Proteoglycans modulate renal inflammation
Zaferani, Azadeh

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Chapter 4

Non-anticoagulant heparinoid reduces renal inflammation in experimental renal transplant dysfunction

Kirankumar Katta, Azadeh Zaferani, Saritha Adepu, Ditmer Talsma, Pramod Kumar Agarwal, Saleh Yazdani, Annamaria Naggi, Giangiacomo Torri, Gerjan Navis, Jan-Luuk Hillebrands, Jacob van den Born
ABSTRACT

The pathogenesis of chronic transplant dysfunction (CTD) is multifactorial and is histologically characterized by interstitial fibrosis and tubular atrophy, transplant vasculopathy and focal segmental glomerulosclerosis, and is also associated by an inflammatory component. To date, no effective therapies are available to treat renal CTD. Heparan sulfate proteoglycans are crucial players in tissue homeostasis via their heparan sulfate polysaccharide side chains. We hypothesized that heparan sulfate proteoglycans could be promising therapeutic targets to limit CTD, by their involvement in cell recruitment and complement cascades. To test this hypothesis, in a rat model for renal CTD, by daily subcutaneous injections, we intervened with vehicle, unfractionated heparin, and the non-anticoagulant heparinoids (N-acetyl heparin and periodate-oxidized, borohydrate-reduced (RO-) heparin) for 9 weeks. Both these non-anticoagulant heparinoids have been described to exert similar anti-inflammatory properties as heparin. On the one hand, RO-heparin treatment ameliorated the cortical tubulo-interstitial accumulation of CD45+ inflammatory cells compared to the vehicle treated group (week 9: 0.8% surface area in RO-heparin group versus 1.8% surface area in vehicle group; p < 0.02). On the other hand, properdin deposition was increased ~2-fold in RO-heparin compared to vehicle (p<0.05), without affecting factor H, C3 and Membrane Attack Complex deposition. A tendency for lower proteinuria and glomerulosclerosis was present in the RO-heparin group, but not in the other heparin(oid) groups. Heparin(oid) treatment did not affect body weight, graft survival, blood pressure, and renal function. These data demonstrate that specifically modified heparinoids can reduce inflammation in experimental CTD, and suggest that carefully selected non-anticoagulant heparan sulfate glycomimetics could have protective effects as adjunct therapy for CTD.
4.1 Introduction

Chronic transplant dysfunction (CTD) is characterized by decline in kidney function over time and is related to progressive tissue remodeling in the transplanted kidney. CTD is the second leading risk for graft loss (after death) and has a histological incidence of >70% already 2 years after transplantation. The pathogenesis of CTD is multifactorial and is clinically associated with progressive hypertension, proteinuria and increased values of triglycerides. At present, besides immunosuppressive therapy, progressive loss of transplant function is only symptomatically treated by anti-hypertensive and anti-proteinuric treatment in combination with lipid lowering drugs. Histologically, CTD is characterized by chronic lesions such as interstitial fibrosis and tubular atrophy (IFTA), transplant vasculopathy (TV) and focal segmental glomerulosclerosis (FSGS). To date, due to lack of knowledge on tissue remodeling and development of these lesions, no effective therapies are available to treat renal CTD. Increasing evidence suggests involvement of complement pathways, especially alternative pathway in CTD.

Recently, we showed increased expression of heparan sulfate proteoglycans (HSPGs) during tissue remodeling in experimental CTD. In native kidney diseases and ischemia-reperfusion injury we demonstrated that HSPGs are critically involved in leukocyte influx and proteinuria-mediated renal injury. However, the precise mechanism behind the involvement of proteoglycans in CTD-associated progressive renal failure is still not unraveled.

After injury HSPGs can act as ligands for adhesion molecules such as L- and P-selectin, MAC-1 and VLA-4, and also as docking molecules for complement factors such as properdin and chemokines. By doing so, HSPGs play pivotal roles in innate immunity. Based on these functions, HSPGs are potential targets for intervention in order to reduce leukocyte recruitment and complement activation. Heparin is well known for its anticoagulant activity and used in the prevention and treatment of thromboembolic complications. In addition heparin has anti-inflammatory, and complement-reducing properties.

Non-anticoagulant heparins and various other heparin derivatives (low-molecular weight heparins, synthetic heparin mimetics and anti-HS antibodies) are regarded as agonists or antagonists of HS function. Non-anticoagulant heparins are obtained from regular heparin by removing and/or changing residues that are essential for high affinity binding to antithrombin.

Beneficial effects of exogenously administered (non-anticoagulant) heparinoids are thought to be associated with altered molecular interaction between HS chains of cell surface and extracellular matrix HSPGs on one hand, and growth factors and their receptors, chemokines, adhesion molecules, and complement factors on the other hand. Thus, these (non-anticoagulant) heparinoids most likely intervene in several processes including selectins blockade, preventing chemokine presentation, interruption of chemokine gradient formation, and down modulation of complement cascades.

Previously, it has been shown that treatment with LMWH reviparin reduces signs of progressive renal failure in experimental renal transplantation. Human renal allograft recipients, receiving unfractionated heparin therapy early after transplantation showed increased risk of hemorrhagic complications. Moreover, heparin therapy can have side effects that include heparin-induced thrombocytopenia type II, a potentially harmful threat to patient and graft survival. In experimental renal ischemia-
reperfusion injury (a non-transplant renal disease), it has been demonstrated that treatment with a synthetic and non-anticoagulant heparin showed reduced inflammation and neutrophil accumulation (29, 30).

Based on our previous data and other heparinoids intervention studies, we hypothesize that HSPGs could be promising therapeutic targets to limit CTD, especially focusing on the potential to inhibit inflammation and complement cascades. To test this hypothesis, in a well-established rat model for renal chronic transplant dysfunction (31), we intervened with vehicle, with unfractionated heparin, and with two different non-anticoagulant heparinoids (N-desulfated/reacetylated (NAc-) heparin and periodate-oxidized/borohydride-reduced (RO-)heparin). Both these non-anticoagulant heparinoids exert similar anti-inflammatory properties as heparin. In addition biostability, activity and specificity of these heparinoids were properly controlled and also showed beneficial effects in several experimental model (24, 32, 33). Besides, these heparinoids can be produced in larger quantities within affordable costs and limited time. Our studies in an experimental CTD model demonstrate beneficial effects of RO-heparin on renal influx of inflammatory cells along with a tendency to lower proteinuria and glomerulosclerosis. However, same RO-heparin increased properdin expression of alternative pathway of complement in CTD.

4.2 Material & Methods

Animals
In this study 52 ten weeks old female inbred Dark Agouti (DA) rats (donors) and 52 ten weeks old male inbred Wistar Furth (WF) rats (recipients) were used. DA and WF rats were obtained from Harlan Nederland (Zeist, The Netherlands) and Charles River Laboratories (I’Arbresle, Cedex, France) respectively. Animals were kept in a temperature controlled room, with a 12:12-h light:dark cycle and fed standard rodent chow and water ad libitum. The local animal ethics committee of the University of Groningen approved all the procedures used in the study and the Principles of Laboratory Animal Care (National Institute of Health publication no. 86-23) were followed.

Kidney transplantation
Kidney allotransplantation was performed from female DA donors to male WF recipients according to standard procedures as described previously (31). Cold and warm ischemia times were 15±3 (mean±SD) and 25 minutes, respectively. After transplantation the recipients were placed in an incubator at 28°C for approximately 6 hours and caged individually. After transplantation all recipients subcutaneously received Cyclosporine A (5 mg/kg BW/day) for 10 days. The native kidney was removed 12 to 14 days after transplantation. Total follow up was 65±4 days (mean±SD). Fourteen rats were excluded from the study because of technical surgery failure (n=3) or acute rejection (n=11) which became evident by inspection of the graft during nephrectomy procedure. Thus, 38 transplanted rats were included in the study.

Experimental groups
In this experiment, an intervention was done with regular, unfractionated heparin (Hep; n=9) and two non-anticoagulant heparinoids derived from regular unfractionated hep-
arin: N-desulfated, N-reacetylated heparin (NAc-Hep; n=10) and periodate-oxidized, borohydride-reduced heparin (RO-Hep; n=9). Production and characterization of these heparinoids have been described before (34). The control transplanted group (Con; n=10) received daily vehicle (physiological saline) injections. One day before transplantation, treatment with the respective formulations was started in both donors and recipients. The above mentioned groups received heparin(oids) daily between 9.00 and 12.00 AM dissolved in physiological salt, injected subcutaneously at 2 mg/kg BW/day until sacrifice. The treatment dose was chosen according to previous studies (25, 26) and is in the physiological range normally used for the treatment of thrombotic complications.

Clinical variables
Animals were weighed every day and observed for signs of decreasing animal welfare reflecting their clinical condition. Upon weight loss of >15% compared to highest measured body weight, animals were sacrificed and regarded as drop outs. Blood pressure was measured non-invasively with tail cuff method (CODA; Kent Scientific, Torrington, CT). Two weeks before transplantation the rats were trained to undergo blood pressure measurements. Rats were placed individually in metabolic cages to obtain 24h urines, food and water intake measurements. Blood pressure, 24h urine sampling and non-fasting blood sampling by orbital puncture were taken before transplantation (baseline), four and eight weeks after transplantation. Urine was analyzed for urea, creatinine and total protein. Blood was analyzed for urea, creatinine and triglycerides. Analyses were performed on a multi-test analyzer system (Roche Modular; F.Hoffmann-La Roche Ltd, Basel, Switzerland) at the central clinical laboratory of the University Medical Center Groningen. Creatinine clearance was calculated from 24h urinary volume, plasma and urinary creatinine.

Quantification of glomerulosclerosis
Kidneys were perfused with saline prior to sacrification. Half of the kidney was fixed in 4% formaldehyde and processed for paraffin embedding and other half was cryopreserved. Paraffin sections (4µm) were stained with periodic acid-Schiff (PAS) to evaluate focal glomerulosclerosis (FGS). The sections were semi-quantitatively scored for focal glomerulosclerosis in a blinded fashion by determining the level of mesangial expansion and focal adhesion in each quadrant in a glomerulus and expressed on a scale from 0 to 4. If the glomerulus was unaffected, it was scored as 0; if one quadrant of the glomerulus was affected, it was scored as 1, two affected quadrants as 2, three affected quadrants as 3 and 4 affected quadrants as 4. In total, 50 glomeruli per kidney were analyzed, and the total FGS score was calculated by multiplying the score by the percentage of glomeruli with the same FGS score. The sum of these scores gives the total FGS score with a maximum of 400.

Immunohistochemistry
Renal tissue was processed for immunohistochemistry. Cryopreserved tissues were fixed in acetone and endogenous peroxidase activity was blocked with 0.1% H₂O₂. Details on antibodies and conjugates are given in Table 4.1. After the staining procedure, random photomicrographs were taken from the cortical region and quantification was done by using the MacBiophotonics ImageJ program (Rasband, W.S., ImageJ, U.S. National Institute of Health, Bethesda, Maryland, USA). Data are expressed as % positive stained
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surface areas.

Statistics
Differences among the groups were tested with a Mann-Whitney U test, p<0.05 was considered statistically significant. The graphs and statistics were done by GraphPad Prism 5.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

4.3 Results

Development of CTD-related renal failure
This study included 38 male WF rats that were transplanted with a female DA kidney. During follow-up from 2-9 weeks, pre-term graft loss occurred in 3 rats in the vehicle treated group (n=10), 5 rats in the normal unfractionated heparin (n=9), 5 rats in the N-acetyl heparin (n=10) and 2 rats in the RO-heparin group (n=9). Renal graft loss was evidenced by clinical signs such as pilo-erected fur, severe body weight loss, disoriented behavior and high blood creatinine values. Graft loss among the various heparinoid groups was not significantly different. The rats that had to be sacrificed before the end of the experiment (before 9 weeks after transplantation) were excluded from all histological and biochemical analyses described later. Accordingly, the following groups with mentioned group size were studied: Allografts treated with Vehicle (n=7), with unfractionated heparin (n=4), with N-acetyl heparin (n=5) and with RO-heparin (n=7). In the plasmas of the rats taken at 8 weeks after renal transplantation, four hours after heparin(oid) injection, we measured the activated partial thromboplastin time. In the saline treated transplanted rats this was 75 sec (median value). In the regular heparin groups this time was 173 sec (saline versus regular heparin: p<0.05), 73 sec in the RO-heparin group, and 69 sec in the N-acetyl heparin group (both non-anticoagulant heparinoids being not different from saline treated rats). These data show that both chemically modified heparin preparations indeed were non-anticoagulant, and clearly different from regular heparin, and not from the saline-treated rats.

No effects of (non-)anticoagulant heparin(oids) on body weight, blood pressure, food and water intake, and urine output
Treatment with heparin and non-anticoagulant heparins had no effect on body weight of the WF recipient rats. In accordance with our previous transplantation data, during the follow-up, the mean arterial pressure increased gradually in recipient WF rats until the end of the experiment without statistical significances among the groups. Similarly, food and water intake and urine output in all the groups were not affected by the treatment (Table 4.2).

Effects of (non-)anticoagulant heparin(oids) on renal function, proteinuria and plasma triglycerides
Vehicle treated groups developed CTD-related renal failure according to previous findings, as evidenced by rise in plasma urea and creatinine, rise in urinary protein excretion and blood pressure, and rise in plasma triglycerides (Table 4.2). The plasma creatinine and urea levels increased in all the groups over time. Although plasma creatinine and urea levels in the RO-heparin group at 8 weeks seems to be higher compared to all other
Table 4.1: Immunohistochemical procedures for various inflammatory components staining.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Marker</th>
<th>Antibody</th>
<th>Conjugate</th>
<th>Quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes</strong></td>
<td>CD45</td>
<td>Mouse anti-rat CD45 antibody (clone OX-1)</td>
<td>Goat anti-mouse IgG PO (Southern Biotech, Birmingham, USA); 1:100, Tetramethylrhodamine System (PerkinElmer LAS Inc)</td>
<td>30 photomicrographs at 200 x magnification</td>
</tr>
<tr>
<td></td>
<td>CD68</td>
<td>Mouse anti-rat CD68 antibody (clone ED-1), (Abd Serotech, Oxford, UK), 1:500.</td>
<td>Rabbit anti-mouse IgPO (DAKO); 1:100, 3-amino-9-ethyl-carbazole (AEC) (DAKO)</td>
<td>30 photomicrographs at 200 x magnification</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td>CD3</td>
<td>Rabbit anti-human CD3; (DAKO, Glostrup, Denmark) 1:100.</td>
<td>Goat anti-rabbit Ig PO (DAKO); 1:100, Tetramethylrhodamine System (PerkinElmer LAS Inc)</td>
<td>30 photomicrographs at 200 x magnification</td>
</tr>
<tr>
<td><strong>T-cells</strong></td>
<td>Properdin</td>
<td>Rabbit anti-human properdin antibody</td>
<td>Anti-rabbit poly HRP (envision kit, DAKO); 3-amino-9-ethyl-carbazole (AEC) (DAKO)</td>
<td>10 photomicrographs at 100 x magnification</td>
</tr>
<tr>
<td></td>
<td>factor H</td>
<td>Goat anti- rat factor H antibody</td>
<td>Rabbit anti-goat HRP (DAKO) 1:100, 3-amino-9-ethyl-carbazole (AEC) (DAKO)</td>
<td>10 photomicrographs at 100 x magnification</td>
</tr>
<tr>
<td><strong>C3</strong></td>
<td>C3d</td>
<td>Rabbit anti-human C3d (DAKO, A0063) antibody C3d 1:4000</td>
<td>Anti-rabbit poly HRP (envision kit, DAKO); 3-amino-9-ethyl-carbazole (AEC) (DAKO)</td>
<td>10 photomicrographs at 100 x magnification</td>
</tr>
<tr>
<td><strong>MAC</strong></td>
<td>C5b-9</td>
<td>Mouse anti-rat-C5b-9 (Hycult HM0333) 1:50</td>
<td>Rabbit anti-mouse HRP (DAKO) 1:100, 3-amino-9-ethyl-carbazole (AEC) (DAKO)</td>
<td>10 photomicrographs at 100 x magnification</td>
</tr>
</tbody>
</table>
**Table 4.2:** Clinical variables from recipients at baseline (pre-Tx) and at 4 and 8 weeks after transplantation. Data shown as Median (25%-75% interquartile range). Abbreviation: Tx-transplantation, *P < 0.05 compared to vehicle treated control group.

<table>
<thead>
<tr>
<th>DA-to-WF allograft</th>
<th>Vehicle (n=7)</th>
<th>Unfractionated Heparin (n=4)</th>
<th>RO-Heparin (n=7)</th>
<th>N-acetyl Heparin (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight (g)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>290 (285-296)</td>
<td>283 (274-291)</td>
<td>270 (263-284)</td>
<td>271 (270-276)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>293 (281-313)</td>
<td>299 (292-302)</td>
<td>284 (281-302)</td>
<td>285 (276-290)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>323 (298-347)</td>
<td>311 (300-320)</td>
<td>298 (280-326)</td>
<td>303 (299-313)</td>
</tr>
<tr>
<td><strong>Food intake (g/24h)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>9 (5-11)</td>
<td>5 (5-7)</td>
<td>5 (4-7)</td>
<td>5 (4-7)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>5 (4-8)</td>
<td>7 (6-9)</td>
<td>2 (1-5)</td>
<td>5 (5-11)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>3 (3-6)</td>
<td>1.5 (1-4)</td>
<td>4 (1-7)</td>
<td>2 (1-6)</td>
</tr>
<tr>
<td><strong>Water intake (ml/24h)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>17 (17-20)</td>
<td>14 (12-17)</td>
<td>13 (10-16)</td>
<td>12 (8-15)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>25 (21-27)</td>
<td>29 (19-37)</td>
<td>18 (16-24)</td>
<td>19 (17-23)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>27 (24-30)</td>
<td>28 (24-31)</td>
<td>22 (17-46)</td>
<td>18 (9-33)</td>
</tr>
<tr>
<td><strong>Plasma creatinine (µmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>19 (18-21)</td>
<td>19 (19-21)</td>
<td>15 (14-17)</td>
<td>15 (15-19)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>73 (59-120)</td>
<td>64 (52-73)</td>
<td>65 (54-168)</td>
<td>81 (53-84)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>96 (73-139)</td>
<td>72 (58-99)</td>
<td>136 (70-207)</td>
<td>73 (58-122)</td>
</tr>
<tr>
<td><strong>Creatinine Clearance (ml/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.0 (2.8-3.8)</td>
<td>2.6 (2.5-3.0)</td>
<td>3.5 (2.7-3.7)</td>
<td>3.0 (2.3-3.0)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>0.7 (0.4-1.1)</td>
<td>1.0 (1.0-1.3)</td>
<td>1.0 (0.5-1.2)</td>
<td>0.7 (0.1-0.8)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>0.6 (0.3-1.0)</td>
<td>0.8 (0.5-1.2)</td>
<td>0.4 (0.2-0.9)</td>
<td>0.9 (0.4-1.0)</td>
</tr>
<tr>
<td><strong>Plasma urea (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>6 (5-7)</td>
<td>6 (5-7)</td>
<td>6 (5-6)</td>
<td>6 (5-6)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>20 (18.3-36)</td>
<td>18 (16-20)</td>
<td>20 (14-39)</td>
<td>19 (15-24)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>28 (21-51)</td>
<td>22 (18-37)</td>
<td>43 (21-64)</td>
<td>27 (24-40)</td>
</tr>
<tr>
<td><strong>Total urinary protein (mg/24h)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>10 (8-11)</td>
<td>7 (6-8)</td>
<td>9 (7-10)</td>
<td>9 (6-9)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>8 (7-12)</td>
<td>8 (7-11)</td>
<td>9 (7-13)</td>
<td>8 (7-10)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>56 (34-93)</td>
<td>61 (57-74)</td>
<td>33 (23-43)</td>
<td>94 (90-184)</td>
</tr>
<tr>
<td><strong>Plasma triglycerides (mmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.6 (0.5-0.6)</td>
<td>0.7 (0.6-0.7)</td>
<td>0.6 (0.5-0.6)</td>
<td>0.6 (0.4-0.7)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>0.6 (0.5-0.7)</td>
<td>0.5 (0.5-0.5)</td>
<td>0.7 (0.5-0.8)</td>
<td>0.6 (0.5-0.6)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>0.8 (0.8-1.0)</td>
<td>0.3 (0.3-0.4)*</td>
<td>0.4 (0.2-0.5)*</td>
<td>1.0 (1.0-1.0)</td>
</tr>
<tr>
<td><strong>Mean arterial pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>118 (111-121)</td>
<td>118 (111-121)</td>
<td>118 (111-121)</td>
<td>118 (111-121)</td>
</tr>
<tr>
<td>4 weeks after Tx</td>
<td>110 (104-123)</td>
<td>118 (106-132)</td>
<td>141 (121-148)</td>
<td>146 (135-148)</td>
</tr>
<tr>
<td>8 weeks after Tx</td>
<td>138 (131-165)</td>
<td>143 (138-150)</td>
<td>149 (135-154)</td>
<td>166 (153-171)</td>
</tr>
</tbody>
</table>
Reduction of renal inflammation by non anticoagulant heparinoids

**Figure 4.1:** RO-Heparin treatment showed a tendency to reduce proteinuria 8 weeks after transplantation (A) and focal glomerulosclerosis at 9 weeks after transplantation (B), however without statistical significant differences. Bars represent mean ± SEM.

groups, these values were not significantly different. In addition, at 8 weeks, unfractionated heparin and RO-heparin treated groups have significantly lower plasma triglycerides compared to vehicle and N-acetyl heparin treated groups (week 8: 0.4±0.26 mmol/l in RO-heparin group versus 0.8±0.2 mmol/l in vehicle group; p< 0.02). RO-heparin treated group showed less urinary protein excretion after 8 weeks follow-up compared to untreated rats (Fig. 4.1A and Table 4.2); however, without reaching the level of statistical significance. Taken together, heparinoids had no effect on renal function and RO-heparin had a tendency to reduce proteinuria; regular heparin and RO-heparin prevented the rise of plasma triglycerides.

**(Non-)anticoagulant heparin(oids) are not effective in reducing CTD associated glomerulosclerosis**

FGS was determined by the PAS staining and was abundantly present in all the groups. Whereas non-transplanted rats had a FGS score close to zero (not shown), animals that were treated with unfractionated heparin and N-Acetyl heparin showed FGS score comparable to the vehicle treated group. These groups approximately scored median value of 3, indicating FGS in three out of four glomerular quadrants. The RO-heparin group showed a lower FGS, scoring median value of 2.5; however, without reaching the level of statistical significance (Fig.4.1B).

**RO-heparin is effective in reducing CTD associated leukocyte recruitment**

Cortical CD45 staining revealed that the leukocyte influx was significantly decreased by ~50% in RO-heparin group compared to the vehicle treated group (p=0.0175; Fig.4.2A-C ). In order to investigate the subtypes of the leukocytes that are reduced in RO-heparin group, we analyzed the macrophage and T-cell influx by morphometric analysis. Glomerular and tubulo-interstitial monocyte/macrophage density (ED-1 positive) were analyzed separately. In the vehicle treated group, the glomerular macrophage influx was substantially lower than in the tubulo-interstitial compartment. Overall, both in glomeruli and
Figure 4.2: RO-heparin treatment significantly reduced leukocyte influx. A: quantification of CD45 positive area for leukocyte influx. RO-heparin treated group showed a significant decrease in leukocyte influx compared to vehicle treated group at 9 weeks after transplantation (*: p value 0.01), Bars represent mean±SE. B-C. Photomicrographs showing less leukocyte (CD45) influx in renal tissue of RO-heparin treated animals. (200x magnification)

in the interstitial areas, there were no major differences among the groups regarding the ED-1 positive cells influx (not shown). We next quantified T-cell infiltration by CD3 staining. The data suggest a lower T-cell infiltration in the RO-heparin group compared to the other groups, however, no statistical significance was reached. We concluded that RO-heparin reduced the influx of inflammatory cells into the transplanted kidney by partially reducing T-cells influx (Fig. 4.3).

Effects of heparin(oid) treatment on renal complement deposition

Since recent data indicate alternative complement pathway involvement in CTD and heparin(oids) have been described to inhibit complement activation, we evaluated renal properdin and factor H (activator and inhibitor of the alternative complement pathway respectively), C3 and MAC deposition in our study. Comparison of healthy donor kidneys with vehicle treated transplanted kidneys revealed clear complement activation evidenced by significant increased deposition of all four complement factors (Fig. 4.4A-H). These data indeed substantiate alternative complement pathway involvement in CTD. Properdin and factor H revealed prominent glomerular staining in a granular fashion along the capillary wall and within mesangial regions. Besides, both properdin and factor H were found in many tubules, mainly in cortical region, tubular basement membranes and granules in the cytoplasm of tubular cells. Apical tubular staining for properdin and factor H was observed on some tubuli as well. Moreover, both factors were diffusely present in interstitial areas (Fig. 4.4A,B,E and F). C3 was localized along the endothelium in the glomeruli, peritubular capillaries and larger vessels. C3 was also seen in many cortical tubular basement membranes and part of these tubules also showed apical C3 deposits (Fig. 4.4C and G). MAC complex was exclusively found along cortical tubular basement membranes and many cortical tubules also demonstrated apical MAC localization (Fig. 4.4D and H). These data show that full complement activation is only seen
**Figure 4.3:** RO-heparin treatment non-significantly reduced the T-cell (CD3) influx. Graphs represent mean±SE at 9 weeks.

**Figure 4.4:** Complement expression in renal tissue. A-H: Immunostaining showed complement activation in rat renal tissue 9 weeks after transplantation. A-D: properdin, factor H, C3 and C5b-9 expression in healthy donor kidney E-H: properdin, factor H, C3 and MAC in vehicle treated rat kidneys after transplantation. Scale bars represent 20µm. I-L: the quantification of complement factors expression in vehicle treated versus heparin(oid) treated animals after transplantation showed a significant increase of properdin expression in RO-heparin group compared to vehicle treated group (*: P value <0.05). Graphs represent median ± IQR.
in tubular compartment, and not along endothelial cells, and confirm an effective complement regulatory system on endothelium, but not on epithelial cells. Quantification of complement deposition in the transplanted kidneys revealed ~2-fold increase in properdin deposits in the heparin(oid)-treated groups, especially in the RO-heparin-treated group (Fig. 4.4I; p<0.05 vs vehicle group). No other significant differences were observed for factor H, C3 or MAC, although the median C3 and MAC values were the highest in RO-heparin group (Fig. 4.4J-L). These data suggest that (RO-)heparin promoted complement deposition, probably via alternative pathway.

4.4 Discussion

In this study we show that a non-anticoagulant heparinoid (RO-heparin) reduced renal inflammation, and plasma triglycerides and exerted modest reducing effects on proteinuria and glomerulosclerosis. However, this heparinoid is not effective in reducing kidney function, graft survival and renal complement deposition in experimental renal transplantation. One of the most striking findings of our study was the fact that the non-anticoagulant RO-heparin was the most active compound with respect to proteinuria, inflammation and triglycerides, even more active than regular heparin. This might be related to the opening of the ring structure of non-sulfated glucuronic acid units of heparin, which results in more flexibility of the polysaccharide chain and consequently a more efficient interaction with most heparin-binding proteins (21). This is an interesting finding, since RO-heparin lacks anti-thrombotic activity, however, is more effective in reducing proteinuria and inflammation. N-acetyl heparin on the contrary, lost one of the sulfate groups by the N-desulfation procedure, and thereby most likely reduces its binding properties to a number of heparin-binding proteins, and consequently did not display any beneficial effects in our study.

Although our study does not explain how the heparinoids exerted their effects, we propose the following mechanisms to be involved. The role of HSPGs in inflammatory leukocyte recruitment is well established (11, 12) and is in particular due to the binding of HSPGs to L- and P-selectins on leukocytes and endothelial cells and the presentation and gradient formation of chemokines. Intervention by heparinoids thus will reduce endothelium – leukocyte interactions (selectin block), and reduces activation and migration of leukocytes by disturbing chemokines. This finding is in line with a number of other studies showing the anti-inflammatory effects of heparin–related compounds (29, 40). Our data furthermore suggest that T-cell recruitment is inhibited to a larger extent than monocyte influx. At present we do not have an explanation for this finding, which however might be related to interruption of certain chemokines such as RANTES (CCL5), that specifically attracts T-cells. The reduction of plasma triglycerides by regular heparin and RO-heparin is most likely explained by the release of lipoprotein lipase, which cleaves plasma triglycerides. This is a well-known and described effect of heparin (41). Due to a lower degree of sulfation, N-acetyl heparin seems not able to release lipoprotein lipase. The apparent attenuation of proteinuria development by RO-heparin coincides with the small reduction in FGS by RO-heparin. We speculate RO-heparin to interrupt glomerular growth factor signaling, resulting in less FGS and improved glomerular filtration function. Unexpectedly, we found the heparin(oids) to increase properdin deposition in the transplanted kidneys. Previously, we showed properdin binding to heparan sulfates and
heparin *in vitro* and to tubular heparan sulfates during proteinuria [13]. So, our expectation was that during heparin(oid) treatment renal properdin binding would be reduced, and as a consequence reduced alternative complement activation would occur. It could be that the heparin(oid) treatment induced intrarenal properdin expression, or alternatively the heparin(oids) themselves bound to renal cells and now act as docking platforms for properdin. Future studies will work this out into more detail. Our study had a number of limitations that should be taken into consideration. First of all, from histopathology (e.g. influx of T-cells) it became evident that our model in fact is a combination of CTD with transplant rejection and not a pure CTD model. Secondly, because of the full HLA mismatch between the donor and recipient there was severe damage to the transplanted kidney, resulting in a high number of dropouts in all the groups independent of treatment which seriously hampered the power of our study to find differences among the groups. We neither can exclude a certain selection bias in the surviving animals.

It remains a question why the heparin(oids) turned out to be ineffective with regard to renal function and graft survival. In other experimental models, treatment with non-anticoagulant LMWH showed prolonged skin, cardiac and renal allograft survival with beneficial outcomes [25, 42–44]. We suggest that the full HLA-mismatch of our DA to Wistar Furth model, without any immunosuppression after day 10 might explain this. The progressive tissue remodeling in our model might be simply too strong to show (partial) down modulation by heparinoids. Besides, it is known that the half-life of unfractionated heparin(oids) as used in our study, is shorter compared to LMW heparinoids. Additionally, animals received 2mg/kg body weight heparinoids per day. This dose was chosen based on previous studies, however, the trend in reduction of proteinuria and inflammation in the RO-heparin group suggests that a higher dose of RO-heparin could be useful for future studies. Based on these consideration we propose a future experiment using anti-inflammatory and complement blocking LMW-heparinoids, in combination with the immunosuppressive agent cyclosporine A.

In conclusion, based on our data we speculate that heparin-like glycomimetics might be promising add- on therapeutic modalities next to immunosuppressants, and blood-pressure and lipid lowering medication. Future studies in experimental models and in human renal transplantation setting have to prove efficacy of this assumption.
References


Reduction of renal inflammation by non anticoagulant heparinoids


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