Proteoglycans modulate renal inflammation

Zaferani, Azadeh

Chapter 1

Heparin/heparan sulfate interactions with the complement system, a target for intervention to reduce renal function loss?

Azadeh Zaferani, Mareike K.S. Richter, and Jacob van den Born

Most used Abbreviations

AP: alternative pathway
APC: antigen presenting cells
CP: classical pathway
CS: chondroitin sulfate
CTD: chronic transplant dysfunction
DC: dendritic cells
DS: dermatan sulfate
ESRD: end-stage renal disease
ECM: extracellular matrix
GAG: glycosaminoglycan
GBM: glomerular basement membrane
GlcNAc: *N*-acetyl-*D*-glucosamine
GalNAc: *N*-acetyl-*D*-galactosamine
GlcA: *D*-glucuronic acid
HS: heparan sulfate
HSPG: heparan sulfate proteoglycan
ICAM: intercellular adhesion molecule 1
IFTA: interstitial fibrosis tubular atrophy
IdoA: *L*-iduronic acid
I/R: ischemia/ reperfusion
MAC: membrane-attack complex
MASP: MBL associated serine proteases
MBL: the mannose-lectin binding pathway
MCP-1: monocyte chemoattractant protein
NDST: *N*-deacetylase/*N*-sulfo-transferases
NK: natural killer
PG: proteoglycans
PMN: polymorphonuclear neutrophils
TGF-β: transforming growth factor
TLR: toll-like receptor
TNF-α: tumor necrosis factor
VCAM-1: vascular cell adhesion molecule 1

1.1 Introduction

The prevalence of chronic renal diseases is increasing worldwide. There is a great need to identify therapies that arrest disease progression to end-stage renal failure (1). A prolonged survival for end-stage renal disease (ESRD) patients was achieved by renal replacement therapy either by dialysis or by renal transplantation. Renal replacement therapy is costly and consumes a large proportion of the health care budget (2). Therefore, the effort should be directed to discover ways to prevent the need for dialysis or renal transplantation in as many patients as possible. ESRD can occur in both native and transplanted kidneys. Chronic transplant dysfunction (CTD), which can develop more than one year after transplantation, is the major complication that leads to late renal graft loss and return of the patient to dialysis. CTD is characterized by the occurrence of interstitial fibrosis and tubular atrophy (IFTA). IFTA can have several causes, both immunologic and non-immunologic (3). In addition, the extent of renal ischemia-reperfusion (I/R) injury, which occurs as the donor organ is reconnected to the recipients blood supply, is a main risk factor for early and late renal graft loss (4). There is growing evidence that proteinuria (in both native and transplanted kidneys) leads to interstitial inflammation and subsequent fibrosis, which thereby contributes to progressive renal function loss and ESRD (2).

In general, progressive renal function loss is associated with immune activation, marked by renal and systemic inflammation. ESRD-associated inflammation is due to activation of innate immune system, mediated by monocytes, macrophages, complement system and chemokines (5). Innate immune-derived inflammation in transplantation has also been shown (6). Proteinuria and I/R are major risk factors for progressive renal function loss in both native and transplanted kidneys (2, 4). Moreover, the role of innate immunity in the pathogenesis of tubular injury in both proteinuria and I/R is established (7, 8).

The complement system is a major player in innate immunity. Three complement pathways can be distinguished based on their activation mechanism: The classical, the alternative and the mannose-lectin binding (MBL) pathway. Several studies have revealed that the complement system and in particular the alternative pathway (AP) is activated in both renal allograft rejection process (9, 10) and I/R (11). In proteinuria as well, the role of the AP is documented (7). Therefore, it would be interesting to target components of the complement system in chronic renal disease patients to reduce the progression of renal function loss.

A class of molecules which could be used for this purpose are the heparan sulfates (HS) and HS-related heparin and/or derivatives. HS are large polysaccharide chains that are sulfated at various positions. They appear mostly in the form of a HS-proteoglycan (HSPG), which means that one or more HS-chains are attached to a core protein (12). Heparin can be regarded as a special HS with a much higher degree of sulfation, which often results in a higher biological activity. Besides a plethora of other proteins, HS and heparins are able to bind to several complement proteins, which normally results in a down-regulation of the complement cascade (13). The knowledge of these interactions can be used for the development of new HS/heparin-based drugs for targeted intervention in renal (transplantation) patients.

This Introduction gives an overview of the involvement of innate immune system, in particular the complement system in progression of renal damage in proteinuria, I/R and

transplantation. In addition, the structure and function of HSPGs is discussed. Furthermore, the current knowledge of complement-heparin/HS interactions is reviewed. Finally, the possibility of using heparin/HS related polysaccharides to inhibit innate immune system will be evaluated.

1.2 Innate immunity

The innate immune system is the first line of defense in all classes of plants and animals. The innate immune system protects hosts against pathogens in a non-specific manner and results in short term immunity. The innate immune system consist of physical barriers, cellular defense and soluble or humoral immune defense (mainly complement system and chemokines) (14). In general, inflammation leads to up-regulation of innate injury molecules, such as toll-like receptors (TLR), complement factors, adhesion molecules and chemokines. TLRs are involved in pathogen and altered-self recognition, and upon binding, TLRs promote the immune response to injury (15). Chemokines act as a chemoattractant to guide the migration of cells. Their release is often stimulated by pro-inflammatory stimuli. Inflammatory chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes, neutrophils and other effector cells from the blood to sites of infection or tissue damage.

Inflammatory cells of innate immunity
The initial capture of circulating leukocytes and the subsequent rolling of the cells along the endothelium are mediated by selectins (P-selectin, E-selectin and L-selectin) interacting with carbohydrate determinants on selectin ligands. Inflammation up-regulates P-selectin and E-selectin on endothelial cells, whereas L-selectin is constitutively expressed on leukocytes. The transition from rolling to firm adhesion requires leukocyte activation (mostly by chemokines) and binding of leukocyte-expressed integrins to endothelial adhesion molecules. The known ligand of integrins on endothelial cells are ICAM-1, ICAM-2, VCAM-1 and RAGE. The signals produced by integrin-ligands interaction, leads to crawling and extravasation of leukocyte to the surrounding tissue. After transmigration, leukocytes follow the chemoattractants gradient to reach the proper site of injury (16).

Cells of the innate immune system include neutrophils, macrophages, DC, monocytes, mast cells, natural killer (NK), and some subsets of T cells. Innate immune cells recruit additional leukocytes to the site of inflammation by releasing cytokines and chemokines. Many innate immune cells can also directly kill invading pathogens (14).

In the early immune response towards invading pathogens, polymorphonuclear neutrophils (PMN) are considered key effectors of innate host defense as they are first recruited into infected tissue. Thereafter, monocytes from the bloodstream and bone marrow, are recruited into the inflamed tissues where they acquire a macrophage phenotype and act together with the residential macrophages to respond against injury (17).

The complement system
The complement system forms one of the humoral parts of the innate immune system and has three main functions: Firstly, defending against harmful bacteria; secondly linking the innate with the acquired immune system; and thirdly disposing waste products like

immune complexes or apoptotic cell debris from tissues (18).

Three different pathways of complement activation can be distinguished: The classical pathway (CP), the mannose-lectin binding pathway (MBL) and the alternative pathway (AP). All of them are activated by different triggers, but they eventually all converge in the activation of C3 and the formation of the cytolytic membrane-attack complex (MAC). Currently, more than 35 different serum and plasma proteins are known to be involved in the complement cascade (19).

The CP is initiated as C1q binds to immune complexes (antigen-antibody complexes). In addition, C1q can also bind directly to pathogenic molecules like bacterial polyanions, viral, fungal and bacterial membranes, and to acute phase proteins like C reactive protein or amyloid P component. Together with two C1r and two C1s molecules, C1q forms the C1 polymer. As C1q binds to an immune complex, its conformation changes which leads to auto-activation of the serine protease C1r. Cr1 subsequently cleaves and activates C1s, the other serine protease of C1. C1s is then able to cleave C4 to generate C4b and C4a. C4b can now covalently bind to bacterial surfaces, were it in turn cleaves C2 to generate C2b and C2a. The latter forms together with the membrane-bound C4b the C3 convertase (C4bC2a) of the CP, which can cleave and activate C3 (18–21).

The MBL pathway cascade starts with the binding of mannose-binding lectin and ficolins to non-self carbohydrates, which contain mannose, glucose, fucose or *N*-acetylglucosamine as terminal hexoses. These types of carbohydrates are mainly present on pathogens like bacteria, viruses and fungi but not in higher animals. The MBL associated serine proteases (MASP) MASP-1 and MASP-2 mediate further complement activation. MASP-1 directly cleaves C3, whereas MASP-2 has C1 like functions since it cleaves C4, which eventually leads to the formation of C3 convertase of the CP (22).

The AP is phylogenetically the oldest branch of the complement system. It is activated when C3(H_2O) or C3b, which are always present in the plasma as a result of a continuous slow hydrolysis of C3, bind to target membranes. There it can bind factor B, which is cleaved to the soluble Ba and the C3b-bound Bb. C3bBb is the active AP C3 convertase complex. Properdin stabilizes and protects this complex from being cleaved by factor I. Moreover, properdin can bind to the cell surface and activate the AP. This explains why the AP is called the properdin pathway as well (23–27). Since the AP does not have any discriminatory nature for bacterial and host cell membrane surfaces, the AP is strictly regulated. For example, the stabilizing protein properdin is to a certain extent selective in the recognition of self or non-self membrane bound C3bBb by recognizing definite microbial targets. C3b more likely attaches covalently to membranes with a polysaccharide and protein structure that differs from the own cells. In addition, the body possesses several regulatory proteins to restrict AP activation. These can be either soluble, like factor I and factor H, or membrane bound, like CD46, DAF/CD55,CD59 and complement receptor type 1 (CR1) (28, 29). Especially factor H is of crucial importance for the regulation of the AP, since it acts as cell surface inhibitor as well as a fluid phase regulator. Factor H can recognize and bind only to self-cell markers like polyanions and proteoglycans (PGs). Once bound to C3b in combination with a self-recognition molecule, factor H accelerates the decay of the AP C3 convertase. In addition, factor H is an important co-factor for the factor-I mediated cleavage of C3b and C4b, which leads to inhibition of complement system activation as well (18, 28).

As described, all three pathways can be involved in the cleavage of C3 to C3b and C3a.

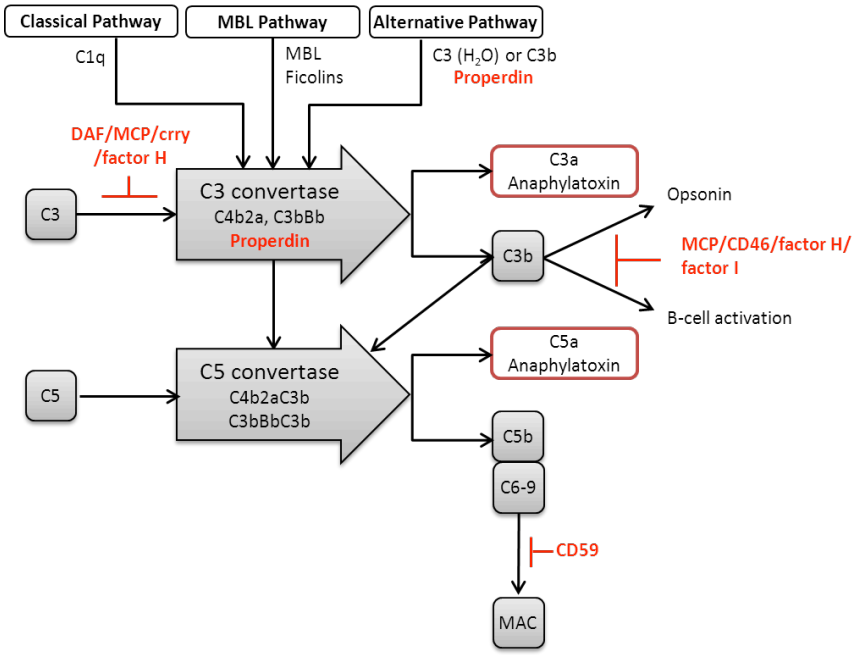

Figure 1.1: Overview of the complement cascade. The complement system activation occurs via three pathways. The classical pathway starts by binding of C1q to immune complexes. Binding of mannose-binding lectin and ficolins to non-self carbohydrates initiate the MBL pathway. The alternative pathway activates by binding of C3b, C3(H_2O) or properdin to target cells. All three pathways generate consecutively C3 convertases, C5 convertase and membrane attack complex. Side products of complement cascade (C3a and C5a) are anaphylatoxins. A number of regulatory factors (DAF, MCP, factor H, factor I, CD59) control complement activation.

C3b can further activate the AP, which leads to an amplification of the danger signal. Moreover, both the AP and CP convertase can bind to an additional C3b molecule to generate a C5 convertase. Subsequently C5 is cleaved into C5a and C5b, which marks the start of the effector phase of complement activation. This results in the assembly of the MAC composed of C5b, C6, C7, C8 and C9. Beside its opsonizing and cytolytic function, the complement system acts chemotactic as well. This role is mediated by the smaller cleavage products C3a, C5a, and C4a. These side products are called anaphylatoxins and they are chemoattractants for inflammatory cells (18). The opsonization of microbes or cells by the complement system occurs by binding of C1q, C3b and C5b to the complement receptors present on phagocytic cells such as macrophages and neutrophils. An overview of all three complement pathways can be found in Fig. 1.1.

Many components of the innate immune system interact with proteoglycans (PGs). This issue will be addressed in detail further in this chapter.

1.3 Proteoglycans

Proteoglycans (PGs) are complex glycoconjugates composed of a core protein and one or more glycosaminoglycan (GAG) chains. GAGs are linear polysaccharides consist of repeating disaccharide building blocks. These disaccharide blocks are built by a *N*-acetyl-*D*-glucosamine (GlcNAc) or *N*-acetyl-*D*-galactosamine (GalNAc) and an uronic acid (*D*-glucuronic acid (GlcA) or *L*-iduronic acid (IdoA)) or a galactose unit. Based on the disaccharide composition, GAGs are classified in four groups: (1) hyaluronan (HA); (2) keratan sulfate (KS); (3) chondroitin sulfate (CS, including dermatan sulfate (DS)); and (4) heparan sulfate (HS, including heparin, which has a higher sulfation/disaccharide ratio and a higher IdoA content than HS) (30).

Taking into account that each PG has a different core protein, different number of GAG chains with different disaccharide composition, variable chain length and various post-translational modifications, it is clear that the diversity of PGs is very complex. PGs are found on the cell surface, as well as in intracellular granules, in the basement membranes of various tissues, and in the extracellular matrix (ECM). PGs are involved in various biological activities mostly via their GAG chains, such as cell–cell and cell–matrix interactions, cell migration and proliferation, growth factor sequestration, chemokine and cytokine activation, microbial recognition and tissue morphogenesis during embryonic development (12, 30, 31). PGs are able to interact with various proteins based on their negative charge, however in some cases a specific sequence or motif on GAG chain is needed for PG-protein interaction. The famous example is the specific pentasaccharide domain on heparin which is able to bind to serine protease inhibitor antithrombin (AT) III and enhance its activity to inhibit the coagulants thrombin and factor Xa (32). Following the discovery of AT III binding pentasaccharide, a lot of effort have been made to investigate other proteins interaction with GAGs.

Biosynthesis of proteoglycans

PGs biosynthesis occurs in the endoplasmic reticulum (ER) and Golgi apparatus. Multiple enzymes are involved in the assembly of a PG. The core protein of PGs is constructed in ribosomes on the rough ER. GAG chain biosynthesis can be divided to chain initiation, chain elongation and chain modification. Chain initiation starts with synthesis of the linker tetrasaccharides added to a specific serine residue of the protein core by xylosyl transferase. Galactosyltransferase I and II, and glucuronyltransferase I are responsible for adding two *D*-galactose (Gal) residues and one GlcA to the *D*-xylose (Xyl) in Golgi and forming the tetrasaccharide linker (GlcA–Gal–Gal–Xyl-*O*-Ser). The type of the GAG (CS/DS or HS/heparin) depends on the fifth saccharide. With a GalNAc as the fifth saccharide, chain elongation will result in CS/DS formation while adding a GlcNAc forms HS/heparin GAG chain. In HS/heparin biosynthesis, chain elongation proceeds by adding GlcA and GlcNAc by glycosyl transferases EXT1 and EXT2. Chain modification occurs during the chain polymerization. The polysaccharide chain can undergo six types of enzymatic modification. The first two are *N*-deacetylation and *N*-sulfation, carried out by four members of *N*-deacetylase/*N*-sulfo-transferase (NDST) family. This step is a prerequisite for all following modifications. The members of NDST-family (NDST 1-4) differ in the ratio of deacetylase and sulfotransferase activity. Since the deacetylated/sulfated regions tend to be clustered on the polysaccharide, it is assumed that the enzymes stay

attached to the chain and move from the initial GlcNAc to the next ones. It is proposed that the length of the sulfated stretch is influenced by two other types of modification, namely epimerization and uronic acid 2-*O*-sulfation. Epimerization of GlcA is carried out by the C5 epimerase. IdoA, the 5' epimer of GlcA, is found in all protein-binding sites of HS and heparin and its generation is therefore a critical process in the arrangement of ligand-specificity. After epimerization, the enzyme 2-*O*-sulfotransferase can add a sulfate group at the C2 of IdoA. Although 2-*O*-sulfotransferase can react with GlcA as well, IdoA is preferably sulfated when both substrates are present (31). The remaining two modifications of the polysaccharide chain are 6-*O*-sulfation and 3-*O*-sulfation of the GlcNS residues. The enzymes that catalyze these reactions are 6-*O*-sulfotransferase, which has 3 isoforms and one splice variant, and 3-*O*-sulfotransferase, which has at least 5 isoforms (33). Due to involvement of multiple enzymes with various isoforms in PG biosynthesis, PGs are highly complex biopolymers. The current understanding of control mechanisms for PG biosynthesis is limited. Moreover their biosynthesis changes during the development and upon environmental stimuli and is tissue specific (30).

Heparan sulfate proteoglycan (HSPG)

HS is the GAG side chain of HSPG consisting of *N*-acetylglucosamine (GlcNAc) or *N*-sulphoglucosamine (GlcNS) together with a glucuronic acid (GlcA) or iduronic acid (IdoA) that are sulfated at various positions. Heparin, which exerts a higher biological activity, has a higher degree of sulfation, whereas HS sulfation varies a lot and generally *N*-acetylation is higher. Moreover, heparin contains a higher percentage of iduronate residues (34, 35).

HSPGs are ubiquitous components of nearly all tissues, found both in the ECM (mainly basement membranes) and on cell surfaces. Based on their distribution, HSPGs can be classified in three categories: membrane-spanning PGs (e.g. syndecans), glycophosphatidylinositol (GPI-) linked PGs (glypicans) and secreted ECM PGs (e.g. agrin, perlecan, collagen XVIII) (12, 36). Due to their high negative charge, HSPGs can specifically interact with several proteins and thereby participate in various biological processes (34). These are mainly providing ECM stability and integrity, mediating cell migration and adhesion and participating in tissue repair, inflammation, vascularization and cancer metastasis (37). To exert these functions, HSPGs can act as co-receptors for signal transduction after binding of several ligands, either insoluble (like ECM proteins) or soluble (like growth factors, chemokines and cytokines). In addition, HSPGs can sequester and store their ligands in the ECM, but also present them in a more active configuration to other receptors (34).

Heparin is produced in mast cells of lungs, intestine and liver. In the cell, 15-20 heparin polysaccharide chains, which are build up from 20-100 monosaccharides each are attached to a serglycin core protein. The animal-derived heparin usually does not have this core protein, likely due to degradation already within the mast cells (35, 36). Heparin is widely used in the clinic as an anticoagulant drug. This function is mediated by binding to AT III via a specific pentasaccharide domain which leads to inhibition of thrombin and factor Xa (32). However, in normal conditions, heparin is hardly detectable in the blood, and the physiological role of heparin is still not completely elucidated. Nevertheless, some functions might be storage of proteases in mast cells and regulation of their release upon inflammation (36). Both heparin and HS can interact with various components of the

complement system, which will be discussed in detail later in this chapter.

Structure and activities of HSPGs
The modifications on HS GAG chains influence their interaction with their various proteins ligands. Most important determinants of HS-protein interaction on HS chains are the density and position of sulfation groups, the length of HS chain, epimerization and 3D conformation of PG (38). Some of the known binding partners of HSPGs are shown in Table 1.1. These various proteins seem to require different HS motifs to interact with HSPGs. But likewise AT III-heparin interaction, there is no clear evidence that the distinctive sequence specificity can be generalized to other GAG–protein interactions. An example of a HS–protein interaction is fibroblast growth factor (FGF) family and their receptors (FGFRs). FGFs modulate cellular proliferation and differentiation through a signaling pathway linked to cancer progression and spreading. FGFs seems to need HSPGs to form a complex with their high affinity receptors. As summerized in Table 1.1, HSPGs can affect cell activities via their interaction with chemokines, cytokines, growth factors, adhesion molecules and ECM proteins.

Regulation of HS GAG biosynthesis and modification dictate HSPG binding capacity.

1.4 Innate immunity and proteoglycans

Leukocytes and proteoglycans
Proteoglycans (PG) are involved at multiple levels in leukocyte influx to the tissue. PGs on the endothelial surface together with leukocyte associated PGs, influence rolling and adhesion of leukocytes to endothelium. These processes are regulated by interaction of PGs with selectin family (P-, E- and L-selectin). Furthermore, activation of leukocytes occurs via PG-bound chemokines resulting in integrin activation which leads to stable adhesion and transmigration of leukocyte to the tissue. PGs in ECM make stable gradients of chemokines to guide the extravagated leukocyte to the target (39). This issue will be discussed in more detail in this chapter.

The complement system and proteoglycans
Already more than 80 years ago, it was discovered that heparins exhibit anti-complementary activities, and since then a lot of research on the different interactions has been done. A large number of complement proteins are able to interact with heparins or HS. Two comprehensive studies on the binding capabilities of heparin to complement proteins have been performed by Sahu and Pangburn, and more recently by Yu *et al* (13, 40). The majority of these interactions have regulatory functions by either augmenting or diminishing the activity of the complement components, and most of these interactions result in the inhibition of the complement cascade. Complement factors interaction with PGs will be discussed in detail further in this chapter.

Heparin and CP components interaction (C1, C1INH, C4BP)
The interactions of C1 with different GAGs have been investigated extensively. It has been shown that heparin can bind to and compete for the limited number of immune complex binding sites on C1q. Thus, activation of CP by C1 can be inhibited with heparin (41).

Table 1.1: Examples of heparan sulfates interaction with proteins. (modified from Dreyfuss *et al.* (38))

Cell surface
L-selectin, P-selectin
PECAM-1 (Platelet Endothelial Cell Adhesion Molecule)
FGF receptor
MAC-1 (Monocyte Adhesion Molecule)
ECM
Collagens
Fibronectin
Laminin
Growth factors
HB-EGF (Heparin Binding- Epidermal Growth Factor)
FGF (Fibroblast Growth Factor)
VEGF (Vascular Endothelial Growth Factor)
TGF-β (Transforming Growth Factor)
Cytokines/Chemokines
IL-1, -2, -3, -4, -5, -7, -8, -10, -12 (interleukin)
CCL-2
MCP-1 (Monocyte Chemoattractant Protein)
RANTES (Regulated on Activation Normal T cell Expressed and Secreted)
TNF-α (Tumor Necrosis Factor)
Wnt (Wingless wg)
Others
Complement factors
DNA and RNA polymerases
Angiogenin
Cathepsin B and G
Neutrophil elastase
Annexin V

Calabrese and colleagues have shown that the C1q component of C1 can recognize the same active heparin fraction as antithrombin. Moreover, C1q is also able to interact with several other GAGs, like low molecular weight (LMW) heparin and dermatan sulfate (DS) (42, 43). It is thought that the recognition of C1q by HS and other polyanions is a physiological regulatory mechanism of the CP that helps to control the inflammatory reaction (44). As shown by Kirschfink *et al,* B cells limit their own complement mediated stimulation by secreting CSPG which interacts with C1q (45).

The interactions with the C1 inhibitor (C1INH), a serine-type proteinase inhibitor of CP, have been studied widely as well (46). Several GAGs are known to potentiate the C1INH mediated CP inactivation. The mechanism of this effect still remains unclear (20, 47). To examine which polyanions could serve as a therapeutical inhibitor for C1, the group of Wuillemin tested several GAGs on their C1INH potentiating capacity. They found that the synthetic GAG, dextran sulfate at high concentration enhanced the C1INH activity 130-fold, whereas from the naturally occurring GAGs, heparin had the highest potentiating action by enhancing C1INH activity 58-fold. Moreover, also DS, HS, *N*-acetyl heparin, and CS were able to increase the inhibitory activity of C1INH (46).

The soluble C4-binding protein (C4BP), which circulates in the blood, is a multifunctional complement inhibitor. C4BP has many mechanisms of action: It can act as cofactor for the regulatory serine protease factor I, it accelerates the decay of the C3 convertase of CP, and it might in high concentrations also exert decay-acceleration activity on the C3 convertase of AP. It has been shown that GAGs present on self-surfaces can help to position C4BP to prevent damage of own tissue (48). Binding of HS chains have been proven to be essential for the concentration of C4BP on the cell surface, but the interaction is also necessary for CD91 mediated endocytosis, which is followed by the degradation of the C4BP. This mechanism reflects that also the action of C4BP is self-limiting (49). The C4BP binding sites for C4b, extracellular DNA and heparin overlap with each other, thus interaction with one of these ligands increases C4BP activity (48). An overview of interactions of complement factors with PGs is shown in Table 1.2.

Table 1.2: An overview of complement system and proteoglycans interaction.

Complement Factor	Pathway	Function	Ligand	Consequence of Complement-Ligand Interaction	Interaction Protective / Damaging	Reference
Antithrombin	MBL	Inhibits MASP-1	Heparin	Heparin increases AT activity	Protective	(50)
MASP-1, MASP-2	MBL	Cleaves C4,C2 to form C3 convertase; MASP-1: unknown	AT, only in presence of heparin	AT inhibits both MASP-1,2 in presence of heparin	Protective	(51)
C4-binding protein	CP & MBL	Complement inhibitor	Heparan sulfate	Cell binding, uptake, intracellular degradation	Damaging	(49)
			Heparin	Inhibits DNA-C4bP interaction, enhances regulatory activity	Protective	(48)
			Heparin	Inhibits C4BP-C4b interaction	Protective	(52)
C1	CP	Initiates CP	Heparin		Protective	(42)
			Heparin, heparan sulfates	Inhibits binding to activator	Protective	(46)
			Heparin	Inhibitory	Protective	(43)
C1 inhibitor	CP	Inhibits C1	Heparan sulfate, heparin	Increases inhibitory function	Protective	(53)
			Heparin, LMW heparin, NOT HS	Increases inhibitory function	Protective	(54)
			Heparan sulfate, heparin	Increases inhibitory function	Protective	(46)
			Heparin	Increases inhibitory function	Protective	(55)
			Heparin	Enhance proteolytic activity	Protective	(20)
			Heparin	Increases activity	Protective	(47)
C1, C4, C2	CP	Form together C3 convertase	Heparin	Inhibit interactions	Protective	(56)
C4b	CP	Part of the C3 convertase	Heparin	Inhibit interactions	Protective	(56)
C1q	CP	Initiates CP	Heparin derived tetrasaccharide (HS-4)	Limited C1 activation (poor activators)	protective	(44)
			Heparan sulfates, heparin	Inhibitory	Protective	(44)

Table 1.2 – *Continued from previous page*

			Heparin, dextran sulfate, heparan sulfate, Proteochondroitin sulfate (CSPG)	CSPG inhibits hemolytic activity C1q and C1	Protective	(45)
C3b	Ap	Part of C3 convertase	Heparin	Inhibits formation C3 convertase	Protective	(56)
C3b	AP		Heparin	Inhibits C3 amplification convertase by inhibiting the binding site for B on C3b	Protective	(57)
fB	AP	active enzyme of C3 and C5 convertase	Heparin	Augments alternative pathway activity by favoring interactions factor B with other constituents (heparin solid phase bound)	Damaging	(58)
fH	AP	Inhibits alternative pathway	Heparan sulfate		Protective	(59)
			Heparin			(60)
			Heparan sulfate agrin, heparin			(61)
			Heparin, dextran sulfate	Augments affinity for C3b	Protective	(62)
			Heparin, heparan sulfates		Protective	(63)
FHR-5	AP	Inhibits C3 converatase activity in fluid phase assay	Heparin	Assists positioning FHR-5 on cell surface		(64)
Properdin	AP	Stabilizes C3 convertase, initiates AP	sulfated glycoconjugates, dextran sulfates, heparin	DS, heparin: Inhibition activated form	Protective	(65)
			Heparin	Prevents formation of properdin-stabilized amplification C3 convertase	Protective	(66)
			Cell surface heparan sulfates	Act as a docking platform for AP initiation by properdin	Damaging	(27)
			Fluid phase heparin and heparan sulfate	Inhibits properdin from binding to surface	Protective	(27)

Table 1.2 – *Continued from previous page*

C1, C4, properdin, factor B	All	Form together/stabilize C3 convertase	Natural sulfated polysaccharides (fucans)	Inhibits formation of C3 convertase	Protective	(67)
C1q, C2, C4, C4bp, C1INH, B, D, H, P, C6, C8, C9	All		Heparin			(40)
C1r, C1s, C3, fI, C5, C7, C3b, Ba, Bb	All		NOT with heparin			(40)
C5a	All	Stimulates inflammation, eosinophil aggregation	Heparin	Binds/inhibits C5a	Protective	(68)
CD11b/CD18	-	Complement receptor, mediates also transepithelial migration	Heparan sulfate of CD44v3 (a glycoprotein)	Facilitates polymorhpnuclear leukocyte recruitment during inflammation		(69)
	All	Integrin receptor, complement receptor	Heparin	Antagonist iC3b	Likely protective	(70)
CFHR1	All	Inhibits C5 convertase, blocks MAC formation	Heparin			(71)
Ecto-protein kinase	All	Phosphorylates C9, which lowers membranolytic activity	Heparin	Inhibitory	Damaging	(72)
Vitronectin	All	Inhibitor assembly MAC	Heparin, heparan sulfates	Neutralizes heparin function on antithrombin		(73)
			Heparin	Binds vitronectin to cell.	Protective	(74)
			Heparin	Vitronectin + heparin + MAC are rapidly removed by the liver	Protective	(75)
			Low molecular weight dextran sulfate	Inhibits complement activation more potent than heparin	Protective	(76)

Heparin and MBL pathway components interaction (Antithrombin / MASP - 1, 2)
Little is known about the interactions of GAGs with MBL or the MASPs. Nevertheless, it has been reported that the anticoagulant antithrombin can inhibit both MASP-1 and MASP-2, provided that heparin is present as well (50, 51, 55). Furthermore, the heparin augmented inhibition of MASP-1 by antithrombin is even stronger than the inhibition achieved by C1INH (Table 1.2) (50).

Heparin and AP components interaction (C3b, factor B, factor H, properdin)
As described above, C4BP is a polyanion-binding protein that regulates both the CP and AP. In addition, there are several other proteins involved in the AP that can interact with GAGs. C3b is one of these proteins, which together with the factor B forms the C3 convertase of AP. Fluid phase heparin can inhibit the formation of this convertase by blocking the C3b binding site for factor B (56, 57).

Factor H is the main fluid phase regulator of AP which helps to distinguish between self and non-self cell surfaces by binding to host cell surface. Essential for this function is the recognition of host cell polyanions like sialic acids and GAGs such as HS. The interaction of factor H with heparins results in an increase of the affinity for C3b. Also polyanions like heparin, dextran sulfate, CS-A, carragenan and sialoglycopeptide, which mimic the naturally occurring GAGs, can enhance the affinity of factor H for C3b. In contrast, bacterial polysialic acid is not able to activate factor H (62, 77). Factor H has two binding sites for GAGs and mutations in these GAG binding regions have been linked to several diseases caused by an overactive complement system, including membranoproliferative glomerulonephritis type II, inherited atypical hemolytic uremic syndrome, and age related macular degeneration (28, 63). Two other proteins that belong to the factor H family have been shown to interact with GAGs as well: the complement factor H related protein 1 (CFHR1), which prevents formation of the C5 convertase of AP, and the complement factor H related protein 5 (CFHR5), which has similar activities as factor H. Both can bind heparin, which assists the proteins to locate host cell surfaces (64, 71).

Properdin is the only positive regulator of AP. It stabilizes the C3 convertase of AP and it can initiate the AP by binding to target cells (26). Interaction of properdin with dextran sulfate and heparin has been shown (65). Kemper *et al* have shown that properdin binds to GAGs on apoptotic T-cells and activates AP (29). Recently, we showed that on tubular cells during proteinuria, HSPG binds to properdin and acts as a docking platform for AP activation (Table 1.2) (27).

Heparin and terminal components of complement cascade interaction (C5a, Vitronectin, receptors: CR3, CR4)
All complement pathways converge in terminal complement activation, which is marked by the production of the anaphylatoxins C3a and C5a and the formation of the MAC. Heparins can also alter these processes. It has been shown that high doses of heparin can reduce C5a-mediated eosinophil aggregation *in vitro*, which might be mediated by binding and neutralizing C5a. The polysaccharide fucoidin, but not DS can have the same action as well (68).

Vitronectin (also known as complement S protein) inhibits complement mediated cell lysis in two ways: It inhibits the binding of C5b-7 to a membrane and prevents C9

polymerization (74). The active, MAC-bound form of vitronectin is able to bind heparin via a GAG-binding domain (78). The main role of this interaction is likely to position vitronectin on self cell surfaces to protect the own tissue from complement induced damage (74). Table 1.2 represents a summary of complement-PGs interaction.

1.5 Proteoglycans in kidney

Normal kidney
In renal tissue, PGs are present in all the basement membranes, ECM and on the surface of the cells (tubular cells, endothelial cells and leukocytes). Agrin, perlecan and collagen XVIII are the three most abundant HSPGs in renal basement membranes of the normal renal tissue during development as well as in the adult kidney (79). Among the cell surface HSPGs, syndecan-1 is found on epithelial cells during development while it is barely present in adult kidney (80). Syndecan-4 has also been shown to be extensively present during kidney development on mRNA levels (81). Moreover, mice deficient for Hs2st (uronyl 2-*O*-sulphotransferase) and HS glucuronyl C5 epimerase showed kidney agenesis (12). Altogether, PGs are important for renal development and basement membrane function.

Inflammation in the kidney
First response of the immune system to inflammation is via innate immune system (see above). Leukocyte recruitment and complement system activation are two essential feature of innate immune system activation. PGs play important roles in both processes. Leukocyte recruitment starts with leukocyte rolling on endothelial cells, followed by activation of leukocytes and their adhesion to vessel wall and transmigration of leukocyte to the target tissue. PGs play an important role in leukocyte recruitment by their interaction with L-selectin (rolling), endothelial bound chemokines (activation of leukocytes), intergrins (adhesion) and making chemokine gradients in inflamed renal tissue (39). It has been shown that upon I/R injury in kidney, microvascular basement membrane HSPGs gain the ability to bind to L-selectin and monocyte chemoattractant protein- 1 (MCP-1) (82). The suggested underlying mechanism is loss of HSulf-1 (one of the extracellular endosulfatases that specifically cleave 6-*O*-linked sulfate residues of HSPGs) expression on endothelium (82). The alteration of HSPGs in various types of renal diseases in the tissue and in urine has been reported (83). An increase in leukocyte recruitment in syndecan-1 knockout mice upon anti-glomerular basement membrane has been shown (84). Fragments of small leucine-rich proteoglycans (such as decorin and biglycan) might activate toll like receptors upon tissue injury and thereby enhance inflammation (85). All these studies together with many others, indicate the importance of PGs specially HSPGs in inflammation in kidney.

1.6 Innate immunity, complement system in kidney

Innate immunity and particularly the complement system participate in renal injury and repair. Our particular interest in renal diseases includes ischemia/ reperfusion (I/R), transplantation and proteinuria. These three conditions have been discussed in more de-

tails below.

Ischemia/Reperfusion
I/R is the leading cause of acute kidney injury in transplanted and native kidneys. Early in ischemia phase, hypoxia and anoxia initiate the cell injury, while in reperfusion phase all innate immune components (complement factors, cytokines, chemokines and inflammatory cells) reach the tissue via blood stream and aggravate the inflammatory responses (8). Many studies have shown the involvement of innate immune system in kidney damage after I/R. Renal I/R induces renal synthesis or activation of pro-inflammatory cytokines, chemokines and complement factors, and recruits leukocytes into the post-ischemic kidneys. Renal tubular cells have been shown to express TLR2 and 4 and they are able of producing a number of complement factors in response to pro-inflammatory cytokines (86, 87). Complement system has been shown to be activated mainly via AP and MBL pathways after renal I/R (11, 88, 89). Macrophages, neutrophils, NK cells and DCs have been shown to be a part of I/R induced renal injury by direct phagocytosis of target cells or by producing inflammatory cytokines (8).

Transplantation
The innate immune system has been shown to play a role in development of chronic kidney disease. Involvement of cellular innate immunity in renal fibrosis has been studied widely. The progression of kidney disease to renal failure correlates with infiltration of mononuclear immune cells into the tubular interstitial compartment. These cells consist of macrophages, DCs, T cells and mast cells and they both initiate and amplify the inflammation. Renal fibrosis and loss of capillaries result in tissue hypoxia and activation of TLRs and innate immunity (discussed above under involvement of innate immunity in I/R) (6).

Moreover, innate immunity is involved in acute and chronic graft rejection in renal transplantation. Activation of the innate immune system can directly lead to organ damage and can also enhance the specific anti-donor immune response of the recipient (90). Rejection process can be initiated by damage-associated molecular pattern molecules which activate TLRs and leads to activation of innate immune system. TLRs activation results in maturation and activation of DCs and promotes acute rejection. Meanwhile, activation of complement system and release of anaphylatoxins (C3a and C5a) leads to lysis of cells and recruitment and activation of APCs and T cells (15). Involvement of AP in chronic allograft rejection has been recently shown by Nakorchevsky *et al.* by a large-scale proteogenomic analysis approach (10).

Proteinuria
Proteinuria is a marker of renal damage regardless of the etiology of the disease. In addition, proteinuria itself leads to kidney function loss and scarring thus progression of renal disease (91).

Next to secondary inflammatory injury and ischemia as mechanisms of injury in the tubulointerstitium, proteinuria itself is now recognized as a pathogenic factor and an independent risk factor for the progression of kidney disease (92).

Proteins in the ultrafiltrate can affect the tubular epithelial cells by direct toxic effect or by stimulating tubular cells to produce pro-inflammatory and pro-fibrotic factors.

Proximal tubular cells challenged with serum proteins showed an overexpression of MCP-1, RANTES, IL-8 and complement factors (7). Stimulation of cultured tubular cells with albumin resulted in increased gene expression and production of TGF-β which is the most important cytokine for renal fibrogenesis (93). These chemokines and anaphylatoxins can attract monocyte/macrophages and lead to enhanced inflammation. Complement factors have been shown to have a major contribution to proteinuria derived tubular injury. This contribution can occur at least via two mechanisms, first by presence of complement factors in ultrafiltrate because of an altered glomerular barrier, and secondly by intra-renal production and activation of complement by tubular cells (7). Tubular cells have been shown to produce a large number of complement factors in normal condition which is enhanced upon stimulation with proteins, immune complexes and cytokines (7, 94, 95). Reduction of proteinuria is now a major therapeutic goal in reducing risk for progression of renal injury.

1.7 Possible targets for intervention in the kidney

Since PGs are involved in various levels in innate immune response, it is logical to think of targeting them for the therapeutic purpose. As summarized in this review, HS, heparin and other GAGs are able to interact with a variety of complement proteins, leukocyte adhesion molecules and cytokines. HSPGs may at times act as pro-inflammatory and at other times as anti-inflammatory, with their function being determined by their location (cell surface or ECM), the heparin-binding molecules they associate with, the presence of modifying enzymes (i.e., heparanase) and the precise structural characteristics of the PG. Based on the pathway of inflammation which is involved in the process of the disease, various heparinoids can be used to target the interaction of the inflammatory factors with PGs. In this approach the pathway of interest can be targeted specifically, therefore the undesired side effects of immune suppression can be avoided to a certain extent. However, before using GAGs as a therapeutic agent in renal diseases, several points have to be taken into account.

Heparin is a molecule which has a very broad spectrum of actions, with the most striking its anticoagulant activity. Thus, patients who are treated with heparin to prevent renal damage, also experience impaired coagulative function, which is undesirable. Moreover, heparin treatment can have side effects like heparin-induced thrombocytopenia type II (HIT II), an acquired auto-immune disease which is due to interaction with platelet factor 4 (96). Therefore, a HS or heparin derivative that specifically binds to proteins of complement system, but not to ATIII, platelet factor 4 or other coagulation ligands would be needed. Due to important role of AP in proteinuria, I/R and renal transplantation, a polysaccharide that interacts only with proteins involved in that pathway would be the drug of choice.

As discussed in this chapter, various components have different heparin/HS binding properties. Thus, the candidate heparinoid for intervention should be chosen wisely, based on the pathway and protein of interest. For achieving an optimal therapeutic result, it is better to know the heparin/HS binding properties of the protein of interest. Otherwise the candidate heparinoid can interact with other inflammatory components and result in undesired consequences.

Up to now, much effort has been made to design heparin derivatives with specific, predictable binding properties for various purposes, including anti-cancer and anti - inflammatory therapy (34). An important finding in the field of renal transplantation has been made by Zhou and colleagues. They used a rat model to demonstrate that *N*-desulfated heparin, which has a lower anticoagulant function than normal heparin, is able to reduce the renal damage after I/R significantly (97). Collino *et al.* have shown that a semi-synthetic *N+O*-sulfated GAG K5 polysaccharide derivative was able to reduce the apoptotic and inflammatory reaction upon I/R in the brain, while it had only limited anticoagulant activity (98). *Escherichia coli* capsular K5 polysaccharide, has identical structure to the non-sulfated precursor of HS and heparin. K5 polysaccharide has the advantage of being modified in several ways (enzymatically and/or chemically) and it is not animal-derived (98). Beside of this semi-synthetic method, it is also possible to chemically synthesize heparin-like polysaccharides de novo, which have the same advantages. For this method, the HS/heparin binding sequence of the target protein and the structural features of the HS/heparin should be known for a rational design of a synthetic drug. Subsequently, a series of penta- or tetrasaccharides can be developed which differ for example in sulfation and methylation. The best new drug candidate is selected by testing the affinity of these compounds for the target protein and for proteins with which no interaction is desired (99, 100). So far, two fully synthetic anticoagulants produced in this way are in use in the clinics, and others are in development (101). The disadvantage of this approach is that it requires a lot of effort and is very expensive. Moreover, only rather small ($\leq$8 monosaccharide units) oligosaccharides can be made synthetically. To reduce the required steps in the synthesis of new HS/heparins, recently a study has been published which aims to set up a HS/heparin library for synthetic oligosaccharides synthesized from disaccharide building blocks. These blocks can be analyzed for their binding properties before using them in a drug (102).

A further possibility is to use other sulfated polysaccharides rather than HS. For example, natural sulfate polysaccharides (fucans) isolated from brown seaweed exert anti-inflammatory and anti-complementary function while they have only little anticoagulant function (67). The semisynthetic glucan, dextran sulfate, can inhibit all three pathways of complement activation and NK-cell activation and it has been shown that dextran sulfate treatment can protect from complement-induced damage in xenotransplantation (103). However, dextran sulfate has strong anticoagulant function as well, and should be chemically modified before being used in the treatment of patients.

In fact, heparinoids have been used for treatment before. The anti-cancer effects of heparinoids are mostly exerted through competition with endogenous HS chains and it has been extensively reviewed before (104). The beneficial effects of non-anticoagulant heparinoids has been shown in I/R and inflammation before (105, 106).

Several investigators have administered heparin compounds, in renal diseases. Striker *et al.* have shown the effect of oral heparinoids on glomerulosclerosis, arteriosclerosis and vascular graft stenosis by using transgenic mice for bovine growth hormone (107). Treatment of mesangioproliferative glomerulonephritis with non-anticoagulant heparinoids was shown to be effective in acute phase and long term (108). The potential nephroprotective effects of GAGs have been recently discussed by other groups (109, 110).

In renal diseases, most of the attention has been paid to treatment of proteinuria in diabetic nephropathy (111–115). Among the heparinoids used for proteinuria treatment,

sulodexide (a mixture of heparin and dermatan sulfate act as a heparanase inhibitor) have been studied extensively. Gambaro *et al.* (114) showed that treatment with sulodexide for 4 months significantly lowered the albumin excretion rate in the type 1 or type 2 diabetic patient with either microalbuminuria or overt nephropathy. They also reported that the decrease in albumin excretion was maintained for 2 months after the drug was stopped. Later on, another trial in type 2 diabetic patients confirmed the results of the previous study and provided enough evidence for a large definitive trial (115). Although recently two large-scale clinical trials failed to show the effect of sulodexide in proteinuria treatment in diabetic patients (116, 117), the interest in sulodexide and other GAGs as potential treatment options in renal disease still exist.

Nevertheless, a large body of literature supports that interaction of PGs with the innate immune system particularly the complement system is of special importance in the pathogenesis of proteinuria and I/R injury in kidney. Thus, these interactions seem to be promising points for interventions. However more research on the details and nature of these interactions is needed to provide additional information for therapeutic targeting.

1.8 Aim of this thesis

In this thesis, we focused on how the interactions between PGs and innate immunity contribute to renal tissue injury in proteinuria, I/R and transplantation. Chapter 1 (general introduction) summarizes innate immune involvement in renal diseases. Additionally, the interactions of PGs with components of innate immune system are reviewed with a focus on the complement system, and an overview of therapeutical potential of heparinoids is discussed. To investigate various aspects of innate immunity in renal diseases, we performed studies on the AP activation and leukocyte influx in proteinuria, I/R and transplantation in this thesis. As described earlier in this chapter, the presence of complement factors in the ultrafiltrate during proteinuria has been shown before (7, 118, 119). Chapter 2 focuses on the mechanism of properdin mediated AP activation on tubular cells. It has been shown that properdin is present on the brush borders of proximal tubular cells in proteinuric patients (120).We aimed to characterize the properdin docking structure on the tubular cells which leads to the AP activation on these cells. We demonstrated that tubular HSPGs act as a docking platform for properdin binding and result in the AP activation. Since, factor H which is the main AP inhibitor, is also shown to interact with HSPGs (61), and factor H is also present in the ultrafiltrate during proteinuria (121), we aimed to compare the binding properties of PGs for properdin and factor H. Therefore, in Chapter 3, we first confirmed the HSPG mediated binding of factor H to the tubular cells. Second, we characterized the nature of properdin and factor H interaction with PGs. We showed that properdin and factor H recognize different binding sites on tubular HSPGs. Thus, the composition of tubular HSPGs plays an important role in the AP regulation in proteinuria and exogenous heparinoids might regulate AP activation on tubular cells. Next, we checked the possibility of using (non-anticoagulant) heparinoids for treatment of innate immune response during experimental chronic transplant dysfunction. In Chapter 4, we used a rodent transplantation model to show that some non-anticoagulant heparinoids modulate leukocyte influx and complement to renal tissue after transplantation. To investigate the role of basement membrane PGs in the leukocyte

influx in more details, in Chapter 5, we used an I/R rodent model. Knockout mice for basement membrane collagen/HSPGs showed a better renal function and less renal tissue damage and inflammatory cells influx after I/R compared to wildtype mice. Furthermore, in Chapter 6, we reported an association between factor H genetic variants with end stage renal disease and acute rejection in a human renal transplant population. The results of Chapters 2-6 have been discussed in Chapter 4. Overall, in this thesis the role of renal HSPGs in innate immune system mediated renal injury is clarified. The findings in this thesis emphasize that PG-innate immune components interaction can be important for targeted therapy in renal diseases.

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