Renal heparan sulfate proteoglycans
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Summary, General Discussion & Future Perspectives
In this thesis we explored the role of heparan sulfate proteoglycans (HSPGs) in renal inflammation, with a focus on leukocyte migration and complement activation. We experimentally showed the crucial role of (sub-)endothelial HSPGs on renal leukocyte infiltration in diabetic kidney disease, ischemia/reperfusion (I/R) and renal transplantation. In a rat renal transplantation model, we demonstrated that treatment with heparin (derivatives) doesn’t lead to improved graft function, despite reduced inflammation, most likely due to increased lymphatic migration of antigen presenting cells as a result of heparin (derivative) treatment. In the second part of the thesis we showed HSPGs to function as docking platforms for complement components. We showed that binding of the alternative complement pathway component properdin to tubular epithelial cells is largely syndecan-1/heparan sulfate dependent, but, in contrast to other sites, does not require initial C3b binding and efficiently initiates the alternative route of complement. We furthermore showed that bound properdin can be dissociated from cellular heparan sulfate by tick protein SALP20, a powerful inhibitor of the alternative pathway of complement. Moreover, we demonstrated the potential of heparan sulfate (HS) and heparin derivatives to specifically inhibit the lectin pathway of complement via MASP enzyme inhibition and that HSPGs might dock the MBL/MASP complex.

About a decade ago, Wang and colleagues elegantly showed that endothelial HSPGs are major contributors to leukocyte recruitment. It was shown that endothelial HSPGs are ligands for L-selectin and play a role in chemokine presentation to high affinity receptors on leukocytes (1). The group of Wang, we and others subsequently explored the role of endothelial HSPGs in various diseases concluding that the interaction between endothelial HSPGs, L-selectin and chemokines can be a target for anti-inflammatory treatment (2-5). In chapter 3 we showed, in line with these results, that glomerular and tubulo-interstitial leukocyte recruitment in experimental diabetic kidney disease is attenuated by endothelial deficient in sulfated HSPGs. Moreover and more importantly, we showed a protective effect of under sulfated endothelial HSPGs on renal fibrosis, which has not been shown before. The relationship between HSPGs and fibrosis has been known for quite some time (6) and our group already demonstrated that HSPG expression is changed under pro-fibrotic circumstances (7). In line with these findings, Alhasan and colleagues showed that fibrosis in chronic transplant dysfunction is accompanied by an increased expression of 6-O sulfated heparan sulfates on tubular epithelium and that 6-O sulfation increases the binding of FGF-2 to tubular epithelial cells, leading to increased pro-fibrotic signaling (8). Although these studies focused primarily on the effect of HSPG changes in epithelial cells, we showed in chapter 3 that under sulfated endothelial HSPGs prevent glomerulosclerosis and interstitial fibrosis. Whether endothelial HSPGs influence the fibrotic process directly or indirectly via prevention of inflammatory leukocyte influx we can only speculate on. It is known that inflammation and fibrosis are closely linked to each other (9). However a direct effect of under sulfated endothelial HSPGs on fibrosis due to a disruption of fibrocyte influx, disruption of
chemokine signaling to myofibroblasts or disruption of the signaling pathways of endothelial to mesenchymal transition could also contribute to the effect seen. Further research on how under sulfated endothelial HSPGs contribute to an anti-fibrotic effect could be interesting both from a clinical and mechanistic point of view.

In the research field of proteoglycans and inflammation there is some controversy on the question whether endothelial basement membrane (BM) or endothelial surface layer (ESL) HSPGs are the major contributors to leukocyte recruitment. From the data in chapter 3, and other publications, it becomes clear that endothelial HSPGs are involved in leukocyte recruitment. However since endothelial cells produce both the ESL and in part the BM HSPGs, no firm conclusions could be drawn from these experiments on the localization of involved endothelial HSPG (2,3). Therefore in chapter 4 we studied in vitro and in vivo the effect of collagen XV and XVIII deficiency on leukocyte recruitment in an ischemia reperfusion (I/R) model. Collagen XV and XVIII are collagen/HSPG hybrid molecules which are present in the abluminal BM zone of blood vessels. We demonstrated that collagen XV and XVIII are essential for macrophage and neutrophil recruitment, indicating that these BM HSPGs play a dominant role in leukocyte transmigration. It is thought that ESL HSPGs are primarily involved in binding of L-selectin and presenting chemokines to high affinity receptors on leukocytes. It has however also been shown that deficiency of syndecan-1, an ESL HSPGs, results in increased leukocyte transmigration (10). Therefore a second theory constitutes that the ESL under normal circumstances is covering E- and P-selectin and adhesion molecules such as ICAM-1 and VCAM-1 on the endothelial cell, but degradation of the ESL under inflammatory stimuli reveals these selectins and CAMs, thereby aiding in leukocyte transmigration. It is for example thought that the anti-inflammatory effect of sulodexide, a mixture of heparan sulfate and dermatan sulfate, is partially because of the inhibition of heparanase-1, thereby inhibiting the degradation of the ESL (11,12). The roles of the ESL in leukocyte recruitment have recently been nicely summarized by Marki et. al. (13). The contribution of BM HSPGs is thought to be more in terms of chemokine gradient stabilization over the entire width of the basement membrane, which is essential to overcome the physical BM barrier for leukocyte extravasation. Thus, in chapter 4 we showed that deficiency of the endothelial BM for HSPGs can obliterate the transmigration of neutrophils and macrophages. This finding is in line with experiments of Celie and colleagues who showed that under inflammatory circumstances, BM HSPGs convert into major chemokine, but also L-selectin binding entities. This indicates that BM HSPGs might be more than just chemokine presenters in leukocyte transmigration (4). Comprehensive models should be developed to study the exact contribution of ESL and BM HSPGs in leukocyte recruitment.

Knockout studies for endothelial and BM HSPGs as shown in chapter 3 & 4 suggest that the interaction between HSPGs and chemokines could be a target for therapy in inflammatory renal diseases. Targeting the chemokine - HSPG
interaction to reduce inflammation has been tested in various experimental models using different methods. Gschwandtner and colleagues used a CXCL8 mutant with high affinity for GAGs but without signaling activity in a model for lung fibrosis, uveitis and urinary tract infection. In all experimental models the treatment with the decoy protein resulted in reduced leukocyte transmigration (14). In experimental renal chronic transplant dysfunction, treatment with low molecular weight reviparin resulted in reduced leukocyte accumulation in the interstitium and improved graft function compared to controls (15). Trials to target the chemokine HSPG interaction in humans have been undertaken by targeting MCP-1. It has been shown in a phase II clinical trial that treatment with a MCP-1 neutralizing spiegelmer (emapticap pegol) can reduce the albumin creatinine ratio in diabetic nephropathy patients (16). These positive results in clinical trials open the opportunity to investigate whether the chemokine HSPG interaction is a viable target in other inflammatory diseases as well.

In chapter 5 we treated allografted rats with heparin and two heparin derivatives to interrupt the chemokine/HSPGs interaction and subsequently reduce leukocyte migration in this model for chronic transplant dysfunction. It had already been shown that heparin derivatives like reviparin could reduce leukocyte migration and allograft dysfunction (15). However these studies were done with anti-coagulant heparin derivatives, which can be a disadvantageous feature in anti-inflammatory treatment when anti-coagulation is clinically undesirable. Therefore, in chapter 5, we included two heparin derivatives lacking anti-coagulant action. One of the non-anti-coagulant heparins, glycol-split heparin, showed a reduction in leukocyte accumulation in the interstitium, however, failed to improve graft function. To the contrary, treatment with glycol-split heparin seemed to reduce graft function, although not significantly. We showed in chapter 5 that this is related to increased antigen presenting cell migration towards the lymphatic system. Migration of antigen presenting cells over the lymphatic endothelium is, like vascular leukocyte migration, chemokine gradient mediated. Two majorly involved chemokines in this process are CCL19 and CCL21. Of the latter we showed that it can bind glycol-split heparin with a higher affinity than perlecan, a major constituent of the (lymph-) endothelial BM and a stabilizer of the chemokine gradient. Together with literature showing that CCL21 binding to glycosaminoglycans (GAGs) results in oligomerization, leading to increased functionality of CCL21 (17), we conclude that the reduction in graft function in glycol-split heparin treated animals may be due to an increased antigen presenting cells migration towards the lymphatic system. This increased migration can then lead to increased antigen presentation in lymph nodes resulting in a stronger allograft response by the acquired immune system. Glycol-split heparin differs significantly from the LMW heparin reviparin in length and molecular flexibility. This could explain the different results in our study compared to other studies using heparin (derivatives) in inflammatory models. The stimulatory effect on lymphatic migration of glycol-split heparin is disadvantageous in treating chronic transplant dysfunction, however could be useful in the cancer research field where it
can be of interest to stimulate antigen presenting cell migration to strengthen the acquired immune response against malignant cells. Therefore stimulating APC migration using heparin derivatives could increase the response to therapy (18).

Since the results from chapter 5 show that high affinity for multiple chemokines can lead to unwanted effects, it can be concluded that specific affinity for one target in the inflammatory cascade should be an important feature of GAG based anti-inflammatory agents. For the use of GAGs in targeting the chemokine HSPG interaction, a library of GAGs should be tested for affinity for multiple chemokines involved, as has been done for the complement system in chapter 8, to prevent unwanted effects as seen in chapter 5. Thereafter the effect of GAG binding on chemokines should be thoroughly investigated since most GAG/chemokine interactions facilitate oligomerization, increasing their effectiveness as has been demonstrated in vivo (19,20). It might be interesting to test whether GAGs can inhibit the oligomerization of chemokines. Small heparin fragments, as have been shown to be specific and functional in chapter 8, might be a suitable candidate to bind the GAG binding epitope on chemokines, without stimulating oligomerization. It has e.g. been shown in vitro that heparin tetrasaccharides have an inhibitory effect on the interaction between CCL5 and CCR1 (21). Other options can be to target leukocyte entry from the vasculature via the chemokine/HSPG interaction. Targeting chemokines using monoclonal antibodies, e.g. against MCP-1, has been discussed before in this chapter. Studies using siRNA to reduce GAG sulfation, via the inhibition of the transcription of sulfotransferases, have shown to reduce macrophage influx and improve respiratory function in elastase induce pulmonary emphysema (22). Concluding from chapter 2, 3, 4 & 5, we think that targeting the chemokine/HSPG interaction can be a viable target in renal inflammatory mechanisms. Multiple methods to target this interaction have been shown to be effective in different experimental models.

One of the interesting findings from chapter 5 is the ability to influence the effectiveness of lymphatic chemokines and thereby increase lymphatic migration of leukocytes. In chapter 5 we unfortunately showed an increase in the lymphatic leukocyte migration while in transplantation a reduction in lymphatic leukocyte migration could be beneficial for the patient. Inhibition of the CCL21/CCR7 signaling has already been shown to improve graft function in corneal transplantation, by reducing dendritic cell migration to the draining lymph node by treatment with a CCR7 blocking agent (23). Whether an inhibiting effect on the CCL21/CCR7 axis can be repeated by a GAG based treatment is unknown. Heparin treatment of CCR7 carrying leukocytes did not influence migration over high endothelial venules (24). Targeting CCL21 with unfractionated heparins to reduce lymphatic leukocyte migration has been proven difficult in chapter 5. Literature shows that binding of heparin to CCL21 induces oligomerization and therefore increased function of CCL21 (25). Although it has been shown in vitro that heparin can also inhibit the function of CCL21 and reduce lymphocyte transmigration across high endothelial venules (24). This indicates that there might be possibilities for GAG based treatment in lymphatic leukocyte migration; however
the exact influence of heparin (derivative) binding on CCL21 effectiveness should be studied first.

Many anti-inflammatory therapeutics released in the past few years like eculizumab and golimumab are monoclonal antibodies and therefore protein based. However these protein based therapeutics have some disadvantages like production costs, susceptibility to proteolysis and, in case of monoclonal antibodies, the necessity of intravenous access and the development of an immunological response against the therapeutic antibody. These disadvantages can be overcome by using GAG based therapeutics, although chapter 5 reveals one of the major problems in GAG based therapy namely specificity. Other factors important in therapeutic application like bio distribution and type of chemical bonding are relatively comparable between glycans and protein based therapies. Concerning ligand specificity, GAGs are synthesized in the Golgi apparatus by polymerization of the monosaccharide units and are subsequently sulfated by a number of enzymes. The degree of sulfation as well as the position of the sulfate groups determines the binding affinity for different ligands. Although a disaccharide unit can be sulfated on the N-, 2O-, 3O- and 6O- positions, leading to a wide variety of GAGs, these polysaccharides are not produced to bind specifically one ligand, unlike antibodies. Antibodies are produced to target one epitope on a ligand and are negatively selected for cross reaction to auto antigens. This difference in synthesis process makes the glycan more prone to have affinity for multiple epitopes and therefore more prone to cross react and subsequently more prone to side effects, as seen in chapter 5. Nevertheless, glycan based therapy has major advances too, e.g. easy and cheap production, and relatively easy administration, although subcutaneous injection is still needed. Decades of experience with heparin as anti-coagulant with relatively minor side effects, shows that GAG based therapy targeting inflammation should be considered a viable option.

Besides leukocyte recruitment we also investigated the role of HSPGs in complement activation in this thesis. The current standings in the treatment of complement using GAGs were reviewed in chapter 6. Literature has shown that properdin, a positive regulator of the alternative pathway (AP), is an important mediator in proteinuria induced tubular epithelial damage (26,27). Subsequently our group revealed that under proteinuric conditions properdin bind to HSPGs on proximal tubular epithelial cells (PTECs). In these studies the epitope requirements for properdin interaction with HS was determined and it was shown that binding of properdin to PTECs was significantly different from binding to endothelial cells (28,29). However, a study by Harboe and colleagues showed that on endothelial cells binding of properdin is dependent on the initial binding of C3b (30). In chapter 7 we convincingly showed that binding of properdin to PTECs is independent of prior C3b deposition and binding experiments with syndecan-1 knockout PTECs reveal that binding of properdin is largely dependent of syndecan-1. These results indicate that under proteinuric conditions, properdin binds to HSPGs on the surface of PTECs and initiates the formation of the alternative C3 convertase. Whether properdin can be a viable target for intervention in
proteinuria mediated tubular epithelial damage should be investigated.

In chapter 7 we furthermore show that Salp20, a tick saliva protein, binds with high affinity to properdin, independent of initial C3b binding, prevents binding of properdin to HSPGs and can dose dependently inhibit the deposition of C3b on PTECs in vitro. The fact that both the binding of C3b and HSPG can be blocked by Salp20, suggests that C3b, HSPGs and Salp20 share the same binding epitope on properdin. However, delicate work by two separate groups showed that the epitopes on properdin for C3b and sulfated glycoconjugates are not the same but positioned close to each other. They showed that C3b and HSPG binding is dependent on the trombospondin-1 repeat 5 of properdin. However trypsin treatment of properdin resulted in an altered trombospondin-1 repeat 5 and thereby abolished C3b binding while sulfated glycoconjugates still bound (31,32). Nevertheless it is interesting that Salp20 can inhibit both the binding of C3b and HSPGs to properdin and this bimodal inhibiting effect could be beneficial for developing an AP blocking agent for proteinuric patients. As discussed in the thesis introduction, many renal diseases are characterized by AP activation and therefore Salp20 mediated inhibition of properdin could be a viable target for treatment of AP mediated renal diseases. In OVA-induced asthma and elastase induced abdominal aortic aneurysm Salp20 has already demonstrated a reduction in AP activation and subsequent injury (33). If the immunogenicity of Salp20 can be tackled, further research should be undertaken to evaluate the clinical potential of this powerful inhibitor.

In chapter 6 and 7 we discuss multiple interactions between GAGs and complement factors, and present literature that shows that heparin can bind a variety of complement factors with high affinity (34). To evaluate whether this interaction can be targeted using heparan sulfate/heparin derivatives, we tested a library of GAGs for their complement inhibiting potential in chapter 8. From the library of GAGs we could not identify a specific classical pathway (CP) or AP inhibitor. However, we demonstrated that small heparin- and heparan sulfate-derived oligosaccharides specifically inhibit the lectin pathway (LP) of complement and that this is most likely by inhibition of the MASP enzymes. The direct effect of GAGs on the MASP enzymes should however be confirmed in experiments with recombinant MASP enzymes. Nevertheless it is an interesting finding since the LP has been identified as a contributor to several renal diseases like I/R and transplantation, but also diabetic nephropathy, as we have shown in chapter 3. Interestingly, inhibition of MASP-2 using monoclonal antibodies has been shown to reduce LP mediated experimental gastrointestinal I/R injury (35) and clinical phase II trials are now performed to evaluate the effect of these monoclonal antibodies in patients suffering from thrombotic microangiopathies. However, complete consensus is not yet reached on the exact mechanism by which the LP causes injury in I/R, either via the canonical LP activation pathway, or via a LP activation independent pathway. Doubt is raised by a delicate study showing that MBL can be internalized by tubular epithelial cells after reperfusion injury and display pro-inflammatory effects separate of LP activation (36). One could
speculate about the mechanism of internalization, but it could well be that the interaction between GAGs and the MBL/MASP complex is a mediator in the internalization process.

MASP enzymes belong to the family of serine proteases. Interactions between GAGs and serine proteases (inhibitors) have long been known, of which the interaction with anti-thrombin III is most studied (37). Studies concerning the interaction of GAGs with anti-thrombin III indicate that a penta-saccharide is the smallest GAGs able to stimulate the effect of anti-thrombin (38). This suggests that the tetra-and hexa-saccharides tested in chapter 8 cannot inhibit anti-thrombin III. Whether this is the case, and whether the tested tetra-and hexa-saccharides can influence the function of other serine proteases (inhibitors) should be tested. Unfortunately our results in chapter 8 indicate that inhibition of the MASP enzymes requires relatively high concentrations of small heparin fragments, although affinity studies should be performed in surface plasmon resonance experiments to confirm this. Nevertheless, this indicates that it might be hard to achieve therapeutically sufficient plasma levels in clinical and experimental treatment. However we do show that it is possible to inhibit a specific complement pathway using small GAGs with a reasonable affinity, which might form the basis for further development of small glycan-based specific MASP inhibitors.

Future perspectives

Classic anti-inflammatory drugs like prednisolone are nonspecific inhibitors of the immune system, inhibiting the activation of pro-inflammatory cellular pathways. This entails that they are powerful inhibitors and are usable in a wide variety of inflammation mediated diseases. The downsides to this however are the non-specific inhibition and multiple immunological and non-immunological side effects, predominantly on the long run. Therefore the need for new, more specific anti-inflammatory drugs is clear. In this thesis we targeted two distinct processes in inflammation namely cellular influx from the vascular system and proteinuria induced complement activation on renal tubular epithelium. We have shown like others that targeting the interaction of HS with inflammatory components is a promising way to reduce inflammation. We attempted to target this interaction using GAGs e.g. modified heparin derivatives. However we have only tested a small amount of the different GAGs available. Sources for GAGs can be animals, plants and bacteria, but semi-synthetic or fully-synthetic GAGs can also be produced. The GAGs derived from these sources have different characteristics and can be chemically modified to represent a specific length and sulfation pattern. These characteristics of GAGs can be very influential in their binding properties as can be seen in this thesis. The use of GAGs as a treatment for inflammation has a number of advantages since they are easy to produce and therefore cheap. Moreover, a lot of experience has been obtained with the use of heparin and LMW-heparins as anti-coagulant drugs which can be used in other
GAG based treatments. However, as discussed in various chapters and above, a downside of GAG based treatment is the nonspecific binding of inflammatory mediator’s by GAGs. Although high affinity protein binding by GAGs has been shown before in the interaction between heparin and antithrombin III, affinity for inflammatory mediators is often in the micro molar range instead of the nano molar range like in protein based treatment, as demonstrated in this thesis.

Based on this thesis and on literature it is fair to state that targeting the interaction between endo-and epithelial cells and chemokines, cytokines, complement factors and lectins is a promising strategy to reduce inflammation. The down modulation of specific sulfotransferases and the use of GAG based therapies in inflammation have potential. The finding that endothelial HSPGs play a role in the development of fibrosis has not been shown before. It would be interesting to unravel in which pro-fibrotic pathway the endothelial HSPGs are involved. Moreover treatment strategies targeting endothelial HSPG synthesis or sulfation could be tested in renal models for fibrosis. Targeting the chemokine/HSPG interaction using fluid phase GAGs has been proven difficult, however other ways to target this interaction can be very interesting. Silencing RNA against sulfotransferases might reduce both the migration of leukocytes trough the vascular endothelium, and might also influence the lymphatic migration, since this process is also reliant on the chemokine/HSPG interaction. This thesis also shows potential for small heparin fragments and Salp20 in treating LP and AP mediated diseases respectively. It would be interesting to study whether small heparin fragments can inhibit LP activation on cells and reduce LP mediated renal injury in e.g. experimental I/R. In case of Salp20 the focus should be on determining the properdin binding epitope and on constructing non immunogenic analogues, which should be tested in vitro and in experimental models for their AP inhibiting potential.
References


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