similarly, experiments using GAG-binding mutant CCL2/MCP-1 and CCL4/MIP-1beta show a reduction in leukocyte transendothelial migration *in vitro*, and leukocyte emigration towards the peritoneal cavity *in vivo* (75-77).

5.2. Chemokine presentation by HSPGs at the endothelial surface

Chemokines are considered to bind to luminal endothelial cell proteoglycans to prevent them from being washed away by blood flow and to enhance clustered presentation (78-80). A number of studies have shown HS-mediated binding of chemokines to the luminal surface of (activated) endothelial cells (81-83). Dermally injected CXCL8/IL-8 was shown to localize to postcapillary venules and small veins, where it is internalized abluminally by endothelial cells, transcytosed to the luminal surface, and presented on endothelial cell projections (83). Importantly, the heparin binding domain of CXCL8/IL-8 is necessary for binding and transcytosis, and *in vivo* activity of the chemokine (83). In line with these findings, binding and transcytosis of CXCL8/IL-8 was significantly impaired in endothelial cells with reduced HS sulfation due to NDST-1 inactivation (45). Hardi *et al.* showed that exogenously added CCL2/MCP-1 is presented apically on endothelial-like cells in a clustered fashion, although HS was present all over the cell surface, indicating preferential binding to a certain HSPG or HS-domain (82). This again stresses that endothelial cells may not necessarily bind a particular chemokine even though they express HSPGs, based on HS-chain dependent selectivity (55,73).

Chemokine binding at the endothelial lumen is generally considered to be mediated by syndecans, although only a limited number of studies show an actual interaction between syndecans and chemokines. Syndecan-1 and/or -2 derived from human umbilical vein endothelial cells were shown to bind CXCL8/IL-8 (32,84). Syndecan-3 was proposed as the dominant HSPG to bind the same chemokine on inflamed synovial endothelium (85). As HSPG core protein expression and HS-chain modification may be different in various vascular beds, it would be interesting to examine more specifically which endothelial HSPGs bind and present which chemokines under inflammatory conditions *in situ* (e.g. in tissue sections) (80).

Apart from presentation of chemokines at the endothelial lumen, it has been suggested that (HS)PG-generated concentration gradients across the endothelium could be important for leukocyte extravasation (79). To our knowledge, a concentration gradient of chemokine in between endothelial cells has not been demonstrated (82). It seems more likely that the presence of immobilized chemokine on one side of the leukocyte would be sufficient to polarize the leukocyte and thereby direct migration (86).

6. BEYOND THE ENDOTHELIUM: HSPGS IN THE VASCULAR BASEMENT MEMBRANE

After passing the endothelial cell layer, extravasating leukocytes encounter the vascular basement membrane. This membrane consists of a dense network of ECM proteins, providing stability to the vascular structure. Leukocyte migration through this barrier is considered to involve local degradation of matrix molecules by specific enzymes, including matrix metalloproteinases (MMPs) and ectoenzymes (87), although specific matrix protein low expression regions have also been identified as preferential exit points for neutrophils (1,88,89).

HSPGs are vascular basement membrane components that help provide structural stability (14). In addition and as described above, subendothelial HSPGs can bind chemokines and L-selectin under inflammatory conditions (55,90), which may enhance extravasation, as well as directed migration by the formation of a haptotactic gradient (91). Cleavage of HSPGs is considered to be necessary for leukocyte migration through the subendothelial matrix (Figure 2C). Leukocytes, endothelial cells and platelets express the HS-specific endoglycosidase heparanase (92-96). Heparanase acts at pH 6 but not at physiologic pH, is resistant to protease activity, and is relocated to the leading edge of migrating leukocytes, supporting its role in HS degradation at the site of inflammation (92,95,97-99). This enzyme specifically cleaves HS-chains within a sequence that has not been defined completely yet, resulting in HS fragments of 10-20 carbohydrate units that retain biological activity (96,98,100). Inhibition of heparanase activity by a polysulfated polysaccharide was shown to inhibit experimental autoimmune encephalitis, although this compound likely interfered with other processes involved in leukocyte extravasation apart from heparanase activity alone (101). The role of heparanase in extravasation has been demonstrated more specifically by Edovitsky *et al*, who locally administrated siRNA to target endothelial heparanase, resulting in decreased delayed-type hypersensitivity inflammatory response *in vivo* (102). Apart from heparanase, leukocytes can also produce reactive oxygen species (ROS), which can cause HS depolymerization (103-105). However, to our knowledge the importance of ROS-induced HS degradation in leukocyte extravasation has not been shown to date.

Degradation of subendothelial HS could both loosen the matrix to facilitate leukocyte migration, and release locally bound cytokines and chemokines, which can

affect cell migration and/or activation (106). Interestingly, fragments of several extracellular matrix proteins have also been shown to activate immune cells directly via interaction with toll like receptors (TLRs), which may function to monitor tissue damage. For example, soluble HS can bind TLR-4 on dendritic cells, which results in dendritic cell maturation (7,107). In addition, HS/heparin di/trisaccharides have been shown to affect T-cell intracellular signaling, adhesion and migration, as well as macrophage function (108-111). The combined roles of ECM HSPGs could thereby significantly influence the inflammatory response, both at the level of cell migration and activation.

7. HSPGS ON LEUKOCYTES

Apart from the endothelium, also leukocytes express HSPGs (112-117). Leukocyte HSPGs may influence transendothelial migration by interacting with P-selectin, or by presenting chemokines to their high affinity receptor in a trimolecular complex on the leukocyte cell surface (*in cis)*. However, no leukocyte-expressed HSPG that binds P-selectin has been reported to date. In addition, GAG-binding mutant chemokines which lack GAG-binding capacity can still efficiently signal through their chemokine receptor and induce chemotaxis in a free diffusion solute gradient, suggesting that presentation of chemokines by HSPGs *in cis* is not of major importance (76,118,119). However, there are studies indicating that binding of chemokines to cell surface HSPGs could directly result in intracellular signaling, independent of G-protein coupled chemokine receptors, as shown for CCL5/RANTES and CD44 (120,121). In addition, T-cell adhesion and migration was shown to be triggered by binding of cyclophilin B to syndecan-1 HS, which enhances syndecan-1 association with CD147 and subsequent intracellular signalling (117,122,123).

In vitro, transendothelial migration was reduced when monocytes were cultured in the presence of chlorate (inhibiting GAG sulfation) or upon treatment with heparitinase (specifically degrading HS) (124). Evidence for a role of leukocyte HSPGs in extravasation *in vivo* has come from syndecan-1 deficient mice, which display increased leukocyte-endothelial adhesion in the ocular vasculature (125). This appeared to contradict the proposed role of HSPGs (including syndecan-1) in enhancing leukocyte rolling and adhesion, but the effect was shown to be mostly due to the lack of syndecan-1 on leukocytes (125,126). Similarly, leukocyte recruitment was increased in syndecan-1 deficient mice in both experimental anti-glomerular basement membrane nephritis, and myocardial infarction (127,128). In the latter, systemic adenoviral overexpression of syndecan-1 reduced the number of inflammatory cells in the infarct area (128). Together, these studies indicate that leukocyte-expressed syndecan-1 can inhibit extravasation, possibly by scavenging chemokines, masking or competing for interactions with adhesion molecules, or simply by increasing cellular negative charge, thereby inhibiting cell-cell interactions. However, the observation that leukocyte extravasation was not affected by inactivation of NDST-1 in leukocytes indicates that leukocyte HS sulfation is of lesser importance for leukocyte extravasation, although it may play a role in T-cell activation (45,129). An alternative explanation for the increased adhesion of syndecan-1 deficient leukocytes could be that the lack of syndecan-1 affects leukocyte adhesion by influencing integrin activation, as functional cooperation between syndecans and integrins *in cis* (on the same cell) has been shown to enhance cell adhesion due to cross-talk in intracellular signaling pathways (130).

8. REGULATION OF HSPGS UPON INFLAMMATION

8.1. Regulation of core protein expression

As HSPGs are constitutively present on the cell surface and in the ECM, regulation of expression and/or binding capacities (section 8.2) of these molecules upon inflammation seems likely (data described below are summarized in Figure 3). Regulation of syndecan core protein expression has been studied most extensively (49,131). *In vitro*, upregulation of syndecan-1, -2 and –4, glypican-1 and the part-time HSPG CD44 has been shown on cultured endothelial cells stimulated with pro-inflammatory cytokines TNF-alpha and/or IL-1beta (47,84,132). *In vivo,* syndecan-1 expression is induced in endothelial cells that revascularize healing wounds and in myocard infarct areas (128,133,134). In addition, endothelial syndecan-3 staining was increased, whereas syndecan-2 and glypican-1 and –4 staining was detected but not different, in inflamed compared to non-inflamed synovium (51,85).

Several studies indicate that syndecan core protein expression can be regulated by the pro-inflammatory NF-kappaB pathway (132,135). In addition, the syndecan-1 gene contains an FGF-inducible response element upstream from the promotor region, which appears to act as an enhancer of transcription (136-138). This clearly implicates regulation of syndecan-1 in inflammation and repair. Interestingly, several HSPGs also have putative target regions for microRNAs ((139) and Celie, unpublished observation (Sanger microRNA database (http://microrna.sanger.ac.uk)), which are small non-coding RNA sequences that can regulate protein expression by inhibiting mRNA translation (140).

In addition to regulation of transcription/translation, syndecans are susceptible to ectodomain shedding, which can affect their function. For example, human umbilical vein endothelial cells constitutively shed syndecan-1/IL-8 complexes under the influence of plasmin under culture conditions (32). This shedding can be inhibited by endothelial PAI-1, which appears to stabilize the syndecan-1/IL8 complex at the cell surface, facilitating neutrophil transmigration (32). Thrombin and EGF were shown to enhance endothelial syndecan-1 and -4 shedding (141). In contrast to stabilizing chemokine/HSPG complexes on the endothelial surface to enhance transmigration, MMP-7 induced shedding of syndecan-1/KC complexes directs transepithelial efflux of neutrophils in the inflamed lung (142). Interestingly, heparanase was also shown to induce syndecan-1 shedding

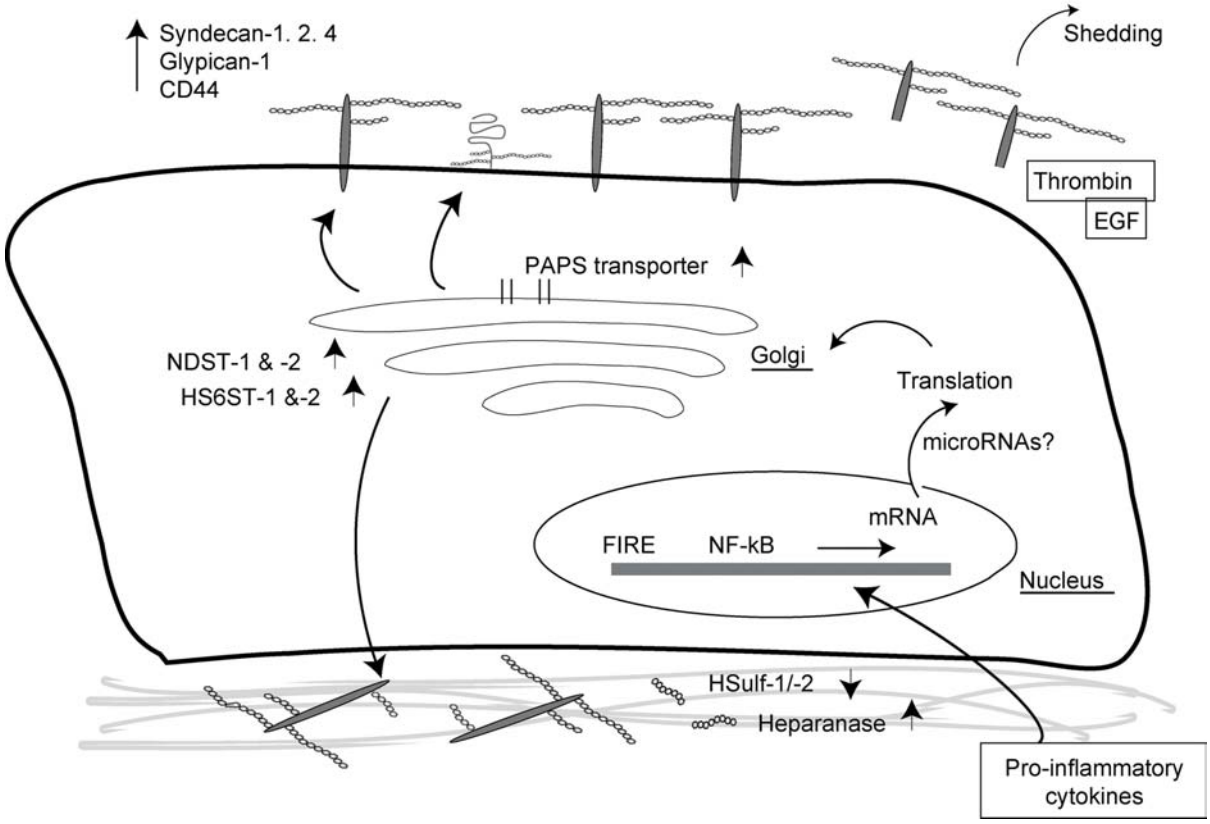

Figure 3. Model for regulation of endothelial HSPG core protein expression and HS GAG modification upon inflammation. Pro-inflammatory cytokines can alter the expression of HSPG core proteins and HS modifying enzymes (located in the Golgi) through activation of NF-κB. Increased expression of PAPS transporter can provide sulfate donors required for increased HS sulfation. Extracellularly, reduced expression of HSulf-1/-2 and increased expression of heparanase results in highly sulfated HS fragments. Thrombin and EGF are known to induce syndecan shedding. FIRE= FGF-inducible response element. See text for further details.

in multiple myeloma and breast cancer cells, and silencing of heparanase gene expression down-regulated syndecan-1 mRNA (143,144). Future studies will further clarify the regulatory pathways involved in HSPG core protein expression upon inflammation.

8.2. Regulation of HS biosynthesis & modification; changes that affect chemokine/L-selectin binding

Regulation of HS biosynthesis and modification can have important effects on HSPG binding capacity, including binding to chemokines and L-selectin. GAG-chain sulfation is an important determinant for both, and there is evidence showing that HS sulfation is altered upon inflammation. NDST-1 and -2 mRNA levels were transiently decreased in microvascular endothelial cells at 4 hours of IFN-gamma or TNF-alpha stimulation, followed by an increase in NDST-1 expression at 16 hours (81). Binding of CCL5/RANTES to the endothelial cells, and transendothelial leukocyte migration paralleled the increase in NDST-1 expression (81). Similarly, TNF-α stimulation was shown to lead to an NF-kappaB-dependent increase in NDST-1 and -2, and 6-*O*-sulfotransferase-1 and -2 mRNA in glomerular endothelial cells (47). In the same cells expression of endo-6-*O*-sulfatase-2 (Sulf2) was decreased, whereas heparanase was increased, suggesting a shift towards more *N*- and 6-*O*-sulfated HS fragments (47). Increased HS-dependent binding of L-selectin and chemokines to endothelial cells has been shown upon stimulation *in vitro*, or when cells are derived from inflamed tissue (44,145). As described above, inflammation-induced L-selectin and chemokine binding to subendothelial HSPGs has been shown *in situ* in synovial tissue and the kidney (55,85,90). This may be at least partially due to modification of HS fine-structure, as no changes in core protein expression could be detected (55). Indeed, increased HS-mediated subendothelial binding of L-selectin and CCL2/MCP-1 coincided with reduced endothelial expression of the extracellular 6-*O*-sulfatase HSulf-1in acute renal allograft rejection (55). Although changes in expression of HSulf-2 were not detected in this study, this enzyme also has the potential to affect chemokine binding (146). Interestingly, mRNA for PAPS transporter, which transports sulfate-donors into the Golgi for use by various sulfotransferases, is increased in capillaries in both experimental heart and kidney transplantation-induced acute inflammation (147). Apart from inflammation, HSPGs have also been implicated in transendothelial migration of stem cells to the bone

Table 1. HSPGs as therapeutics or targets in anti-inflammatory strategies; various HS-like or HS-targeting compounds with anti-inflammatory effect in indicated settings

	Compounds	Setting
HS (PGs) as therapeutics	Heparin, low molecular weight heparin	Cerebral ischemia (38), peritonitis (39,155,172), delayed type hypersensitivity (39), meningitis (173), allergic encephalomyelitis (174), airway (allergic) inflammation (175-179), hepatic ischemia/reperfusion (180), cutaneous inflammation (178,181), myocarditis (182), renal ischemia/reperfusion (183,184), renal allograft rejection (183,185)
	Synthetic heparin mimetics	Renal ischemia/reperfusion (186,187)
	Small HS-mimetics	Untested (159)
HSPGs as targets	Anti-HS antibodies	*In vitro* leukocyte rolling & adhesion (47)
	GAG-binding deficient chemokines	Thioglycollate-induced peritonitis, allergic airway inflammation, allergic encephalomyelitis (77,188)
	Small inactive chemokine fragments	Untested (164)

marrow, via presentation of the stem cell chemokine stromal derived factor-1 and adhesive interaction with L-selectin (48,91,148). Interestingly, bone marrow endothelial cells were shown to express more highly sulfated HS than human umbilical vein endothelial cells, favoring both chemokine and L-selectin binding (149). Studies into the transcriptional regulation of HS biosynthetic enzymes involved, and resulting GAG-chain properties, would be interesting to gain more insight into the formation of these apparently constitutive 'extravasation-enhancing' HS-types.

In the recent years, interest has focused on the notion that not only HS sulfation, but also the resulting 3D-conformation of HS-chains, spatially positioning certain sulfate groups in more or less flexible regions of the GAG-chain, is important in determining binding activity and specificity (20,150,151). In addition, environmental factors, including pH and the presence of particular cations (including Zn^{2+} contained in platelets), can contribute to HS-chain conformation and/or binding properties, making the situation even more interesting, but also even more complex (21,151).

9. HSPGS AS THERAPEUTICS OR TARGETS IN ANTI-INFLAMMATORY STRATEGIES

Based on the variety of functions of HSPGs, these molecules could be interesting therapeutic agents, or possibly targets, in anti-inflammatory strategies (Table 1). This would be especially relevant in chronic inflammation and auto-immune diseases (e.g. artherosclerosis, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis). Other situations in which HSPGs are considered as therapeutic agents or targets include oncology, especially focusing on the potential of inhibiting growth factor signaling, angiogenesis and heparanase-induced degradation of ECM by tumor cells, which precedes metastasis (152-154). Heparin has been shown to bind and inhibit several molecules involved in leukocyte extravasation, including selectins, chemokines, leukocyte integrins, and heparanase (36,39,40,46,64,96,99,155), which can likely explain the anti-inflammatory effects observed after (low molecular weight) heparin administration in various models (Table 1). Interestingly, also tumor cells have been shown to exploit integrin- and selectin-mediated adhesion during metastasis (156-158). Nowadays, interest is focusing on the possibility to produce small HS-mimetics, which may more specifically target a particular component of HS/heparin bioactivity (154,159). For this, it will be necessary to obtain pure, homogeneous oligosaccharide preparations, which can probably only be achieved by chemical synthesis. This approach has proven particularly challenging, but significant progress has been made in the last years, for example by the generation of heparin-glycan arrays to study heparin-protein interactions and the production and clinical use of Fondaparinux (synthetic heparin-like pentasaccharide) (73,160-163). Factors known to potentially influence the biological activity of these compounds, including multivalent linkage and 3D conformation, may be taken into consideration to find a balance between biostability, activity and specificity.

The use of HSPGs as targets, for example using antibodies that recognize and thereby block specific HS-motifs/domains, may also have clinical potential. This strategy has been exemplified *in vitro* by the demonstration that 6-*O*-sulfate specific anti-HS antibodies produced in a phage-display library can inhibit leukocyte rolling and firm adhesion to glomerular endothelial cells, whereas anti-HS antibodies with different specificities do not (47). GAG-binding deficient chemokines can be used to specifically inhibit cell migration (164). In addition, small inactive chemokine fragments can be generated that block the HSPG-binding sites of their *in vivo* active counterparts (164). Together, there are many options for the use of HSPGs in therapeutic strategies, although many need further development and proof of efficacy *in vivo*.

10. CONCLUSIONS & PERSPECTIVES

In this review, we have described the current knowledge regarding the role and regulation of HSPGs in different steps of leukocyte extravasation. There are clear indications to show that in the non-inflamed endothelial glycocalyx HSPGs have anti-adhesive properties, whereas endothelial HSPGs can promote leukocyte extravasation upon inflammation through their interaction with different proteins, including L-selectin and chemokines. This may be explained by the observation that upon inflammation (part of) the anti-adhesive endothelial glycocalyx is shed. Theoretically, it is possible that pro-adhesive HSPGs are continuously expressed but masked by the glycocalyx, although this seems unlikely as the cellular machinery for HS synthesis and modification is considered to be shared by all HSPGs expressed by a certain cell, which should

result in similar HS profiles. Accumulating evidence shows inflammation-induced changes at the level of core protein synthesis, but importantly also of HS-chain modification, which may account for the change in HSPG properties from anti-adhesive to pro-inflammatory. Possibly, changes in HSPGs over time can help define and contain the inflammatory response, affecting the type of leukocyte that extravasates (71,72), the vascular bed through which extravasation occurs (165), and the transition from acute to chronic (or resolution of) inflammation. Interestingly, chemokine-induced leukocyte migration can be inhibited by the family Slit-proteins, and these proteins have been shown to bind HS (166-169). Emerging *in vivo* experiments show that Slit-proteins can inhibit leukocyte infiltration in various inflammatory models (170,171). Further research may direct at these topics, e.g. specifically examining HSPG binding properties or HS modification at different timepoints and under different inflammatory conditions. In addition, it would be interesting to examine whether HSPG subsets are located in membrane domains, where they may function to enhance the interaction between endothelial cell and leukocyte. A remaining challenge in this context is to prove causality between changes in expression of HSPG core proteins/modifying enzymes based on the high potential for redundancy (multiple core proteins as well as modifying enzymes) and post-translational regulation (enzyme activity, shedding of core proteins). In addition, HSPGs are known to be important in development and structural stability of tissues, and therefore tissue-specific and/or inducible knockout animals for different core proteins and/or modifying enzymes may significantly contribute to our understanding of the role of HSPGs in specific settings.

Interestingly, HSPG binding properties may not only play a role at the endothelial surface but also in the underlying vascular basement membrane, affecting directional cell migration and activation. Degradation of HS by heparanase can both allow cell migration through this barrier and release bioactive HS fragments. The ability to interfere specifically with any of the above-mentioned roles of HSPGs may have significant therapeutic implications, and considerable effort is being invested in this direction. Typically, HS modifying reactions do not go to completion *in vivo*, and it would be interesting to consider the existence of inhibitors of the modifying enzymes *in vivo*, either at the transcriptional or translational level, or the development of compounds that block or skew away from a particular (undesired) reaction.

In conclusion, there is compelling evidence that HSPGs play an important role in leukocyte extravasation. Future studies of the mechanisms, regulation and specificity of HSPG-mediated interactions in different inflammatory reponses will further increase our understanding of these intricate but intriguing molecules, and can open important new therapeutic possibilities.

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Abbreviations: HSPGs: heparan sulfate proteoglycans; GAGs: glycosaminoglycans; HS: heparan sulfate; CS: chondroitin sulfate; DS: dermatan sulfate; KS: keratan sulfate; ECM: extracellular matrix; NDST-1: *N*-deacetylase *N*-sulfotransferase-1; MMPs: matrix metalloproteinases; TLRs: Toll-like receptors

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