Renal heparan sulfate proteoglycans
Talsma, Ditmer Tjitze

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Endothelial Heparan Sulfate Strongly Contributes to Inflammation and Fibrosis in Murine Diabetic Nephropathy

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*Laboratory Investigation, Submitted*
Abstract

Inflammation has been revealed to play a vital role in the development of diabetic nephropathy, but the underlying regulatory mechanisms are only partially understood. Our previous studies demonstrated that, during acute inflammation, endothelial heparan sulfate (HS) contributes to the adhesion and transendothelial migration of leukocytes into perivascular tissues by direct interaction with L-selectin and the presentation of bound chemokines. In the current study we aim to assess the role of endothelial heparan sulfate on chronic renal inflammation and fibrosis in a diabetic nephropathy mouse model.

To induce diabetes, age matched male $Ndst1^{+/+}/Tie2Cre^{-}$ (wild type) and $Ndst1^{+/+}/Tie2Cre^{+}$ (specific $Ndst1$ deletion under Tek2 promotor in endothelium) mice on C57Bl/6J background were injected intraperitoneally with streptozotocin (50 mg/kg) on five consecutive days ($N=10-11$ group). Urine and plasma were collected. Four weeks after diabetes induction the animals were sacrificed and kidneys were analyzed by immunohistochemistry and qRT-PCR.

Compared to non-diabetic controls, diabetic $Ndst1^{+/+}/Tie2Cre^{-}$ mice showed increased glomerular macrophage infiltration, mannose binding lectin complement deposition and glomerulosclerosis however these pathological reactions were prevented significantly in the diabetic $Ndst1^{+/+}/Tie2Cre^{+}$ animals (all three $p<0.01$). In addition, the expression of podocyte damage marker desmin was significantly higher in the $Ndst1^{+/+}/Tie2Cre^{-}$ group ($p<0.001$), although the diabetic $Ndst1^{+/+}/Tie2Cre^{-}$ and $Ndst1^{+/+}/Tie2Cre^{+}$ animals had comparable podocyte numbers. In the cortical tubulo-interstitium, similar analyses show decreased interstitial macrophages accumulation in the diabetic $Ndst1^{+/+}/Tie2Cre^{+}$ animals compared to the diabetic $Ndst1^{+/+}/Tie2Cre^{-}$ mice ($p<0.05$). Diabetic $Ndst1^{+/+}/Tie2Cre^{+}$ animals also showed reduced interstitial fibrosis as evidenced by reduced density of αSMA-positive myofibroblasts ($p<0.01$), diminished collagen III deposition ($p<0.001$) and reduced mRNA expressions of collagen ($p<0.001$) and fibronectin ($p<0.001$).

Our studies indicate a pivotal role of endothelial HS in the development of renal inflammation and fibrosis in diabetic nephropathy in mice. These results suggest that HS might be a possible target for therapy in diabetic nephropathy.
Introduction

Despite improvements in blood glucose control, progression towards diabetic nephropathy remains a major burden for diabetic patients and society (1,2). Inflammation is increasingly recognized as a key initiator of the histological changes seen in diabetic nephropathy leading to the hallmark histological changes i.e. glomerulosclerosis, basement membrane thickening and interstitial fibrosis (3). Pro-inflammatory cytokines, chemokines and growth factors like IL-1, IL-6, IL-18, TNF, MCP-1, RANTES and TGF-β are expressed in diabetic nephropathy and are thought to be involved in macrophage influx and play a role in fibrosis induction (3). Targeting some of these chemokines and cytokines has already been shown to be effective in reducing clinical symptoms and histological changes in (experimental) diabetic nephropathy. MCP-1 deficiency in diabetic db/db mice resulted in less albuminuria, reduced interstitial macrophage influx and reduced renal fibrosis (4). Early clinical trials on targeting inflammation in diabetic kidney disease reveal promising results (5). As has been shown in general and for diabetic nephropathy specifically, the development of fibrosis is largely dependent on initiation by an inflammatory response, as has been reviewed by Kanasaki et. al (6). It is thought that sustained inflammatory cell activation leads to priming of local fibroblasts, resulting in deposition of ECM components (7). This entails that inflammation is a major trigger and activator for the fibrotic response and indeed MCP-1 deficiency, amongst others, results in reduced renal fibrosis in an animal model for type 2 diabetes (4). Another important cytokine signaling between inflammatory cells and fibrotic cells is TGF-β and targeting TGF-β has been demonstrated to reduce fibrosis and kidney hypertrophy in animal models for diabetic nephropathy (8-10). Both MCP-1 and TGF-β bind HS with high affinity (11,12), which might indicate an important role for HS in the development of fibrosis in diabetic nephropathy and suggesting HS as a potential therapeutic target in diabetic nephropathy (11,12).

Inflammation is characterized by the influx of leukocytes from the circulation to areas of tissue injury. During hyperglycemic conditions, like in diabetes, tissue damage results from alternative glucose metabolism, leading to the activation of endothelial cells in the glomerulus and the interstitium (13). Activated endothelial cells produce ligands for L-selectin, which is expressed on leukocytes, causing leukocyte rolling and adhesion to the endothelium (14). Our previous study identified HS expressed on the endothelium as a ligand for L-selectin and reduction of sulfation of endothelial HS diminishes the binding of L-selectin to these cells, which, in consequence, results in a reduced adhesion of leukocytes to the endothelium and reduced neutrophil migration (15). We also reported that binding of L-selectin in inflamed renal tissue occurs predominantly on the sub endothelial basement membrane (12). The involvement of sub endothelial HS in leukocyte transmigration was proven in renal ischemia reperfusion experiments in which macrophage influx was reduced in mice deficient for the basement
membrane hybrid HSPGs/collagens XV and XVIII (16). Another important factor in leukocyte transmigration is the stimulation of leukocytes by binding of chemo-
kines presented on the endothelium to G-protein coupled receptors on leuko-
cytes. The role of endothelial HS on chemokine mediated leukocyte activation
seems to be multifactorial (17). There is evidence that chemokines immobilized
on endothelial HS, but not soluble chemokines, induce LFA-1 transformation on
leukocytes enabling binding to endothelial expressed ICAM-1 and thereby facil-
itate firm adhesion (18). It has also been shown that a chemotactic gradient of
chemokines bound to endothelial HS facilitates leukocyte migration across the
endothelium (19). Furthermore it has been shown that chemokine binding to HS
results in chemokine oligomerization, which might increase the leukocyte activat-
ing potential of chemokines (20). Finally, HS has been demonstrated to be in-
olved in the transcytosis of chemokines across the endothelial layer (15). These
chemokines are largely produced by perivascular leukocytes and for presentation
on the luminal side of the endothelial cells they need to be transported through
the endothelial layer. Studies, including our previous finding (15), have shown
that this process is HS dependent (19,21). Taken together, these findings show
the role of endothelial HS on L-selectin and chemokine-mediated recruitment of
inflammatory cells.

In HS biosynthesis, the enzyme N-deacetylase/N-sulfotransferase-1
(Ndst1) initiates modification reactions to generate binding site for protein li-
gand. Lack of the Ndst1 enzyme has been shown to result in an undersulfated
and therefore biologically less active HS (22). Ndst1 appears to be essential for
mammalian development. Conventional knockout of Ndst1 in mice results in
early post-natal death with abnormal lung and forebrain development defects
(23,24). In previous studies, we generated a conditional Ndst1 mouse (Ndst1f/f)
and crossbred with a transgenic mice expressing Cre recombinase under the
TEK tyrosine-kinase promoter (Tie2Cre) to generate the Tie2Cre+Ndst1f/f mice in
which Ndst1 is specifically ablated in endothelial cells (15,25,26). In the current
study we induced diabetes in the Ndst1f/f Tie2Cre+ mice and their littermate wild-
type control Ndst1f/f Tie2Cre- mice to directly assess the role of endothelial HS in
development of renal inflammation and fibrosis in the streptozotocin-induced
diabetic nephropathy mouse model.

Materials & Methods

Experimental animals

The Ndst1f/f Tie2Cre+ mice were generated by breeding Ndst1f/f mice with
transgenic Tie2Cre mice as previously described (15,26). All experimental mice
were fully backcrossed to C57BL/6 background and handled according to guide-
lines of the University of Georgia Institutional Animal Care and Use Committee.

Induction of diabetes
Baseline plasma and 24 hour urine samples were collected approximately one week before induction of diabetes. To induce diabetes, 7-11 week old male mice received either 50mg/kg streptozotocin for diabetic animals (DB) or citrate buffer for healthy controls (HC) intraperitoneally on 5 consecutive days (27). Animals were monitored for 2 weeks, where after 24 hour urine was collected and blood glucose was measured. Animals responsive to the administered streptozotocin with a blood glucose level over 300 mg/dl were included in the study. Four groups of mice were included in the study: HC/Ndst1<sup>f/</sup>fTie2Cre<sup>+</sup> (N=3), HC/Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> (N=3), DB/ Ndst1<sup>f/</sup>fTie2Cre<sup>+</sup> (N=10) and DB/Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> (N=11). The animals were monitored on a daily basis for weight loss, activity and fur condition. After four weeks of monitoring, the animals were sacrificed and the organs were harvested (six weeks after diabetes induction). Before sacrifice plasma and 24 hour urine were collected.

**Immunohistochemistry**

Four µm frozen and formalin-fixed paraffin embedded kidney sections were used for immunohistochemical stainings. Details on fixation, antigen retrieval, antibodies and conjugates are given in Table 1. All controls (omitting primary and/or secondary antibodies in various combinations) proved to be negative (not shown). For interstitial quantification of macrophages, T-cells, myofibroblasts and neutrophils 10 photomicrographs per animal were randomly taken at a 200x magnification. Quantification of macrophages, T-cells and myofibroblasts was performed using the MacBiophotonics ImageJ software (Rasband, W.S., ImageJ, U.S. National Institute of Health, Bethesda, Maryland, USA). Data was expressed as % of area stained for F4/80 (macrophages) and CD3 (T-cells) and fold increase compared to control for αSMA (myofibroblasts). Nimpr14<sup>+</sup> neutrophils were counted manually and data was expressed as number of neutrophils per mm<sup>2</sup> of tissue. To quantify WT-1<sup>+</sup> podocytes 10 cortical photomicrographs were taken randomly of every animal. Podocytes (by positively stained nuclei) were counted manually and data was expressed as mean number of podocytes per glomerulus. Glomerular macrophages were quantified using the basement membrane HSPG agrin double staining to identify glomeruli. Ten photomicrographs per animal were randomly taken from the cortical region of the kidney at 200x magnification. Intraglomerular macrophages were counted manually and data was expressed as mean number of macrophages per glomerulus.

Deposition of collagen III, complement MBL-C and C3 was quantified by analyzing 10 randomly taken photomicrographs of the cortical region of the kidney per animal, at a magnification of 200x, with MacBiophotonics ImageJ software. MBL-C was co-stained with basement membrane HSPG agrin to better study the distribution of MBL-C in the glomerulus. Data was expressed as mean % of area stained for the respective marker. Collagen III deposition in the glomerulus was quantified using 15 randomly taken photomicrographs of glomeruli at 400x magnification and analyzed with the MacBiophotonics ImageJ software. Data was expressed as the mean % of glomerular area stained for collagen III.
<table>
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<th>Tissue processing</th>
<th>Marker</th>
<th>Antibody</th>
<th>Conjugate(s) + Visualization</th>
</tr>
</thead>
<tbody>
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<td>Agrin + F4/80</td>
<td>Rat anti-mouse F4/80 (eBioscience, CA, USA) 1:1500 + Sheep anti-Agrin (GR14, own stock), 1:1500</td>
<td>Goat anti-Rat IgG HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) 1:500 + Rabbit anti-Sheep IgG FITC (Abcam, Cambridge, UK); 1:50. Tetramethylrhodamine System (PerkinElmer LAS Inc)</td>
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<td>Formaldehyde fixed, paraffin embedded, deparaffinization, Tris/EDTA (pH 9.0)</td>
<td>CD3</td>
<td>Rabbit anti-Human CD-3 (cross-reactive with mouse; DAKO, Glostrup, Denmark) 1:100</td>
<td>Goat anti-Rabbit Ig HRP 1:100. Rabbit anti-Goat Ig HRP (DAKO, Glostrup, Denmark) 1:100. Tetramethylrhodamine System (PerkinElmer LAS Inc)</td>
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<td>Mouse anti Rabbit anti-SMA 1A4 (Sig- ma, Zwijndrecht, The Netherlands) 1:4000</td>
<td>Goat α Mouse IgG2a HRP (Southern Biotech, Birmingham, USA) 1:100 + Rabbit anti-Goat Ig HRP (DAKO, Glostrup, Denmark) 1:100. Tetramethylrhodamine System (PerkinElmer LAS Inc)</td>
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<td>Collagen III</td>
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<td>MBL-C</td>
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<td>Goat anti-Rat Ig G HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) 1:500 + Rabbit anti-Sheep IgG FITC (Abcam, Cambridge, UK); 1:50. Tetramethylrhodamine System (PerkinElmer LAS Inc)</td>
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<td>Complement activation</td>
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<td>Rat anti-Mouse C3b (Hycult biotech, Uden, the Netherlands) 1:100</td>
<td>Rabbit anti-Rat Ig HRP 1:100 + Goat anti-Rabbit Ig HRP (DAKO, Glostrup, Denmark) 1:100. 3-amino-9-ethyl-carbazole (AEC)</td>
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ELISA

Albumin concentration in the urine was determined using the following ELISA procedure. 96 wells plates were coated overnight with a goat anti-mouse albumin antibody (BETHYL Laboratories, Inc. Montgomery, Texas, USA) diluted 1:100 in 0.05M Carbonate-Bicarbonate at pH 9.6. After blocking with 50mM Tris, 0.14M NaCl, 1% BSA, pH 8.0, urinary samples were incubated for 1 hour in various dilutions. Bound albumin was detected using an HRP conjugated goat anti-mouse albumin antibody diluted 1:75,000 (BETHYL Laboratories, Inc. Montgomery, Texas, USA). Binding was visualized using 3,3',5,5'- tetramethylbenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands) where after 1M H$_2$SO$_4$ was added and absorbance was measured at 450nm (Benchmark Plus microplate spectrophotometer, BIORAD). Urinary albumin concentration was determined using a standard curve with known albumin concentrations.

Urinary creatinine was measured using the creatinine colorimetric assay.

Figure 1. Validation of the model: FGF-2 binding, hyperglycemia & rising ACR. Binding of exogenous FGF-2 (in red fluorescence) was reduced in the Nd$s^t$Tie2Cre$^+$ animals compared to the Nd$s^t$Tie2Cre$^-$ mice (A+B). After administrating streptozotocin for 5 consecutive days plasma glucose starts to rise from baseline, reaching approximately 500 mg/dL at 6 weeks after the diabetes induction (C). No differences in blood glucose levels were observed between Nd$s^t$Tie2Cre$^-$ and Nd$s^t$Tie2Cre$^+$ diabetic animals. (B) The urinary albumin/creatinine ratio (ACR) starts to increase after induction of diabetes reaching significant level at 6 weeks (P<0.05 and p<0.01), showing albuminuria is developing. No significant difference in ACR, both at 2 and 6 weeks post diabetes induction, was seen between the Nd$s^t$Tie2Cre$^-$ and Nd$s^t$Tie2Cre$^+$ group. In healthy control Nd$s^t$Tie2Cre$^+$ animals, no albuminuria is developing.
kit (Cayman Chemicals, Ann Arbor, Michigan, USA) according to manufacturer’s instructions. Albumin values were adjusted for creatinine values by dividing the urinary albumin concentration by the urinary creatinine concentration resulting in the albumin creatinine ratio (ACR).

**qRT-PCR**

RNA was isolated from 10 mg pieces of frozen kidney using the FavorPrep RNA Purification kit (Favorgen Biotech Corp, Vienna, Austria) according to the manufacturer protocol. The RNA concentration and integrity were determined by spectrophotometry (Nanodrop Technologies, Wilmington, DE). For quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis, total RNA was reverse transcribed using the Qiagen reverse transcription kit (Venlo, the Netherlands) in accordance to the manufacturer’s protocol. qRT-PCR was performed in 384 wells plates (Applied Biosystems, Foster City, CA) with a final reaction volume of 10 µl consisting of 3 µl of cDNA, 5 µl of 2x SYBR Green Master (Bio-Rad, Veenendaal, The Netherlands), 0.08 µl 50 µM primer mix (0.4 µM) and 1.92 µl RNA free H₂O. All reactions were performed in triplicate. The primers used: desmin forward CAGGATCAACCTCCTATCC, reverse CTGTCTTTTTGGTATGGACTTC, fibronectin forward CCTATAGGATTGGAGACACG, reverse GTTGGAATAATAGCTGTCCGG and collagen I forward CTATGCAACACTCAAG, reverse GAAGCAGTTTCTTCTCAAG were all purchased from Sigma (Sigma, Zwijndrecht, The Netherlands). qRT-PCR reaction was performed using a ViiA7 Real-Time PCR system (AB applied Biosystems). Differences in the expression of a gene of interest was determined by normalizing the mean Ct-value against the mean Ct value of ribosomal 36B4 housekeeping gene (forward: AAGCGCGTCCTGGGATTGTC and reverse: GCAGCCGCAAATGAGATGG) using ΔCt-method: ΔCt = Ct gene of interest – Ct mean 36B4. Relative expression of gene of interest was calculated as 2^(-ΔCt).

Control non-diabetic samples of both strains did not show differences for any of the transcripts and were represented in one group.

**Statistics**

Data is expressed as mean ± SEM unless otherwise specified. Statistical analysis was performed using a two sided, Student’s t-test to compare the DB/Ndst1+/−/Tie2Cre+ and DB/Ndst1+/−/Tie2Cre− group. When the variances were not equal a Welch’s correction was applied. P<0.05 was considered statistically significant.

**Results**

**Validation of the animal model**

To verify the under sulfated status of the endothelial HS in the Ndst1+/−/Tie2Cre+ animals, an FGF-2 binding assay was performed. The growth factor FGF-2 binds HS with a high affinity and the binding requires HS to be modified with...
Figure 2. Endothelial Ndst1 deficiency protects from glomerular macrophage influx, collagen deposition, MBL deposition and podocyte damage in STZ-diabetic kidney disease. (A-D) Glomerular macrophage influx (green)(white arrows) was markedly reduced in the Ndst1^{+/+}Tie2Cre^{−} animals compared to the Ndst1^{+/−}Tie2Cre^{−} group (p<0.001). Basement membrane HSPG agrin (red) was used to identify glomeruli. (E-H) MBL-C deposition (green)(white arrows) in the kidney was reduced in the Ndst1^{+/−}Tie2Cre^{−} group compared to the positive Ndst1^{+/+}Tie2Cre^{−} group (p<0.01). Basement membrane HSPG agrin (red) was used to identify glomeruli. However, C3b deposition was minor and not different among the groups (O). Staining for collagen III revealed a strong reduction in the deposition in the Ndst1^{+/−}Tie2Cre^{−} group compared to the fibrotic Ndst1^{+/+}Tie2Cre^{−} group (p<0.001) (I-L). Podocyte damage was measured by the mRNA expression of desmin and was reduced in the Ndst1^{+/−}Tie2Cre^{−} group compared to the Ndst1^{+/+}Tie2Cre^{−} group (p<0.001) (M). However podocyte number did not differ among the groups. (N). Representative photomicrographs were taken at 400x magnification.
Chapter 3

Figure 3. Endothelial Ndst1 deficiency reduces interstitial inflammation under diabetic conditions. Kidney sections were stained for macrophages (F4/80), T-cells (CD3) and neutrophils (Nim-pR14). (A-D) Macrophage (green) influx in diabetes is increased in diabetic animals compared to health controls. However the increase is reduced in the Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> group compared to the Ndst1<sup>f/f</sup>Tie2Cre<sup>−</sup> group (p<0.05). Agrin staining (red) was used to clarify the renal histology. T-cells (E) and neutrophil (F) influx were unchanged between the Ndst1<sup>f/f</sup>Tie2Cre<sup>−</sup> and Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> group. Moreover compared to healthy control animals T-cell influx was not increased in the diabetic groups. Representative photomicrographs were taken at 200x magnification.
N- and 2-O-sulfation (28,29). \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} mice clearly showed strong FGF-2 binding, predominantly with the peritubular capillaries (Fig. 1A). In contrast, FGF-2 binding was markedly reduced in the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} mice (Fig. 1B). This observation demonstrated that sulfation modification of endothelial HS in the peritubular area is reduced in the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} kidney. After induction of diabetes, plasma glucose gradually increased over time, but no difference in glucose levels between the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} and \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} animals were seen (Fig. 1C), indicating that deficiency of endothelial HS does not prevent diabetes development in the mice. The mice were characterized by increased 24h urinary volume accompanied by increased water intake (not shown). Urinary albumin excretion developed over time as shown by an increased albumin creatinine ratio (ACR) between 2 and 6 weeks follow-up (p<0.01). However, these parameters were not different between the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} and the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} animals (Fig. 1D), indicating endothelial \textit{Ndst1} deficiency could not prevent nor significantly reduce albuminuria. No increase in ACR was seen over time in HC mice, including both the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} and the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} animals.

**Glomerular inflammation and fibrosis in experimental diabetes is dependent on endothelial HS**

Macrophage staining (F4-80 antibody) revealed that compared to healthy controls, glomerular macrophage influx was markedly increased in diabetic \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} mice, while no increase was observed in the diabetic \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} animals (Fig. 2A-D). To more precisely localize the infiltrated macrophages in the glomerulus, macrophages were co-stained for heparan sulfate proteoglycan agrin, a useful marker for the glomerular basement membrane. The co-staining revealed that macrophages were predominantly situated in the outer mesangial areas.

Several reports have shown a role for Mannan Binding Lectin (MBL) pathway of complement-mediated damage in diabetes both in experimental models and human diabetes (30-32). In our study, staining for MBL-C deposition indeed showed an increase in MBL-C deposition mainly in the glomeruli of diabetic mice compared to the healthy controls. Moreover, the \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} diabetic group showed a reduction in the MBL-C deposition compared to \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} diabetic animals (p<0.01)(Fig. 2H). In the healthy control animals, less MBL-C staining was seen in glomeruli and more specifically in the glomerular mesangium (Fig. 2E). The strongest signals were seen at the base of the glomeruli, where the efferent and afferent arterioles invade the glomerulus. In diabetic animals the distribution of the MBL-C was more widely distributed in the glomerulus (Fig. 2F+G). As a measure of complement activation, C3 deposition was assessed. However the increase in MBL-C deposition in the diabetic \textit{Ndst1}\textsuperscript{f/f} \textit{Tie2Cre} group was not accompanied by increased C3 deposition as no differences were observed between both genotype diabetic groups after staining for C3 (Fig. 2O). C3 depositions were seen in both the glomerulus and in peri-tubular area, most probably in peri-tubular capillaries.
Figure 4. Endothelial Ndst1 deficiency abolishes interstitial fibrosis. To assess the interstitial deposition of fibrotic components, sections were stained for myofibroblast marker αSMA and collagen III. (A-D) Although there was some variability, the increase in αSMA expression (grey) was significantly reduced in the diabetic Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> group compared to the diabetic Ndst1<sup>f/f</sup>Tie2Cre<sup>-</sup> group (p<0.05). Data was expressed as mean increase of αSMA expression compared to control. (E-H) Collagen III deposition was increased in the Ndst1<sup>f/f</sup>Tie2Cre<sup>-</sup> group, but the increase was diminished in the Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> group (p<0.001). (I+J) At mRNA level both collagen I and fibronectin were expressed higher in the Ndst1<sup>f/f</sup>Tie2Cre<sup>-</sup> group compared to the Ndst1<sup>f/f</sup>Tie2Cre<sup>+</sup> group (p<0.01 and p<0.001 respectively). The diabetic Ndst1<sup>f/f</sup>Tie2Cre animals and healthy controls had comparable mRNA levels of collagen I and fibronectin. Representative photomicrographs were taken at 200x magnification.
Mesangial collagen III deposition was determined as a measure for glomerulosclerosis. Increased glomerular collagen III depositions were completely absent in the Ndst1/^+/Tie2Cre^+ group in contrast to the Ndst1/^+/Tie2Cre^- animals (p<0.001) (Fig. 2I-L). To evaluate podocyte number and damage, podocytes were counted manually (WT-1 positive nuclei). No podocyte loss was observed in the diabetic animals compared to the healthy controls and no differences between Ndst1/^+/Tie2Cre^- and Ndst1/^+/Tie2Cre^+ groups was seen (Fig. 2N). However, more sensitive assessment of podocyte injury was done by measuring mRNA levels of the podocyte damage marker desmin and revealed an increase in expression in diabetic Ndst1/^+/Tie2Cre^- animals, which was lower in the Ndst1/^+/Tie2Cre^+ mice (p<0.001) (Fig. 2M).

Collectively, these data show that glomerular influx of macrophages, deposition of lectin complement component MBL and glomerulosclerosis in STZ-diabetic kidney disease in the mouse were completely prevented by Ndst1 deficiency in endothelial cells.

Tubulo-interstitial macrophage influx and fibrosis are reduced by endothelial HS deficiency in experimental diabetes

To evaluate the effect of the endothelial Ndst1 deficiency on tubulo-interstitial leukocyte influx under experimental diabetic conditions, density of macrophages, T-cells and neutrophils in the interstitium were quantified using immunofluorescent staining. Diabetic conditions resulted in a significant influx of macrophages in wild type mice. Interestingly, interstitial macrophage staining was significantly reduced in the diabetic Ndst1/^+/Tie2Cre^- group compared to the diabetic Ndst1/^+/Tie2Cre^- group (p<0.05) (Fig. 3A-D). Most macrophages were found in the peri-tubular compartment, while only a few macrophages were intra-tubular. Diabetic condition was not associated with accumulation of T-cells and neutrophils in Ndst1/^+/Tie2Cre^- mice and no differences were seen between Ndst1/^+/Tie2Cre^- and Ndst1/^+/Tie2Cre^- animals (Fig. 3 E+F).

Hyperglycemia-induced interstitial fibrosis is prevented by endothelial HS deficiency

To assess whether or not endothelial HS is required for development of hyperglycemia-induced interstitial fibrosis, the kidney tissue sections from the diabetic mice were stained for expression of α- SMA, a key marker of myofibroblasts. Quantification showed that the accumulation of myofibroblasts was abundant in the interstitium of the Ndst1/^+/Tie2Cre^- group, but was completely absent in the Ndst1/^+/Tie2Cre^- mice (Fig. 4A-D). Myofibroblasts are known producers of interstitial collagens. To find further support for the decrease of fibrosis in Ndst1/^+/Tie2Cre^- mice, collagen III deposition was determined. Anti-collagen III staining revealed a reduction in collagen III deposition in Ndst1/^+/Tie2Cre^- mice compared to the fibrotic Ndst1/^+/Tie2Cre^- animals (p<0.001) (Fig. 4E-H). In agreement with these findings, we also observed higher mRNA expressions of collagen I and f-
bronectin, two additional fibrotic markers, in the diabetic Ndst1\textsuperscript{+/−}Tie2Cre\textsuperscript{−} mice than in the diabetic Ndst1\textsuperscript{+/−}Tie2Cre\textsuperscript{+} mice (both p<0.001) (Fig. 4I+J). Taken together, these data show that not only glomerular changes, but also tubulo-interstitial inflammation and fibrosis were prevented by endothelial Ndst1 deficiency.

Discussion

In this study we demonstrate the crucial role of endothelial HS in diabetes-induced chronic renal inflammation and fibrosis. Endothelial Ndst1 deficiency resulted in a reduced macrophage accumulation both in the glomerulus and in the tubulo-interstitium in diabetic animals. The reduction in cellular inflammation was accompanied by a reduced diabetes-induced glomerular MBL-C deposition in the Ndst1\textsuperscript{+/−}Tie2Cre\textsuperscript{−} mice. In addition, endothelial Ndst1 deficiency also associated with reduced renal fibrosis under diabetic conditions as shown by the decreased deposition of collagen III both in the tubulo-interstitium and in glomeruli and a reduced accumulation of myofibroblasts in the tubulo-interstitium.

It is generally accepted that endothelial HS proteoglycans play a pivotal role in leukocyte migration under inflammatory conditions where they act as a ligand for L-selectin and as docking structures for chemokines and cytokines, presenting them to high affinity receptors of leukocytes. As has been shown in other inflammatory disease models (33,34), endothelial Ndst1 deficiency leads to a reduced macrophage accumulation in our study as well. Since we showed reduced glomerular and tubulo-interstitial macrophage accumulation, this finding strongly support a direct role of endothelial HSPGs on macrophage migration. Previously performed \textit{in vitro} experiments have shown a strong reduction in macrophage, monocyte and neutrophil transmigration over Ndst1 deficient endothelium (15,34,35). \textit{In vivo} studies demonstrated that neutrophil transmigration is hampered in endothelial specific Ndst1 deficient mice in an acute inflammatory model (15). However our chronic STZ-induced diabetes model is not characterized by neutrophil influx as can be seen in figure 3F. The difference is thought mostly due to different sets of chemokines that are involved in the various experimental conditions, such as MCP-1 is critical for macrophage transmigration (4), whereas IL-8, MIP-2 and KC are more potent for neutrophil migration (15).

HSPGs produced in the endothelial cell are mainly deposited in the abluminal basement membrane but are also expressed on the apical side of endothelial cells (28). We have shown before that predominantly basement membrane HSPGs are involved in L-selectin and chemokine binding under inflammatory conditions (12). We also showed before that mice lacking two dominant endothelial basement membrane HSPGs, namely proteoglycan/collagen XV and XVIII hybrids, which are decorated mainly by HS side chains, showed strongly impaired neutrophil and macrophage transmigration in a renal ischemia/reperfusion model (16). Therefore we suggest that the protective effect of endothelial Ndst1 deficiency on diabetic inflammation is predominantly due to the expression of undersulfat-
Endothelial HSPGs in inflammation & fibrosis

Endothelial HSPGs in the abluminal basement membrane of endothelial cells, although we cannot exclude a potential role of luminal expressed HSPGs.

The role of inflammation as a trigger and activator of renal fibrosis is rather well established (7,36). Interestingly, in our model, compared to the inhibitory effect on inflammation, a far stronger effect of endothelial Ndst1 deficiency was seen on ECM production, both in the tubulo-interstitium as in the glomerulus. And although endothelial Ndst1 deficiency has been shown before to reduce inflammation in a number of different models, such a clear anti-fibrotic effect of endothelial Ndst1 deficiency has not been shown before (15,25,33,34,37). It is known that activated macrophages are key regulators of fibroblast differentiation to collagen producing myofibroblasts in renal pathology (38) and this could be an explanation for the reduction in myofibroblasts seen in the Ndst1 deficient mice in our study. However, since the inhibitory effect of endothelial Ndst1 deficiency on fibrosis is far stronger compared to the effect on macrophage influx so we suggest other HS-dependent mechanisms play roles as well.

It has long been thought that renal myofibroblasts originated predominantly from resident fibroblasts, however recent work showed a significant contribution of circulation derived and CXCL16 dependent fibrocyte influx to ECM expansion in a renal fibrosis model (39). Although the role of endothelial HSPGs on fibrocyte influx is unknown, CXCL16 has been demonstrated to be a heparin-binding chemokine, suggesting a role for endothelial HSPGs in CXCL12-mediated fibrocyte transmigration (40). This could explain the dramatically decreased interstitial myofibroblast number and fibrosis in the endothelial HSPG deficient animals in our experiment. In addition, the endothelial cell itself is a source of ECM deposition. Under stimulus of heparan-binding factors, like TGF-β1, produced under hyperglycemic conditions, endothelial cells have been shown to undergo endothelial to mesenchymal transition (EndMT) (41). Although the role of endothelial HSPG on EndMT is unknown, an inhibition in EndMT due to Ndst1 deficiency might be a possible explanation for the reduced ECM deposition observed in our study. Which of these mechanisms leads to the reduced ECM deposits in our study has not been determined yet. However the severity of the reduction in ECM deposits might indicate involvement of endothelial HSPG in multiple pro-fibrotic processes under diabetic conditions.

Despite strong effects of endothelial Ndst1 deficiency on inflammation and fibrosis, we could not show a significant improvement in urinary albumin excretion, which might be due to the relatively short follow-up. In recent years the paradigm that glomerular endothelial HSPG are directly involved in the glomerular filtration barrier has been challenged (42). In agreement with this, our study did not find a significant protective effect of endothelial Ndst1 deficiency on proteinuria. However, we are aware of the evidence that Ndst1 deficient-endothelial cells still express undersulfated HS, and HS in basement membranes and ECM is also contributed by other type of cells, such as podocytes in the glomerulus and pericytes and/or vascular smooth muscle cells in non-glomerular vessels. Further studies, such as complete elimination of HS expression in endothelial
Chapter 3

cells and podocytes, will enable to clearly address the role of HS in glomerular filtration barrier. Nevertheless, our studies clearly demonstrate that endothelial HS plays a major role in development of diabetes-induced renal inflammation and fibrosis, highlighting endothelial HS is a potential target for therapies to limit progression of diabetic nephropathy.
Endothelial HSPGs in inflammation & fibrosis

References


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