

Progressive glomerulosclerosis in type 2 diabetes is associated with renal histone H3K9 and H3K23 acetylation, H3K4 dimethylation and phosphorylation at serine 10

Sufyan G. Sayyed^{1,*}, Anil Bhanudas Gaikwad^{1,2,*}, Julia Lichtnekert¹, Onkar Kulkarni¹, Dirk Eulberg³, Sven Klussmann³, Kulbhushan Tikoo^{2,*} and Hans-Joachim Anders^{1,*}

¹Nephrological Center, Medizinische Poliklinik, Ludwig-Maximilians-University, Munich, Germany, ²Department of Pharmacology and Toxicology, National Institute of Pharmaceutical, Education and Research, SAS Nagar (Mohali), Punjab, India and ³NOXXON Pharma AG, Berlin, Germany

Correspondence and offprint requests to: Hans-Joachim Anders; E-mail: hjanders@med.uni-muenchen.de

*Equal contribution to the results of the study.

Abstract

Background. Distinct histone modifications regulate gene expression in certain diseases but little is known about histone epigenetics in diabetic nephropathy. The current study examined the role of histone epigenetics in development and progression of nephropathy in db/db mice.

Methods. We studied kidney damage in 6-month-old non-diabetic mice and type 2 diabetic db/db mice that underwent either sham surgery or uninephrectomy at 6 weeks of age which accelerates glomerulosclerosis in db/db mice via glomerular hyperfiltration. Histone H3K9 and H3K23 acetylation, H3K4 and H3K9 dimethylation and H3 phosphorylation at serine 10 was explored by western blotting of renal histone extracts.

Results. Uninephrectomy in C57BL/6 mice or onset of diabetes in type 2 diabetes reduced renal H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10. In contrast, H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10 were significantly increased in uninephrectomized db/db mice. The disease pattern of these mice is characterized by an increased glomerular cell proliferation, severe glomerulosclerosis, albuminuria and glomerular filtration rate reduction. Treating uninephrectomized db/db mice with a Mcp-1/Ccl2 antagonist prevented the histopathological damage and the aforementioned histone modification abnormalities of advanced diabetic glomerulosclerosis.

Conclusion. We conclude that advanced diabetic nephropathy is associated with increased renal H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10 that enhance chromatin unfolding and gene expression.

Keywords: chemokine; diabetic nephropathy; epigenetics; histone H3; MCP-1

Abbreviations: DN; diabetic nephropathy; GFR; glomerular filtration rate

Introduction

Diabetic nephropathy (DN) is a common complication of type 1 and type 2 diabetes and has become the leading cause worldwide of chronic and end-stage renal disease [26]. Histopathologically, DN is characterized by progressive remodelling of the glomerular structure, i.e. thickening of the glomerular basement membrane, activation of mesangial cells that produce increasing amounts of diffuse and nodular mesangial matrix deposits and podocyte damage altogether leading to glomerulosclerosis [9]. These structural alterations cause progressive hyperfiltration of the remaining glomeruli and albuminuria. As glomerulosclerosis become severe, glomerular filtration rate (GFR) declines, and DN progresses to end-stage renal disease. Numerous molecular pathways have been identified that link the metabolic abnormalities of diabetes to glomerular hyperfiltration and glomerular cell activation including hyperglycaemia and the generation of glycation end products [21], JAK/STAT expression [4], p38 MAPK [1], local TGF- β production [12,23], endothelial dysfunction [25], oxidative stress [6] and activation of NF- κ B-dependent inflammatory genes [27].

Nuclear translocation of transcription factors represents the link between activation of outside-in signaling pathways and the expression of target genes involved in DN. However, nuclear translocation of transcription factors is required but not sufficient for gene activation. The accessibility of chromatin transcription factor binding sites is determined by the position and compaction of histones, proteins that wind up the double-stranded DNA in nucleosomes. Whether the nucleosomes are packed in the chromatin more or less tightly is determined by the activity of a number of enzymes that regulate the covalent modification of distinct amino acids in histones through processes such as acetylation, methylation and phosphorylation [3,13]. For example, histone H3 acetylation at lysines 9, 14, 18 and 23 or mono- or dimethylation at lysine activate chro-

matin for transcription factor binding while histone H3 di- or trimethylation at lysines 9 and 27 rather silence chromatin by inhibiting transcription factor accessibility [32]. An increasing number of reports describe the association of distinct histone modifications with carcinogenesis [31] and autoimmunity [29], but little is known about its role in diabetes. Patients with type 1 or/and type 2 diabetes display increased H3 acetylation at TNF- α and COX-2 promoters in human blood monocytes [16]. Indirect evidence for a role of histone acetylation in diabetes is referred to in a study that describes trichostatin A, a histone deacetylase inhibitor, as an agent preventing glomerulosclerosis in diabetic rats (Ha H *et al.* *Nephrol Dial Transpl* 2006; 21(Suppl 2): iv33).

We hypothesized that the evolution of diabetic kidney disease is associated with distinct histone H3 modifications. We performed Western blotting of renal histone extracts obtained from db/db mice with type 2 diabetes to evaluate histone H3K9 and H3K23 acetylation, dimethylation of H3K4 and H3K9 and H3 phosphorylation at serine 10. Furthermore, we hypothesized that preventing the progression of kidney disease in db/db mice could potentially revert abnormal histone modification patterns, an assumption that is supported by our results.

Material and methods

Animal studies

Male 5-week-old C57BLKS db/db or C57BLKS wild-type mice were obtained from Taconic (Ry, Denmark) and housed in filter top cages with a 12-hour dark/light cycle and had unlimited access to food and water throughout the study. At the age of 6 weeks, uninephrectomy (1K mice) or sham surgery (2K mice) was performed through a 1-cm flank incision as previously described in db/db and wild-type mice [20]. In mice of the sham surgery groups, the kidney was left *in situ*. At the age of 3 months, 1K db/db mice were divided into four groups of 12 mice each that received subcutaneous injections three times per week as follows: (i) 5% glucose (vehicle, 10 ml/kg) from month 3–6 of age; (ii) 50 mg/kg of the anti-Ccl2 Spiegelmer mNOX-E36 (PEGylated at the 3' end with a 40-KD-branched PEG) in vehicle from month 3–6 of age; (iii) 50 mg/kg PoC-PEG, a non-functional control Spiegelmer with a scrambled sequence in vehicle from month 3–6 of age; (iv) anti-Ccl2 Spiegelmer from month 5–6 of age. At the end of month 6, tissues were obtained for histopathological evaluation 3–4 hours after the last injection. The binding specificity, *in vitro* and *in vivo* potential of mNOX-E36 to inhibit murine Ccl2 (5'-GGCGACAUUGGUUGGGCAUGAGGCGA-GGCCUUUGAU GAACCGCGGCCA-3') and the non-functional control Spiegelmer (5'-UAAGGAAACUCGGUCUGAUGC GGUAGCG-CUGUGCAGAGCU-3') have been characterized *in vitro* and *in vivo* with regard to their binding affinity and specificity to mCCL2 and concerning their respective pharmacokinetics in uninephrectomized db/db mice [14,19]. All experimental procedures had been approved by the local government authorities.

Assessment of diabetes and renal function

Blood glucose levels were monitored at monthly intervals (Accu check sensor, Roche, Mannheim, Germany), and urine samples were collected at monthly intervals for the analysis of urinary albumin (ELISA: Bethyl Labs, Montgomery, TX, USA) and urinary creatinine levels (Jaffé reaction: DiaSys Diagnostic Systems, Holzheim, Germany). GFR was determined by clearance kinetics of plasma FITC-inulin (Sigma-Aldrich, Steinheim, Germany) 5, 10, 15, 20, 35, 60 and 90 minutes after a single bolus injection [20]. Fluorescence was determined with 485-nm excitation and read at 535-nm emission. GFR was calculated based on a two-compartment model using a non-linear regression curve-fitting software (GraphPad Prism, GraphPad Software Inc., San Diego, CA).

Histopathological evaluation and immunostaining

From each mouse, parts of the kidneys were fixed in 10% formalin in PBS and embedded in paraffin. Two-micrometre sections were stained with periodic acid–Schiff reagent following the instructions of the supplier (Bio-Optica, Milano, Italy). Glomerular sclerotic lesions were assessed using a semiquantitative score by a blinded observer as follows: 0 = no lesion, 1 = <25% sclerotic, 2 = 25–49% sclerotic, 3 = 50–74% sclerotic, 4 = 75–100% sclerotic, respectively, as described [19]. Fifteen glomeruli were analysed per section. All immunohistological studies were performed on paraffin-embedded sections as described [19]. The following rat and rabbit antibodies were used as primary antibodies: rat anti-Mac-2 (glomerular macrophages, Cederlane, Ontario, Canada, 1:50) and anti-Ki-67 (cell proliferation, Dianova, Hamburg, Germany, 1:25).

Histone extraction and immunoblotting

Kidneys from mice were dissected, and histone extraction and immunoblotting were performed as described [28]. Briefly, kidneys were homogenized and processed for nucleus isolation. Nuclei were suspended in low salt buffer (10 mM Tris, 10 mM NaCl, 10 mM EDTA, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM phenyl methane sulfonyl fluoride). Histones were extracted in 0.25 M HCl and precipitated by adding 20% trichloroacetic acid. The precipitate was washed initially with acetone with HCl (0.25 M) followed by acetone wash. After quantification, equal amounts of proteins (measured in micrograms) were loaded in each well of a 14% SDS–polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore, Eschborn, Germany). Immunoblot analysis was performed by using anti-acetylated histone H3 at lysine 23 (rabbit 1:5000), anti-phosphorylated histone H3 at serine 10 (rabbit 1:5000), dimethylated histone H3 at lysine 4 and 9 (rabbit 1:5000), anti-histone H3 (rabbit 1:5000) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (all antibodies from Cell Signaling Technology, Danvers, MA). Enhanced chemiluminescence was used for developing blots (Amersham, Freiburg, Germany).

Statistical analysis

Data are presented as mean \pm SEM. Comparison of groups was performed using ANOVA and *post hoc* Bonferroni's correction was used for multiple comparisons. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Early glomerulosclerosis in db/db mice is associated with reduced histone H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10

At 6 months of age, nephropathy of obese db/db mice was characterized by mild to moderate glomerulosclerosis but neither with a significant decline in GFR (Figure 1) and only little albuminuria (supplementary Figure 1) as compared to age-matched C57BL/6 mice. We questioned whether early glomerular remodelling in db/db mice is associated with alterations in epigenetic histone modification patterns in kidneys of db/db mice, hence, we extracted histones from kidneys of db/db and C57BL/6 mice at 6 months of age. Western blotting of histone extracts revealed that early glomerulosclerosis of db/db mice was associated with a decrease in H3 acetylation at lysine 9 and 23, H3 dimethylation at lysine 4 and H3 phosphorylation at serine 10 (Figure 2). In contrast, H3 dimethylation at lysine 9 was not affected by type 2 diabetes (Figure 2). These abnormalities developed between 3 and 6 months of age as no such differences could be detected at 6 or 12 weeks (supplement Figure 2A and C). Thus, early glomerulosclerosis of

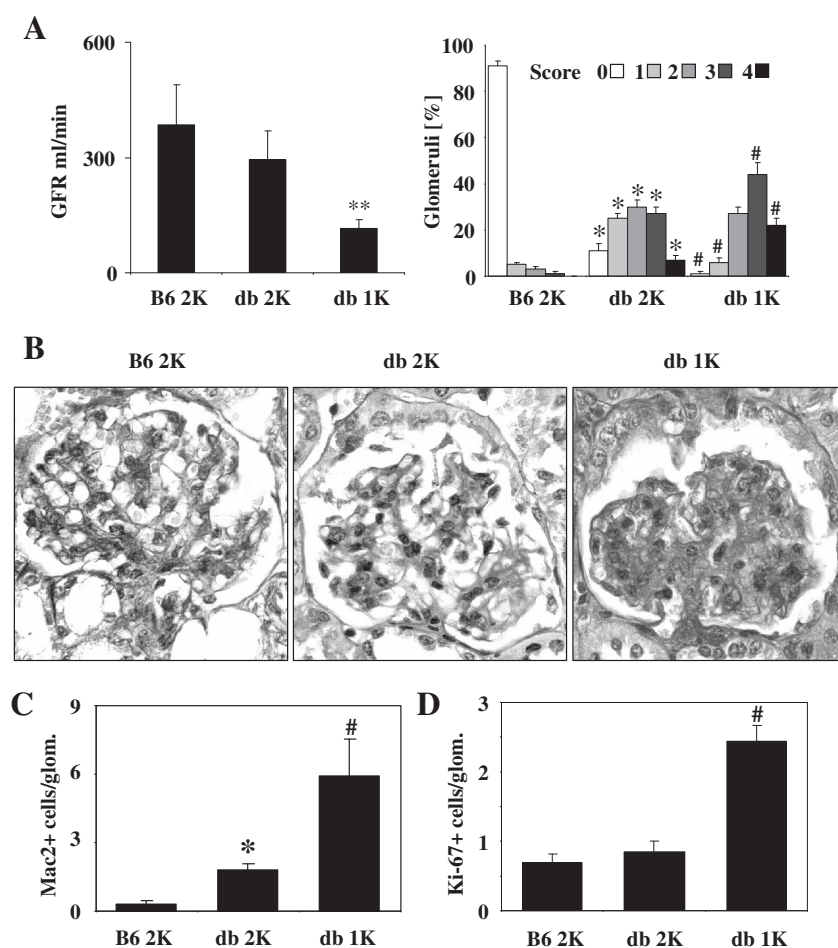


Fig. 1. Kidney disease in db/db mice. (A) Glomerular filtration rate (GFR) was measured by FITC–inulin clearance kinetics in 6-month-old C57BL/6 wild-type mice (B6), sham-operated db/db mice (db 2K) and in uninephrectomized db/db mice (db 1K). Data represent means \pm SEM from five to 10 mice. ** $P < 0.01$. Renal sections from mice of the different groups were stained with periodic acid–Schiff (PAS) and scored for the extent of glomerulosclerosis as described in methods. The graph illustrates the mean percentage of each score \pm SEM from all mice in each group ($n = 7–10$). Note that uninephrectomy was associated with a shift towards higher scores of glomerulosclerosis. (B) Images show representative PAS-stained glomeruli as indicated (original magnification $\times 400$). (C and D) Mac2 macrophages (C) and Ki67-positive proliferating cells (D) were identified by immunostaining on renal section from 6-month-old mice of all groups ($n = 7–10$). Data represent means \pm SEM from 15 cortical glomerular cross-sections. * $P < 0.05$ versus B6 2K, # $P < 0.05$ versus db 2K.

db/db mice is specifically associated with decreased H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10.

Uninephrectomy accelerates glomerulosclerosis in db/db mice in association with increased H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10

The metabolic changes of type 2 diabetes and glomerular hyperfiltration both contribute to the progression of early to late DN. Consistent with our previous reports [18,19], uninephrectomy performed at 6 weeks of age enhanced the progression of kidney disease as documented by a significant decline in GFR and increased presence of severe diffuse glomerulosclerosis at 6 months (Figure 1A and B). The progression of glomerulosclerosis was associated with increased numbers of glomerular leukocytes and Ki-67-positive proliferating glomerular cells as compared to age-matched 2K db/db or C57BL/6 mice of the same

age (Figure 1C and D). We questioned whether progressive glomerulosclerosis in 1K db/db mice is associated with further alterations in the aforementioned epigenetic histone modification pattern observed in kidneys of 2K db/db mice. Western blotting revealed that advanced glomerulosclerosis in 1K db/db mice was associated with an increase of H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10 as compared to age-matched 2K db/db mice (Figure 2).

The increase of H3K23 acetylation and phosphorylation at serine 10 was more pronounced in 1K db/db mice as compared to age-matched C57BL/6 mice whereas H3K9 acetylation and H3K4 dimethylation were not significantly different (Figure 2).

Uninephrectomy has opposite effects on histone modifications in non-diabetic mice

These aforementioned observations were specific for the combination of uninephrectomy-induced hyperfiltration

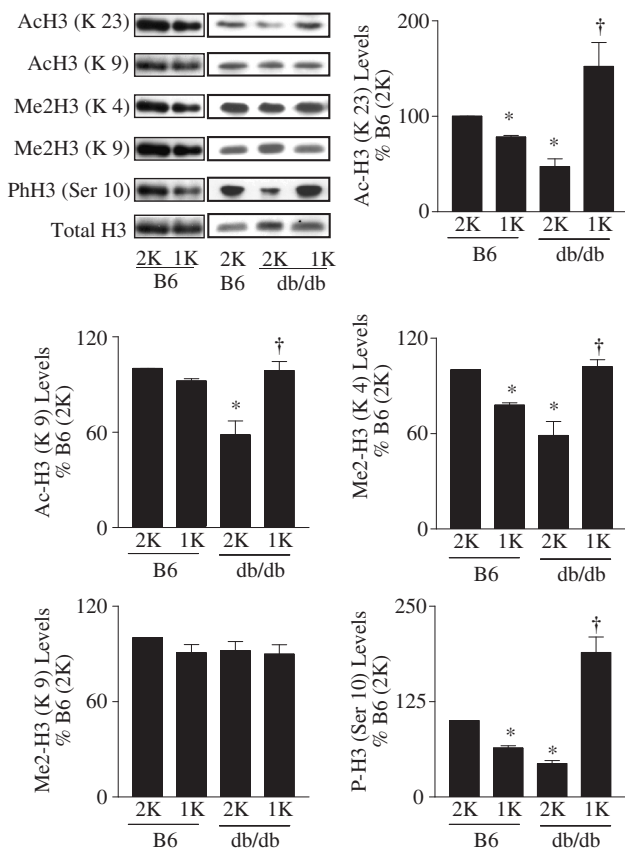


Fig. 2. Effect of uninephrectomy and diabetes on histone modifications in kidneys. H3 acetylation (Lys 23 and 9), methylation (Lys 4 and 9) and phosphorylation (Ser 10) was determined by Western blot in total kidney histone isolates from sham-operated (2K) and uninephrectomized (1K), wild-type (B6) as well as db/db mice at the age of 6 months. The blots shown are representative of three different blots from histone isolates of different mice of each group. For quantitative analysis, each band of each isolate was normalized to the respective total histone H3 content (lowest blot). The values in the histograms represent means \pm SEM from three separate blots. * $P < 0.05$ versus 2K WT and † $P < 0.05$ versus 2K db/db.

and type 2 diabetes since uninephrectomy in non-diabetic B6 mice rather reduced renal H3K23 acetylation, H3K4 dimethylation and phosphorylation at serine 10 and had no effect on modifications at H3K9 (Figure 2). Thus, advanced diabetic kidney disease in db/db mice is specifically associated with increased H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10 in kidneys.

Preventing the progression of glomerulosclerosis by Ccl2 blockade reverses H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10

As distinct renal histone modifications correlate with the progression of DN, preventing disease progression with suitable interventions should revert this histone modification pattern. We have previously shown that Mep-1/Ccl2 blockade with a suitable inhibitor (Spiegelmer mNOX-E36) from month 4–6 prevents diffuse glomerulosclerosis and improves GFR in 1K db/db mice by blocking glomerular recruitment of chemokine receptor Ccr2-positive

macrophages [19]. Here, we initiated Ccl2 blockade in 1K db/db mice either at month 3 or 5 of age and assessed renal function, renal histopathology and histone epigenetics at 6 months of age. Independent of the treatment duration, Ccl2 blockade in 1K db/db mice improved GFR to the same degree (Figure 3A) and reduced albuminuria and glomerulosclerosis consistently (Figures 3B, C, D) without affecting blood glucose levels or body weight (supplement Figure S3). Immunoblotting of renal nuclear extracts revealed that Ccl2 blockade significantly reduced the enhanced H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10 for the treatment period months 3–6 (Figure 4). For the shorter treatment period (months 5–6), only H3K23 was significantly reduced whereas other modification sites displayed only a trend towards a reduced modification. In addition, the longer treatment also led to a reduction of H3K9 dimethylation although this site was unchanged in the comparison of 2K db/db mice vs. wild-type and 1K db/db, respectively. Could that observation simply relate to Spiegelmer exposure? Treating age-matched 2K db/db mice with the same Spiegelmer for 3 months did not affect histone H3 modifications which excluded this possibility (supplement Figure S2B and C). Thus, preventing renal disease progression reverts the histone H3 modifications that are associated with uninephrectomy-accelerated glomerulosclerosis in db/db mice with type 2 diabetes.

Discussion

Our data confirmed not only our hypothesis that the evolution of DN is associated with distinct histone H3 modifications in db/db mice but also that these modifications no longer persist when diffuse glomerulosclerosis is prevented by Ccl2 blockade. Thus, histone H3K9 and H3K23 acetylation, H3K4 dimethylation and phosphorylation at serine 10 correlate with the severity of advanced glomerulosclerosis in type 2 diabetes.

Covalent modifications of histone residues determine chromatin structure dynamics and transcription factor accessibility, i.e. epigenetic control of chromatin replication and gene transcription [5,13]. Not much is known about histone epigenetics in diabetes but a recent study suggested that high glucose levels activate the histone acetylases CBP and p/CAF to increase H3 acetylation which in turn leads to an elevated transcription of inflammatory genes in cultured monocytes [16]. Our results demonstrate that advanced diabetic glomerulosclerosis is associated with increased renal histone H3 acetylation at H3K9 and H3K23. We found histone H3 acetylation only increased in 1K db/db mice in which early uninephrectomy enhanced the progression to diffuse glomerulosclerosis. As uninephrectomy itself rather decreased histone acetylation, the combination of type 2 diabetes and glomerular hyperfiltration seems to specifically foster histone H3K9 and H3K23 acetylation. Both of these modifications have been described to activate chromatin and enhance gene transcription [3,13]. In fact, gene array analyses of renal biopsies from patients with advanced diabetic nephropathy demonstrated the increased expression of a large number of dif-

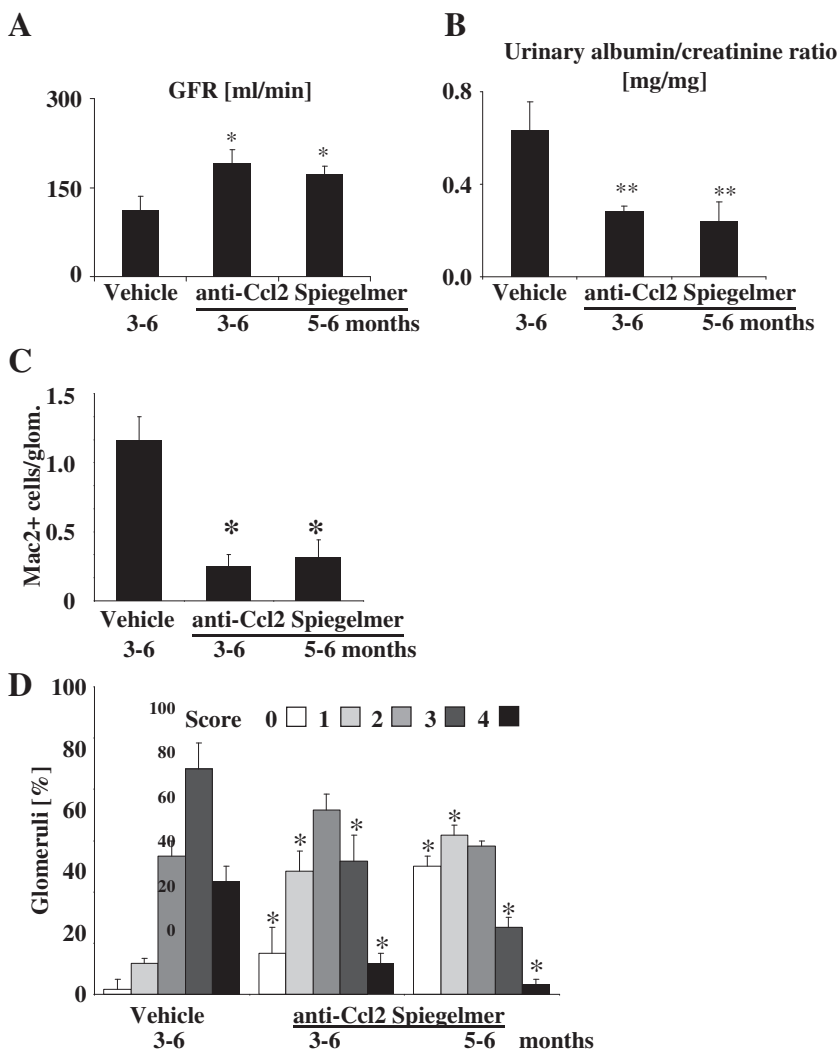


Fig. 3. Ccl2 blockade improves kidney disease in db/db mice. Uninephrectomized db/db mice were treated either with vehicle or anti-Ccl2 Spiegelmer from months 3–6 or Spiegelmer from months 5–6, respectively. **(A)** The glomerular filtration rate (GFR) was measured by FITC–inulin clearance kinetics at 6 months of age. Data represent means \pm SEM from 10 mice. * $P < 0.05$ versus vehicle group. **(B)** Proteinuria was assessed by calculating the ratio of urinary albumin and creatinine in spot urine samples taken at 6 months of age. Data represent means \pm SEM from 10 mice. ** $P < 0.01$ versus vehicle group. **(C)** Glomerular macrophage numbers were assessed by counting Mac2-cells in 15 glomeruli of renal sections. **(D)** Renal sections from mice of all groups were stained with PAS and scored for the extent of glomerulosclerosis. The graph illustrates the mean percentage of each score \pm SEM from all mice in each group ($n = 10$). * $P < 0.05$ versus vehicle group.

ferent gene families [27]. Furthermore, we observed increased cell proliferation in the glomerular and tubulointerstitial compartment of 1K db/db mice which is consistent with the concept that histone H3K9 and H3K23 acetylation also foster gene replication and transcription in diabetic kidney disease. Histone H3K4 dimethylation has a similar permissive effect and enhances NF- κ B-dependent gene expression of inflammatory genes in macrophages of diabetic mice [15]. By contrast, H3K9 dimethylation represses gene transcription [13]. A recent study reported increased H3K9 dimethylation in lymphocytes from patients with type 1 diabetes but also reported considerable cell type specificity in histone methylation [17,18]. This may explain why we could not observe any effect of either diabetes or uninephrectomy on renal H3K9 dimethylation. By contrast, we observed significantly less H3K4 dimethylation in kidneys of diabetic mice as compared to non-diabetic mice.

However, uninephrectomy-induced progressive glomerulosclerosis was associated with increased H3K4 dimethylation consistent with the respective H3K9 and H3K23 acetylation patterns. H3K4 methylation is associated with activation of vascular smooth muscle cells under diabetic conditions and contributes to metabolic memory and sustained pro-inflammatory phenotype [24,30]. This may also play a role in diabetic glomerulosclerosis because glomerular mesangial cells share many structural and functional characteristics with vascular smooth muscle cells [26] and significantly contribute to the remodelling process of the glomerular matrix [23]. Histone H3 phosphorylation of Ser-10 is another marker of chromatin relaxation and gene expression [3,22], but its role in diabetes has not yet been explored. Our data generate first evidence that three distinct H3 modifications, all known to promote nucleosome unwinding and gene expression, are consistently associated

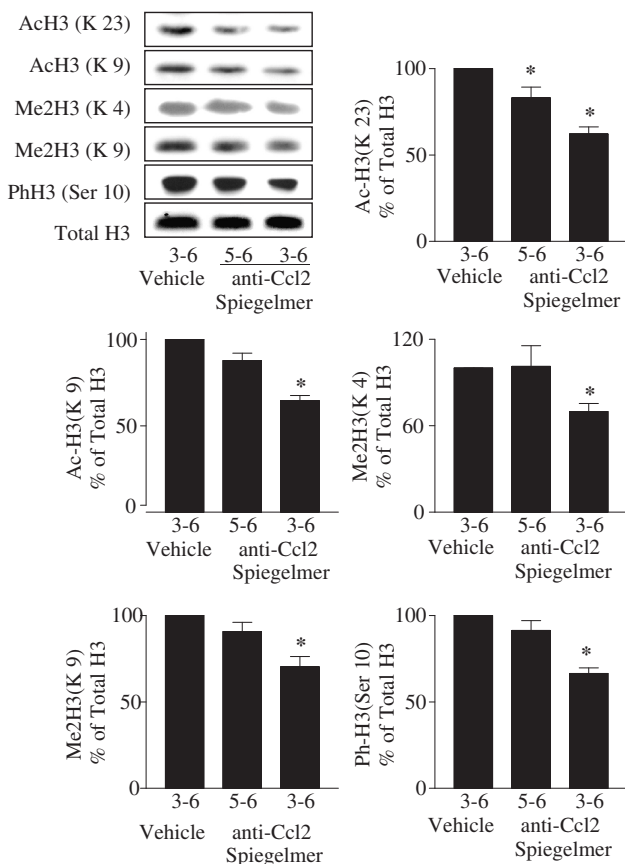


Fig. 4. Histone modifications in kidneys of db/db mice treated with the Ccl2 antagonist. Uninephrectomized db/db mice were treated either with vehicle or anti-Ccl2 Spiegelmer from months 3–6 or Spiegelmer from months 5–6, respectively. H3 acetylation (Lys 23), methylation (Lys 4) and phosphorylation (Ser 10) was determined by western blot in total kidney histone isolates. The blots shown are representative for three different blots from histone isolates of different mice of each group. For quantitative analysis, each band of each isolate was normalized to the respective total histone H3 content (lowest blot). The values in the histograms represent means \pm SEM from three separate blots. * $P < 0.05$, ** $P < 0.01$ versus vehicle.

with glomerular cell proliferation during the progression to advanced diabetic glomerulosclerosis in db/db mice.

As a second finding, Ccl2 blockade reverted these histone modifications together with preventing progression of DN. Ccl2 promotes (diabetic) glomerulosclerosis by mediating the recruitment and intrarenal activation of macrophages and by direct activation of glomerular mesangial cells [2,11]. Vice versa, loss-of-function mutations in the Ccl2 gene prevent macrophage recruitment, inflammation and glomerulosclerosis in mouse models of type 1 and 2 diabetes [7,8]. The present study extends our previously published data [19] by showing that a Ccl2 antagonist started as late as at 5 months of age is sufficient to prevent diffuse glomerulosclerosis in 6-month-old 1K db/db mice. Remarkably, Ccl2 blockade also reduced histone H3K9 and H3K23 acetylation, H3K4 dimethylation and H3 phosphorylation at serine 10, suggesting a role for Ccl2 in the regulation of histone epigenetics. However, the shorter (months 5–6) compared to the longer (months 3–6) treatment period did not lead to a statistically significant reduction of the

histone modification pattern, although both treatment periods showed equally strong effects on the GFR, the urinary albumin/creatinine ratio and the extent of glomerulosclerosis. Yet it remains unclear whether these histone modifications represent only markers of disease progression or whether they directly promote the transcription of pathogenic genes. Evidence for the latter concept comes from a preliminary report describing beneficial effects of the histone deacetylation inhibitor trichostatin A on glomerulosclerosis in diabetic rats [10].

In summary, the progression of DN in db/db mice with type 2 diabetes is associated with global renal histone H3K9 and H3K23 acetylation, H3K4 dimethylation and phosphorylation at serine 10. Future studies will have to define how single modifications regulate the expression of pathogenic genes in distinct glomerular cell types and whether modifying histone epigenetics, e.g. with specific enzyme inhibitors, may represent a novel strategy to prevent DN.

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Conflict of interest statement. None declared.

Supplementary data

Supplementary data is available online at <http://ndt.oxfordjournals.org>.

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Imatinib ameliorates fibrosis in uraemic cardiac disease in BALB/c without improving cardiac function

Marcus Baumann¹, Kirsten Leineweber², Marion Tewiele³, Kun Wu³, Tobias R. Türk³, Song Su³, Mario Gössl⁴, Thomas Buck⁵, Benjamin Wilde³, Uwe Heemann¹, Andreas Kribben³ and Oliver Witzke³

¹Department of Nephrology, Klinikum rechts der Isar, Technische Universität München, Germany, ²Department of Pathophysiology, University Hospital Essen, University of Duisburg-Essen, Germany, ³Department of Nephrology, University Hospital Essen, University of Duisburg-Essen, Germany, ⁴Department of Internal Medicine, Mayo Clinic College of Medicine, Rochester, Minnesota, USA and ⁵Department of Cardiology, University Hospital Essen, University of Duisburg-Essen, Germany

Correspondence and offprint requests to: Oliver Witzke; E-mail: oliver.witzke@uk-essen.de

Abstract

Background. Cardiovascular disease is one of the major causes of mortality and morbidity in patients with end-stage renal disease (ESRD). It is characterized by multiple left ventricular abnormalities, referred to as ‘uraemic cardiomy-

opathy’. The aim of the study was to investigate uraemic cardiac disease in a mouse model of chronic renal failure induced by subtotal nephrectomy and to evaluate the impact of the tyrosine kinase inhibitor imatinib and its antifibrotic as well as functional properties on the extent of the disease.