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Glycosaminoglycans and infection

Rafael S. Aquino[1], Pyong Woo Park[1,2]

[1]Division of Respiratory Diseases, [2]Division of Newborn Medicine, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

TABLE OF CONTENTS

1. ABSTRACT

Glycosaminoglycans (GAGs) are complex linear polysaccharides expressed in intracellular compartments, at the cell surface, and in the extracellular environment where they interact with various molecules to regulate many cellular processes implicated in health and disease. Subversion of GAGs is a pathogenic strategy shared by a wide variety of microbial pathogens, including viruses, bacteria, parasites, and fungi. Pathogens use GAGs at virtually every major portals of entry to promote their attachment and invasion of host cells, movement from one cell to another, and to protect themselves from immune attack. Pathogens co-opt fundamental activities of GAGs to accomplish these tasks. This ingenious strategy to subvert essential activities of GAGs likely prevented host organisms from deleting or inactivating these mechanisms during their evolution. The goal of this review is to provide a mechanistic overview of our current understanding of how microbes subvert GAGs at major steps of pathogenesis, using select GAG-pathogen interactions as representative examples.

2. INTRODUCTION

In the last century, environmental, prophylactic, and therapeutic interventions have significantly reduced the incidence, morbidity and mortality of infectious diseases. However, infection continues to be a major public health threat. A recent analysis of causes of global mortality indicates that diseases of infectious origin killed approximately 11.6. million people in 2010, accounting for 22% of all deaths, with lower respiratory infections, AIDS, infectious diarrheal diseases, and tuberculosis ranked as the number 4, 6, 7, and 10 causes of death (1). Infections can significantly damage tissue structure and function and dysregulate the host immune and tissue repair responses, resulting in organ dysfunction and failure, systemic shock, or secondary chronic diseases. The emergence of new pathogens (*e.g.,* Ebola virus, SARS, avian influenza), the re-emergence of pathogens thought to be under control (*e.g.,* Dengue virus), resurgence of old pathogens (*e.g., Vibrio cholera*e), and the emergence of drug-resistant strains (*e.g.,* multi-drug resistant tuberculosis, MRSA) are adding to the threat of infectious diseases. Microbial pathogens perform various functions to promote their growth and survival in the host environment. Infection begins with microbial colonization of host tissues, but colonization by most microbes does not lead to infection. In fact, many microbes are supportive of host well-being and survival. Infection occurs when several pathogenic microbes breach the protective barriers of the host, enter the body, multiply, damage cells, and disrupt normal tissue functions. Fortunately, even after pathogens penetrate the protective borders, disease usually happens in only a small proportion of infected people because the host rapidly mounts an effective defense mechanism to eradicate them. However, several microbes have either adapted or evolved clever strategies to shift the balance of host-pathogen interactions to favor pathogenesis.

The outcome of a microbial infection is largely governed by the ability of pathogens to subvert

host components and their activities. Among the host components, glycosaminoglycans (GAGs) are apparently prime targets of pathogens. Early work from the 1960's studying infection of primary human amnion cells by herpes simplex virus (HSV) unexpectedly showed that human blood inhibits infection (2). Analyses of different blood samples showed that HSV infection is inhibited by heparinized blood but not by those anticoagulated with EDTA or citrate, suggesting that heparin (HP) was responsible for the anti-infective activity. The anti-infective effect of HP was later confirmed *in vivo* in a rabbit model of skin infection (3). Later studies in the 1970's examining the anti-infective effects of HP showed that this highly sulfated GAG also inhibits the initial attachment of other pathogens to host cells, such as *Neisseria meningitides* (4) and *Chlamydia trachomatis* (5).

These early studies with HP clearly suggested that GAGs play an important role in the initial attachment of pathogens to host cells, and led to a surge of studies examining the role of various GAGs in infections in the last three decades. We now know that a large number and a wide variety of pathogens, including viruses, bacteria, parasites and fungi, also subvert GAGs for virtually all major steps of pathogenesis (6-9). For example, many intracellular pathogens use cell surface heparan sulfate (HS) for host cell attachment and invasion. Several extracellular pathogens secrete factors that release GAGs from cell surfaces and extracellular matrices (ECMs) and exploit the ability of these solubilized GAGs to inhibit antimicrobial factors. Some pathogens coat their surfaces with solubilized GAGs to escape immune recognition. Yet several virulence factors co-opt cell surface GAGs as receptors for their pro-pathogenic activities. Using select examples, this review will discuss the diverse GAG subversion strategies of pathogens.

2.1. Primer on GAG biology

GAGs are complex linear polysaccharides ubiquitously expressed inside, on, and in the surrounding environment of most, if not all, cell types. The five types of GAGs are HS/HP, chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and hyaluronic acid (HA). GAGs are defined by the composition of their repeating disaccharide units and chemical linkage of the amino sugar and uronic acid monosaccharides in the disaccharide unit (10-13). The signature disaccharide repeat of HS/HP is $(GlcA/IdoA\beta1\text{-}4GlcNAc\alpha1\text{-}4)_n$, CS is $(GlcA\beta1\text{-}3GalNAc\beta1\text{-}4)_n$, DS is $(GlcA/IdoA\beta1\text{-}3GalNAc\beta1\text{-}4)_n$, KS is $(Gal\beta1\text{-}4GlcNAc\beta1\text{-}3)_n$, and HA is $(GlcA\beta1\text{-}3GlcNAc\beta1\text{-}4)_n$. Except for KS and HA, GAGs are attached to and polymerized on certain Ser residues of a Ser-Gly dipeptide sequence often repeated two or more times. All GAGs except for HA exist as proteoglycans *in vivo* and are synthesized in the ER and Golgi where the unmodified disaccharide units are elongated through the action of glycosyltransferases and modified by epimerases and sulfotransferases. In contrast, HA chains are synthesized during its transit through the plasma membrane by several HA synthases. KS chains are sulfated poly-*N*-acetyl lactosamines and classified by their core protein linkages. KS I is *N*-linked to specific Asn via high mannose type precursor oligosaccharide, KS II is *O*-linked to Ser or Thr via GalNAc, and KS III is *O*-linked to Ser or Thr via 2-*O*-mannose. Because the polymerization and modification reactions of GAGs do not go to completion, the biosynthetic process generates an exceptionally diverse array of GAG structure, both in length and extent of modification. Adding to the structural complexity is the fact that GAG polymerization and modification varies with cell type and tissue source. Mature HS/HP can also be processed by sulfatases (14) and heparanases (15) in the extracellular environment. The interaction of GAGs with ligands depends on their 3D structure, their sulfate substitution pattern, carboxyl groups, and rotations around the glycosidic linkage. The flexibility of the pyranose rings in the IdoA residues of HS/HP and DS also allows these GAGs to assume conformations that facilitate specific ligand binding.

3. GAG SUBVERSION MECHANISMS AT PORTALS OF ENTRY

Microbial pathogens must overcome several anatomical barriers to enter the host and survive the innate and adaptive immune attacks to cause disease. These host defense mechanisms are usually very effective in protecting the host from infections. However, in some cases, the protective barriers are accidentally breached by tissue injury, such as those caused by incisions, punctures, chemicals, and burns. These conditions allow access to the pathogens for entry and predispose the host to infection. Very young or old age, immunological disorders (*e.g.,* AIDS), chemotherapies, and several chronic conditions (*e.g.,* COPD, diabetes) can also compromise host defense and increase the susceptibility to infection. The primary portals of entry for the pathogens are the skin, ocular surface, and mucosal epithelium of the lung, gut and urogenital tract. Pathogens can also be delivered directly into the circulation by transfusion of contaminated blood products, invasive medical procedures, and i.v. drug abuse. Several infected arthropods also serve as vectors to transmit pathogens to humans. Infections at the primary portals of entry are the major cause of human morbidity and mortality. For example, pneumonia and diarrheal diseases remain as leading causes of death, and ocular surface infections are significant causes of blindness worldwide. Thus a better understanding of the evolving mechanisms of how pathogens subvert host components, such as GAGs, at the portals of entry is needed for the development of new and effective antimicrobials.

3.1. Respiratory tract

The human lung possesses a large surface area, approximately the size of half of a tennis court,

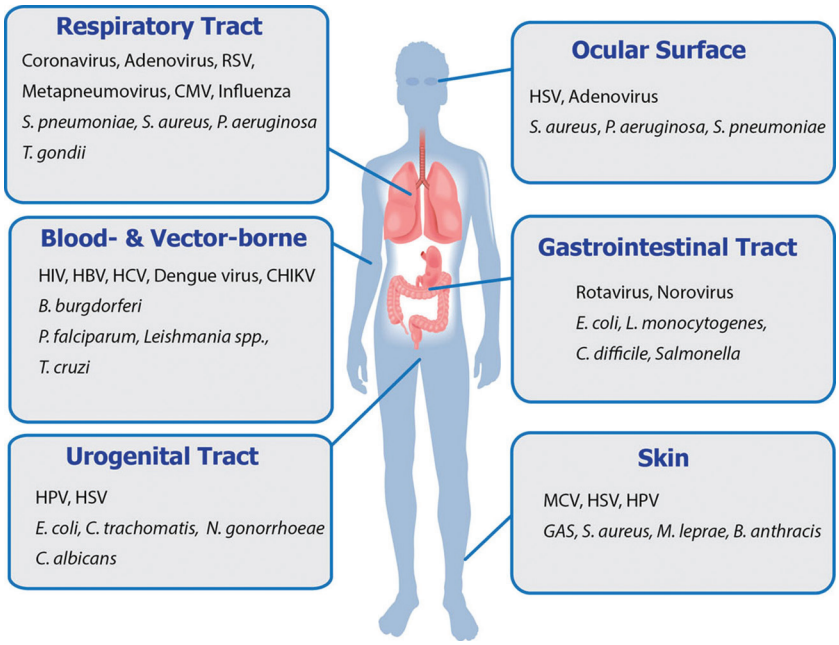

Figure 1. Pathogens that subvert GAGs at major portals of entry. A partial list of pathogens that subvert GAGs at the respiratory tract, gastrointestinal tract, urogenital tract, skin, and ocular surface, and those that are blood- or Vector-borne and co-opt GAGs for their infection are shown.

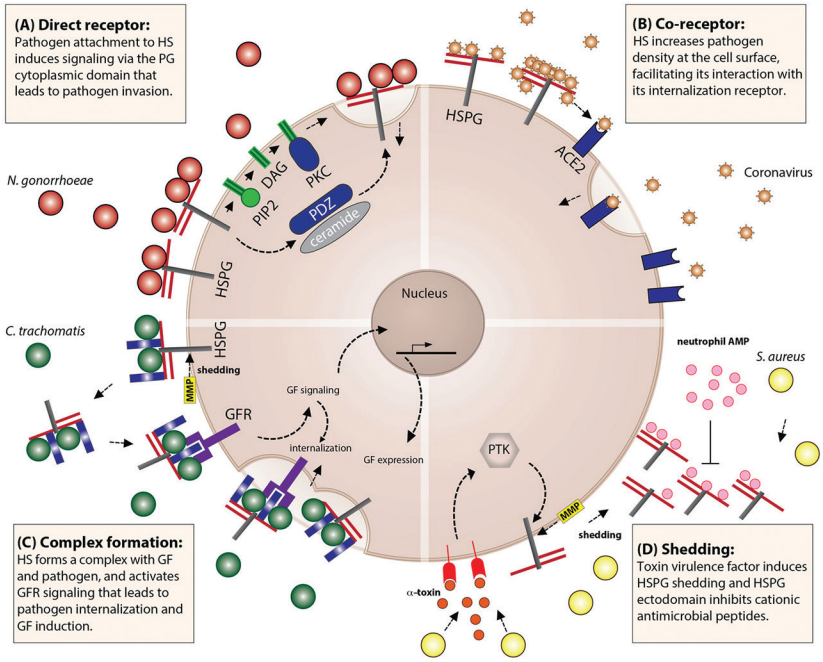

Figure 2. Mechanisms of GAG subversion. Select examples of several major mechanisms of GAG subversion are shown. A) Direct receptor: *N. gonorrhoeae* binds to the HS moiety of HSPGs (syndecan-1 and -4) and activates an intracellular signaling pathway via the cytoplasmic domain of the core protein that leads to gonococcal internalization. B) Co-receptor: binding of coronavirus to cell surface HS increases the local virus density, enabling coronavirus to interact with its entry receptor ACE2. Without HS, coronavirus does not interact efficiently with ACE2. C) Complex formation: cell surface HS forms a multimeric complex with *C. trachomatis* and growth factors (GFs), and the complex gets shed by MMPs and signals via GF receptors (GFR), leading to bacterial internalization and GF overexpression. D) Shedding: *S. aureus* α-toxin induces HSPG (syndecan-1) shedding and HS chains of HSPG ectodomains bind to and inhibit cationic antimicrobial peptides secreted by neutrophils, promoting *S. aureus* survival.

making the respiratory tract a common entry route for infection. The rapid and tight attachment of microbes to epithelial cell surfaces or ECM components exposed in damaged airways is a critical first step to avoid being trapped and swept away by mucociliary clearance, ejected by coughing and sneezing, or rapidly killed by innate defense mechanisms, such as alveolar macrophages, antimicrobial peptides, and surfactant

proteins. Many pulmonary pathogens express GAG-binding proteins that mediate their attachment to GAGs on the cell surface and in the ECM. For example, the respiratory pathogen human metapneumorivus (HMPV) uses GAGs as an attachment receptor. HMPV fusion proteins G (16) and F (17) bind to cell surface GAGs and HMPV attachment to host cells is significantly reduced in the absence of GAGs. Treatment of cells with heparinase I and III significantly inhibits HPMV adhesion, while treatment with chondroitinase ABC has no effect, indicating that HS chains are specifically targeted during HPMV infection. Similarly, respiratory syncytial virus (RSV), a major cause of lower respiratory tract infections in infants and children, uses its F glycoprotein to bind to cell surface HS (18). A later study examining the effects of unsulfated, *N*-, *O*-, or *N*- and *O*-sulfated *E. coli* heparosan showed that RSV binding to HEp-2 human epithelial cells requires *O*-sulfated motifs in HS (19), indicating that RSV recognizes specific sulfate modifications in HS to attach to host cells.

Cell surface GAGs also serve as co-receptors by increasing the local concentration of pathogens so that they can interact more efficiently with their entry receptors. For example, coronavirus NL63 (CoV-NL63) and the severe acute respiratory syndrome coronavirus (SARS-CoV) use angiotensin-converting enzyme 2 (ACE2) as a primary receptor for their entry (20, 21). However, ACE2 expression is not sufficient for infection as directed expression or selective cleavage of ACE2 has no effect on virus attachment. Instead, both CoV-NL63 and SARS-CoV initially bind to cell surface HS and virus entry is dependent on the HS interaction (22). These findings suggest that cell surface HS increases virus density at the cell surface and facilitate the interaction between CoV-NL63 and SARS-CoV and ACE2 for viral entry.

Respiratory bacterial pathogens also exploit GAGs for their infection. The Gram-negative bacterium *Pseudomonas aeruginosa*, a significant cause of nosocomial pneumonia, has been shown to co-opt HS during several steps of its pathogenesis. *P. aeruginosa* infects human lung epithelial cells via binding to two distinct receptors that are expressed in a polarized manner (23, 24). *P. aeruginosa* type IV pili bind to *N*-glycans on the apical surface, while flagella bind to HS on the basolateral surface of lung epithelial cells. *P. aeruginosa* flagella binding to cell surface HS activates epidermal growth factor receptors and phosphatidylinositol 3-kinase (PI3K)/Akt signaling, and induces bacterial internalization at the basolateral surface. These results suggest an interesting mechanism where *N*-glycans serve primarily as an adhesion receptor, whereas cell surface HS functions as an internalization receptor.

P. aeruginosa also stimulates the ectodomain shedding of syndecan-1, the major cell surface HS proteoglycan (HSPG) of epithelial cells. Shedding is induced by LasA, a virulence factor for *P. aeruginosa* lung infection (25). LasA enhances syndecan-1 shedding by stimulating a host cell mechanism that is dependent on PTKs and metalloproteinase sheddases. Importantly, ablation of syndecan-1 in mice was found to be a gain of function mutation that enables these mutant mice to significantly resist *P. aeruginosa* lung infection relative to control wild type mice (26). Furthermore, airway administration of a sheddase inhibitor inhibited, whereas purified syndecan-1 ectodomains enhanced *P. aeruginosa* lung virulence in mice, suggesting that activation of syndecan-1 shedding is an important virulence activity of this bacterial pathogen. The infection promoting activity of syndecan-1 ectodomains was traced to the ability of ectodomain HS chains to inhibit cationic antimicrobial factors (26). Together, these studies suggest that *P. aeruginosa* uses the HS moiety of syndecan-1 for both its invasion of host cells and evasion of innate host defense.

3.2. Urogenital tract

The urogenital tract is normally well protected by the mucosal epithelial barrier, flow of urine, low pH environment, mucus layer, epithelial secretions, and intrinsic antimicrobial defense mechanisms. However, tissue injury and underlying pathologic conditions predispose the urinary tract to infections, such as injury to the mucosal epithelium, obstructive or neurological diseases of the urinary tract, diabetes, pregnancy, and aging. Similar to the respiratory tract, the binding of microbial pathogens to GAGs on the mucosal epithelium or in the underlying ECM is considered as an important pathogenic mechanism.

The obligatory intracellular Gram-negative bacterium *Chlamydia trachomatis* is the leading cause of bacterial sexually transmitted disease (STD). *C. trachomatis* was initially suggested to bind to cell surface HS for its attachment to host cells (27, 28). Later studies revealed a more complex mechanism where *C. trachomatis* exploits surface HS to form a ternary complex of HS, growth factors (GFs) and *C. trachomatis*, and activate GF signaling to promote its infection (29). Treatment with EGFR or FGFR inhibitors prevented *C. trachomatis* infection of HaCaT human keratinocytes, suggesting that the formation of an HS-GF-*C. trachomatis* complex is not only important for bacterial attachment but also for activation of GF signaling and subsequent invasion. Furthermore, *C. trachomatis* infection induced the expression and secretion of FGF-2, suggesting a positive feedback infection loop. Interestingly, metalloproteinase inhibitors interfered with the isolation of the HS-GF-*C. trachomatis* complex from the culture medium. This observation indicates that the complex is shed in a metalloproteinase-dependent manner, and suggests the involvement of cell surface HSPGs that are shed from the cell surface by metalloproteinases, such as syndecans.

Cell surface HS is co-opted by *Neisseria gonorrhoeae* for its attachment and invasion of host cells. Along with *C. trachomatis*, *N. gonorrhoeae* is one of the two most common causative agents of bacterial STD. Available data suggest that HS functions as both co-receptors and direct internalization receptors for this Gram-negative bacterial pathogen (30, 31). As a co-receptor, gonococci use HS to bind to fibronectin, and use it as a molecular bridge to bind to b1 integrins, which then mediate *N. gonorrhoeae* internalization. Binding to HS is key in this mechanism since fibronectin is unable to enhance gonococcal invasion in cells that had been treated with heparinase III (31).

As direct entry receptors, *N. gonorrhoeae* has been shown to not only bind to the HS moiety of syndecan-1 and -4 for its attachment, but also for activation of signaling required for host cell invasion. Binding to syndecan-1 and -4 via its outer membrane protein Opa_{HSPG} is essential for infection *in vitro* as overexpression of syndecan-1 or -4 in HeLa cells increased *N. gonorrhoeae* infection (32, 33). However, *N. gonorrhoeae* attached but did not invade HeLa cells overexpressing syndecan-1 or -4 constructs lacking the cytoplasmic domain. Expression of a syndecan-4 mutant construct lacking the dimerization motif in the cytoplasmic domain that binds to protein kinase C (PKC) and phosphatidylinositol 4,5-bisphosphate (PIP2), and a syndecan-4 mutant lacking the invariant C-terminal Glu-Phe-Tyr-Ala PDZ binding domain also did not support gonococcal invasion (33). These findings suggest that the cytoplasmic domains of syndecan-1 and -4 play a critical role in the activation of an intracellular signaling mechanism, possibly involving PKC, PIP2 and PDZ proteins, that is essential for gonococcal invasion. However, other signaling pathways may also participate since *N. gonorrhoeae* binding to cell surface HSPGs induces the generation of phosphatidylcholine-specific phospholipase C, diacylglycerol, acidic sphingomyelinase, and ceramide (30). A key question that remains unanswered is how engagement of the HS moiety transmits signals to the cytoplasmic domain of the HSPG core protein to activate various signaling mechanisms.

Human papilloma virus (HPV) is a non-enveloped, double-stranded DNA virus that infects mucosal epithelial cells and hijacks the host cell cycle to cause both benign and malignant cervical tumors. HPV uses cell surface HS as an attachment receptor (34, 35). Induced expression of syndecan-1 in erythroleukemia cells significantly increased HPV attachment and entry compared to the induced expression of syndecan-4 or glypican-1 (36), suggesting that syndecan-1 is the primary HSPG that provides the HS binding site for HPV. Furthermore, HPV attachment and entry were inhibited by heparinase III treatment of host cells and by antibodies against the cationic HPV capsid protein L1 (36). Subsequent studies revealed that L1 binding to cell surface HS induces a conformational change in the minor capsid protein L2, exposing residues in L2 that bind to a secondary entry receptor, which mediates HPV internalization (37, 38). These observations suggest a mechanism where HPV attaches to host cells via the L1-syndecan-1 HS interaction, which leads to the activation of L2 and L2-mediated HPV entry. Interestingly, L2 is cationic and binds to HP (39). Pre-incubation of HPV with HP was found to promote HPV attachment to HS-deficient cells (40), raising the possibility that HP-like highly sulfated S-domains in HS may also bind to and directly activate L2-mediated HPV entry.

3.3. Gastrointestinal tract

A large number of physical barriers and innate defense mechanisms protect the gastrointestinal (GI) tract from infections. These include peristalsis, acidic pH of the stomach, alkaline pH of the intestines, mucus coating, rapid turnover of GI epithelial cells, normal intestinal flora, and antimicrobial activity of intestinal secretions, bile, pancreatic enzymes, secretory IgA, and resident leukocytes. However, several conditions promote GI infections, such as consumption of contaminated food and water containing a large concentration of pathogens, drug-induced slowing of peristalsis and alteration of normal flow, malnutrition, prematurity and immunological deficiencies, among others. Viruses are the most common cause of GI infections, although bacterial infections are also quite common. It is unknown if conditions that predispose individuals to GI infections regulate the expression or activity of GAGs to favor pathogenesis, but this is an interesting area for future investigation.

Several enteric viral (*e.g.,* Rotavirus, Norovirus) (41, 42) and bacterial pathogens (*e.g., E. coli*, *Salmonella*) (43, 44) bind to GAGs and use this interaction for their attachment to host cells and tissues. Rotavirus also uses GAGs to promote the activity of its enterotoxin NSP4 (45). Rotavirus infection is a major cause of severe diarrhea in infants and young children, especially in developing countries with poor hygienic conditions. NSP4 is a transmembrane glycoprotein that is secreted as soluble oligomers from infected intestinal epithelial cells despite retention of its hydrophobic transmembrane domain. NSP4 binds to the cell surface of HT-29 human colon epithelial cells in a GAG-dependent manner. NSP4 binding to HT-29 cells is inhibited by the addition of HS or HP, and also by treatment of HT-29 cells with heparinase I and III, indicating that this enterotoxin binds to cell surface HS. NSP4 induces Ca^{2+}-dependent epithelial secretions and causes diarrhea, but it is not known if NSP4 binding to cell surface HS enhances signaling required for these processes.

Similarly, cell surface HS is exploited by *Yersinia enterocolitica* for the delivery and translocation of its virulence factor *Yersinia* outer proteins (Yops) (46).

Y. enterocolitica is widespread in nature, though most environmental isolates are avirulent. However, several strains found in animal sources are pathogenic for humans and consumption of contaminated food (especially undercooked pork) can cause enterocolitis and diarrheal disease. A major cause of these symptoms is Yops, which are translocated from the cell surface to the cytosol where they inhibit signaling initiated by innate immune receptors and subsequent host defense responses (47). A component of the Yop virulon, LcrG, was found to bind to host cells in a GAG-dependent manner (46). Addition of HP and treatment of host cells with heparinase I, but not chondroitinase ABC, inhibited uptake of LcrG, indicating that LcrG binds to sulfated S-domains in cell surface HS. LcrG also binds directly to HP and contains cationic residues reminiscent of an HP-binding motif. Translocation of the effector protein YopE was also specifically inhibited by HP and heparinase I treatment. These observations suggest that the LcrG-HS interaction is essential for the delivery of Yop virulence factors and suppression of host defense.

Listeria monocytogenes is an intracellular Gram-positive food-borne pathogen that crosses the intestinal mucosa and enters the systemic circulation where it can cause bacteremia and meningitis in immunocompromised hosts, pregnant women, and the very young and elderly. Listeriosis is a rare disease, but it carries a high mortality and is associated with several major outbreaks from the consumption of contaminated food. For example, a listeriosis outbreak that was traced to contaminated cantaloupes in the US in 2011 caused 147 illnesses, 33 deaths, and 1 miscarriage. *L. monocytogenes* expresses two adhesins that bind to cell surface HS. One is internalin protein B (InlB), which also binds to the hepatocyte growth factor (HGF) receptor Met and complement factor C1q in addition to HS. C1q mediates the uptake of *L. monocytogenes* in professional phagocytes, whereas Met mediates bacterial internalization in hepatocytes by inducing the mono-ubiquitination of Met and subsequent clathrin-dependent endocytosis (48). Cell surface HS is thought to enhance Met-mediated internalization of *L. monocytogenes* by acting as a scaffold that clusters both InlB and Met at the cell surface, and by stabilizing the InlB-Met complex during bacterial internalization (49, 50). Because HS also binds to HGF, formation of the HS-InlB-Met complex and subsequent bacterial internalization may be facilitated by this HS/HP-binding growth factor, but this issue has not been directly examined. In addition to InlB, *L. monocytogenes* ActA binds to cell surface HS (51). ActA is best known for its capacity to manipulate the actin cytoskeleton to allow bacterial migration within and between host cells (52, 53). However, ActA binding to HS is thought to affect bacterial invasion of epithelial cells, possibly through microvilli. The significance and relevance of this dual HS binding mechanism are not clearly understood, but based on the available data, the ActA-HS interaction may be important for the infection of intestinal epithelial cells, whereas the InlB-HS interaction may promote infection of hepatocytes by disseminated *L. monocytogenes*. The specific HSPGs that bind to both InlB and ActA have not been identified, but syndecan-1 is a possible candidate because both intestinal epithelial cells and hepatocytes abundantly express syndecan-1 in a polarized manner.

3.4. Skin

The skin, with a thick outer layer of dead cells, is arguably the most important physical barrier against pathogens. Most microbes cannot penetrate the skin unless it is breached by injury or insect bites. Hair follicles provide some open areas for entry, but the low pH and antimicrobial factors present in hair follicle secretions effectively inhibit the growth of many pathogens. However, when the outer skin barrier is breached, pathogens gain access to many GAGs that are abundantly expressed on the surface of epidermal and dermal cells, and in the dermal ECM. As such, several major dermal pathogens, including viral (*e.g.,* HSV, MCV) (54, 55), bacterial (*e.g.,* Group A Streptococci, *S. aureus*) (56, 57), parasitic *(e.g., Leishmania*) (58) and fungal pathogens (*e.g., Candida*) (59), have been shown to bind to GAGs. For example, Merkel cell polyoma virus (MCV) primarily binds to cell surface HS, and to a lesser extent CS, for its initial attachment to host cells (55). MCV is a circular double-stranded DNA virus that is thought to be the causative agent of Merkel cell carcinoma, a rare but highly lethal form of skin cancer. Although early studies showed that MCV binds to sialylated glycoproteins and glycolipids, these interactions were found to be dispensable for its attachment to cultured human cell lines. Instead, addition of HP or CS, treatment of cells with heparinase and chondroitinase, and inhibition of GAG sulfation by chlorate significantly inhibited MCV infection, indicating that both HS and CS are important (55). However, studies with various CHO cell lines deficient in HS, CS, HS and CS, or several HS modification enzymes showed that cell surface HS is the dominant receptor and that 6-*O*-sulfation and possibly also *N*-sulfation and epimerization of HS are required for MCV entry (55). These data indicate that structural features of HS are important, but these features probably do not explain the tropism of MCV for the skin since *N*- and 6-*O*-sulfation and epimerization are quite common among all HS. Interestingly, although MCV readily binds to cell lines deficient in sialic acid, it cannot enter these mutant cells, suggesting that HS is a co-receptor that facilitates MCV binding to its sialylated entry receptors. Thus, the coordinated action of cell surface HS and sialylated receptors may underlie the skin tropism of MCV.

Another example where structural characteristics of GAGs influence the outcome of infection is Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) skin infection. GAS is a Gram-positive pathogen that frequently infects the skin and throat, and

cause diseases such as impetigo and pharyngitis (Strep throat). GAS can also occasionally cause severe and life threatening invasive disease (*e.g.,* necrotizing fasciitis, also known as flesh-eating bacteria syndrome). GAS expresses HA GAGs, which are abundant in the skin, and this molecular mimicry is thought to protect GAS from host defense attack. A recent study showed that the size of HA chains influence how they function in GAS infection (56). Experiments with high and low molecular mass HA prepared by enzymatic digestion or custom synthesis showed that high molecular mass HA inhibits GAS phagocytosis by macrophages and increases GAS survival in a mouse model of GAS skin infection, whereas low molecular mass HA enhances GAS phagocytosis and attenuates GAS infection. These observations suggest that the conversion of HA into short GAG chains, which is typically observed during skin injury, may be a host defense mechanism. How low molecular mass HA chains promote macrophage phagocytosis and host defense remains to be clearly defined, but these data suggest that other GAGs may also contain cryptic bioactive domains that are released during microbial infections.

3.5. Ocular surface

The ocular surface is constantly challenged by many microbial pathogens, but is also effectively protected by both passive and active defense mechanisms. These include blinking and flow of tear fluids that flushes pathogens, the lipid layer of tear film and mucus layer that block access to the epithelial surface, the tight stratified epithelial barrier that blocks access to subepithelial and stromal compartments, and the high concentration of antimicrobial factors (*e.g.,* lysozyme, lactoferrin) in tear fluids that kills or slows the growth of pathogens (60). Susceptibility to infection increases when one or several of these passive and active defense mechanisms are disrupted, giving pathogens the opportunity to interact with host determinants that can promote infection, such as GAGs.

One of the first identified viruses to bind to GAGs is the alphaherpesvirus HSV. The two most virulent serotypes of HSV are HSV-1 and HSV-2. Both can infect the cornea, but HSV-1 is the dominant serotype that causes infections above the waist, whereas HSV-2 is a major cause of genital diseases. HSV glycoproteins gB and gC have been shown to bind to cell surface HS to promote viral attachment (61-63). Viral fusion is mediated by gD, which has been shown to bind to nectin-1 and -2, herpes virus entry mediator proteins (members of TNFα receptor superfamily), and a rare 3-*O*-sulfated form of HS (54). Thus, cell surface HS is subverted by HSV for both its attachment and entry. However, during a productive infection, the HS interaction can trap exiting HSV progenies and inhibit their release and viral spread. To circumvent this problem, HSV apparently induces the expression of heparanase, an endoglycosidase that degrades the polysaccharide backbone of HS/HP (64). Because retention of viral particles is a potential common problem for all HS-binding viruses, a similar heparanase-dependent mechanism may be used by other viral pathogens. Alternatively, because all GAGs except for HA are found conjugated to core proteins and exist as proteoglycans *in vivo*, proteases that shed cell surface proteoglycans could in principle achieve the same effect. Consistent with the fact that HSV preferentially targets epithelial cells and that syndecan-1 is a major epithelial HSPG, overexpression of syndecan-1 was found to promote viral fusion and cell-to-cell spread (65). However, rather unexpectedly these activities were determined to be mediated by the core protein and not by the HS moiety of syndecan-1, suggesting that the HS chains that support HSV attachment and entry are provided by a yet to be identified HSPG.

In contrast, syndecan-1 was found to promote *S. aureus* corneal infection in an HS-dependent manner (66, 67). *S. aureus* is one of the leading causes of bacterial keratitis, accounting for 10-25% of confirmed cases (68-70). However, despite the observations that *S. aureus* binds to HS (57) and expression of syndecan-1 enhances *S. aureus* adhesion to several types of host cells (71), purified syndecan-1 did not bind to *S. aureus* and was dispensable for *S. aureus* adhesion to both human and mouse corneal epithelial cells (66). Instead, *S. aureus* induces syndecan-1 shedding from the surface of corneal epithelial cells and syndecan-1 ectodomains promote *S. aureus* corneal infection by inhibiting the staphylocidal activity of neutrophil-derived CRAMP, a cationic antimicrobial peptide, in an HS-dependent manner. The significance of this mechanism is supported by the findings that ablation of syndecan-1 or inhibition of shedding significantly reduces *S. aureus* corneal virulence, whereas addition of purified syndecan-1 ectodomains or HS significantly enhances virulence (66), and *S. aureus* induces syndecan-1 shedding through α-toxin (72), a major virulence factor for *S. aureus* keratitis (73, 74). Interestingly, inhibition of CRAMP by syndecan-1 HS was found to be dependent on 2-*O*-sulfate groups (67). Because other major corneal bacterial pathogens, such as *Streptococcus pneumoniae* and *P. aeruginosa*, also induce syndecan-1 shedding in epithelial cells (25, 75), subversion of 2-*O*-sulfated domains in syndecan-1 ectodomain HS to inhibit cationic antimicrobial peptides may be a broadly used pathogenic strategy in bacterial corneal infections.

3.6. Blood- and Vector-borne

Exposure to bloodborne pathogens can occur through several mechanisms, such as contaminated needles or other medical devices, blood, semen, vaginal fluids, saliva, and amniotic fluid. HIV and hepatitis viruses are the leading causes of bloodborne infections, and AIDS and hepatitis are significant global burdens of morbidity and mortality, with AIDS killing approximately 1.5. million and cirrhosis and liver cancer secondary to hepatitis

killing approximately 1.1. million globally in 2010 (1). HIV and hepatitis viruses possess several mechanisms to interact with GAGs. For example, hepatitis C virus (HCV) binds to cell surface HS via two distinct mechanisms. One is through E1 and E2 envelope glycoproteins, which bind to HS and promote HCV attachment and entry (76). E1 and E2 glycoproteins bind more avidly to HS extracted from the liver, consistent with the observations that these glycoproteins bind preferentially to highly sulfated HS, liver HS contains a higher density of sulfate groups compared to HS from other tissues, and that HCV primarily infects hepatocytes (77). A second mechanism is a bridging mechanism where HCV binds to apoliprotein E (apoE) and uses apoE to bind to cell surface HS for their attachment and entry (78).

Structural features of HS also regulate HCV interactions with HS. For example, studies with HP oligosaccharides, modified HP, and gene silencing of HS biosynthetic enzymes showed that *N*- and 6-*O*-sulfation, but not 2-*O*-sulfation, and a minimum of HP decasaccharide are required for HCV cellular infection (79). E1 and E2 envelope glycoproteins also bind in an *N*- and 6-*O*-sulfate-dependent manner (77), suggesting that E1 and E2 interactions with HS may predominate in HCV infection *in vitro*. However, the abundance of lipoproteins and apolipoproteins in the circulation suggest that HCV may efficiently bind to apoE4 and use the ability of apoE4 to be endocytosed by syndecan-1 (80) to enter hepatocytes *in vivo*. However, the uptake of lipoproteins by syndecan-1 in hepatocytes is mediated by 2-*O*-sulfated, and not *N*- or 6-*O*-sulfated HS domains (81), illustrating a clear difference with E1- and E2-mediated mechanisms. Furthermore, while expression of syndecan-1 was shown to promote HCV infection (82), a recent study indicated that syndecan-4 is also important (83). Thus, HCV may use two distinct attachment and entry mechanisms *in vivo* where E1 and E2 envelope glycoproteins mediate HCV infection via syndecan-4 HS, whereas apoE4 mediates infection via syndecan-1 HS. Several other hepatitis viruses, such as HBV (84), HDV (85) and HEV (86), have also been shown to use cell surface HS for their attachment and entry, suggesting that HS subversion may be a common virulence mechanism of hepatitis viruses.

HIV binds to cell surface HS of macrophages, dendritic cells (DCs), endothelial cells, and epithelial cells through gp120 (87-90). Syndecans are the major cell surface HSPG receptors because HIV does not attach to K562 erythromyeloblastoid cells deficient in syndecans, but does attach when K562 cells are transfected with syndecan-1 (90). Similarly, HIV shows increased binding to Burkitt lymphoma-derived Namalwa B cells transfected with syndecan-1, -2, -3 or -4 (88), suggesting that HS chains expressed by all syndecans can support HIV attachment. However, syndecan-2 and -3 are apparently the major HIV receptors on DCs (88, 89). DCs facilitate HIV transmission to CD4-positive T cells, where rapid viral replication occurs (91). Syndecan-3, together with one of the entry receptors DC-SIGN, prolong the infectivity of HIV, increase infectivity of DCs (*in cis)*, and promote transmission to CD4-positive T cells.

HIV Tat, a small cationic polypeptide that is released from HIV-infected cells, also binds specifically to cell surface HS (92). Tat binding to HS depends on the size and sulfation of HS (93, 94). WiDr human colon carcinoma cells, which lack all HSPGs except perlecan, are permissive for Tat internalization (95), suggesting that perlecan is one of the HSPG receptors. Importantly, Tat bound to HS/HP is protected from proteolytic degradation (96) and Tat binding to HS is necessary for Tat internalization and subsequent activation of transcription (92, 97). Similar to several HS-growth factor interactions, HS binding facilitates Tat oligomerization. A Tat homodimer binds to cell surface HS expressed on both infected lymphoid cells and endothelial cells, leading to the formation of an HS-Tat-Tat-HS tetrameric complex that physically links lymphoid cells to the endothelium, which is thought to promote HIV extravasation (98). Together, these data illustrate the many ways HIV subverts HSPGs for its attachment, entry, cell-cell transmission, and dissemination.

Vector-borne infections are transmitted by the bite of infected arthropods, such as mosquitoes, ticks, and sandflies. Vector-borne infections account for more than 17% of all infectious diseases, of which malaria caused by the parasite *Plasmodium* spp. kills more than 1.1. million people annually worldwide (1). Malaria is a mosquito-borne infectious disease. Five species of *Plasmodium* can cause malaria, though most are caused by *P. falciparum* (~75%) and *P. vivax* (~25%), and lethal malaria is mostly caused by *P. falciparum*. *Plasmodium* spp. travel from the skin, through the blood, and to the liver where they differentiate, reproduce, and rupture hepatocytes to escape once again into the circulation to infect red blood cells. The sequestration of infected red blood cells (iRBCs) in the microvasculature is thought to cause end organ damage and lead to death of the host (99). A unique case arises in pregnant women where their placenta expresses low sulfated CS-A, which serves as a receptor for *Plasmodium* VAR2CSA induced on the surface of iRBCs (100-102). Analysis of recombinant VAR2CSA binding to placental tissue extracts showed higher affinity for CS-A chains present on the placental cotyledon (103, 104), which may underlie the observed tropism of *P. falciparum* for the placenta in pregnant women. The tissue-specific sulfate modifications of HS may also have important ramifications in how *P. falciparum* travels from the skin to the liver (105). *P. falciparum* circumsporozoite protein (CSP) detects the level of HS sulfation in tissues that it encounters while traveling from the skin to the liver. Low sulfated HS on dermal and endothelial cells bind to CSP, but they do not

promote *P. falciparum* invasion of these cells. Instead, *P. falciparum* only invades cells expressing highly sulfated HS, such as hepatocytes, suggesting that this mechanism may allow *P. falciparum* to navigate through different tissues to successfully reach and infect the liver.

Tick-borne *Borrelia burgdorferi* is the causative agent of Lyme disease. *B. burgdorferi* expresses two adhesins that bind to the DSPG decorin (106). Mutant *B. burgdorferi* that does not bind to DS showed decreased adhesion to HEp-2 cells *in vitro* (107). More importantly, mice deficient in decorin significantly resisted *B. burgdorferi*-induced arthritis, consistent with the observation that decorin is abundantly expressed in joints (108). Furthermore, longer-term studies suggested that in decorin-rich tissues, *B. burgdorferi* decorin adhesin expression and decorin binding prevent immune-mediated clearance (109). Thus, these data indicate that *B. burgdorferi* exploits DS chains of decorin to attach to host cells and to evade host defense.

Dengue virus is a mosquito-borne RNA virus that causes the tropical disease dengue fever. Dengue fever is usually not life threatening, but in a small but significant number of patients, dengue infection can lead to lethal dengue hemorrhagic fever and shock syndrome. The precise mechanism of dengue disease progression is not understood, but the accumulation of the secreted dengue NS1 glycoprotein on microvascular endothelial cells can lead to immune-mediated vascular injury and leakage, which are the major symptoms of dengue hemorrhagic fever. Interestingly, NS1 glycoprotein binds to cell surface HS and CS-E (110), suggesting that these GAGs may mediate the accumulation of NS1 on microvascular endothelial cells. Furthermore, dengue virus infects endothelial cells by binding to cell surface HS (111). Inhibition of integrins and other potential cellular receptors was unable to reduce dengue infection of human endothelial cells, but pretreatment of endothelial cells with heparinase III or addition of HP or HS significantly inhibited dengue infection, and dengue virions bound specifically to HP affinity resins. Thus, dengue virus appears to use cell surface HS for multiple purposes, *i.e.,* for its attachment, entry, and induction of immune-mediated endothelial damage through the accumulation of secreted NS1 glycoprotein.

4. SUMMARY AND FUTURE PERSPECTIVES

GAGs are expressed in intracellular compartments, on the cell surface, and in the extracellular *milieu* where they bind to growth factors, ECM components, adhesion receptors, antimicrobial factors and other bioactive molecules to regulate many molecular and cellular activities. GAGs are essential for normal development as ablation of EXT1 required for HS synthesis (112) or glucuronyltransferase I required for HS, CS and DS synthesis (113) results in embryonic lethality. GAG modifications are also essential as mice lacking HS 6-*O*-sulfotransferase I show late embryonic lethality, whereas those lacking HS *N*-deacetylase *N*-sulfotransferase I (114, 115), C5 epimerase (116), or 2-*O*-sulfotransferase (117) show early postnatal lethality. Furthermore, mice lacking syndecan-1 develop normally, but show abnormal responses to infectious and inflammatory stimuli. Importantly, these phenotypes can be rescued by HS/HP (26, 67, 118-120), indicating that HS functions are also important post-developmentally. Studies in the last several decades have established that biological functions of GAGs also include modulation of microbial pathogenesis and host defense mechanisms. As discussed above, a wide variety of viral, bacterial, parasitic, and fungal pathogens subvert GAGs at all major portals of entry and at almost every major step of pathogenesis. Perhaps this comes as no surprise given the ubiquitous nature and versatile functions of GAGs, and the multitude of virulence mechanisms elaborated by pathogens. However, what is surprising is the ingenious ways pathogens subvert GAGs. Pathogens have either adapted or evolved to exploit fundamental biological activities of GAGs, such as serving as a cell surface scaffold that increases ligand density, serving as cell adhesion and internalization receptors, facilitating ligand or receptor oligomerization, inducing conformational changes, activating signaling pathways, and forming multimeric complexes with ligands and receptors. This clever subversion strategy targeting fundamental GAG functions likely prevented the removal or inactivation of GAGs and their activities during human evolution, despite the fact that they clearly promote microbial pathogenesis.

However, these features also suggest that GAG subversion mechanisms are potential targets for the development of both highly specific and broadly effective antimicrobial therapies. In fact, several engineered GAG mimetics, sulfated compounds, cationic compounds, GAG-digesting enzymes, and selective knock-down of GAG biosynthetic enzyme or proteoglycan core protein genes have been shown to effectively inhibit infection in cell-based and pre-clinical animal models (121-128). Toxicity and potential off-target effects are currently major hurdles for usage of these compounds *in vivo*. Furthermore, precise details of how pathogens subvert GAGs *in vivo* are still incompletely understood. Future studies should aim to more clearly define the essential GAG modifications, both made during synthesis and post-synthesis, identify specific proteoglycans that carry the subverted GAGs, and determine exactly when and where GAGs are subverted by pathogens using relevant animal models of infection. In addition, although available data suggest that many microbes preferentially subvert HS for their infection, the role of other GAGs in microbial pathogenesis and host defense is clearly understudied. The need for new and effective antimicrobial agents is greater than ever because of the emergence of multidrug resistance in highly virulent pathogens and the rapid

emergence of new pathogens. In principle, essential host genes do not evolve as rapidly as those of microbial pathogens, suggesting that pathogens will not rapidly develop resistance to therapeutic agents and approaches that target fundamental activities of GAGs.

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Send correspondence to: Pyong Woo Park, Children's Hospital, Harvard Medical School, 320 Longwood Avenue, Enders-461, Boston, MA 02115, USA, Tel: 617-919-4584, Fax: 617-730-0240, E-mail: pyong.park@childrens.harvard.edu