Acquired loss of renal nuclease activity is restricted to DNaseI and is an organ-selective feature in murine lupus nephritis.

By

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Running title: Shut-down of DNaseI in lupus nephritis

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Abstract

An acquired loss of renal DNaseI has recently been shown to promote transformation of mild mesangial lupus nephritis into membrano-proliferative end-stage organ disease. In this study, we analyzed expression profiles of DNaseI in other organs of lupus-prone (NZBxNZW)F1 mice during disease progression to determine if silencing of the renal DNaseI gene is an organ specific feature or if loss of DNaseI reflects a systemic error in mice with sever lupus nephritis. Our results demonstrate normal or elevated levels of DNaseI mRNA and enzyme activity in liver, spleen and serum samples of (NZBxNZW)F1 mice throughout all stages of lupus nephritis. DNaseI activity was dramatically reduced only in kidneys of mice with sever nephritis and was the only nuclease that was down-regulated, while 6 other nucleases (DNaseIl1-3, caspase activated deoxyribonuclease, Dnase2a, and endonuclease G) were largely normally expressed in kidneys, liver and spleen. Loss of renal DNaseI was not accompanied by changes in serum DNaseI activity, suggesting an independent mechanism of DNaseI regulation in circulation and in kidneys, and an absence of compensatory up-regulation of serum DNaseI activity in case of renal DNaseI deficiency. Thus, silencing of renal DNaseI is a unique renal feature in membrano-proliferative lupus nephritis. Determination of mechanism(s) responsible for DNaseI down-regulation is a future step in generation of new therapeutic targets to treat and prevent progressive lupus nephritis.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by production of a variety of autoantibodies to nuclear antigens.[1] Formation and sequential deposition of immune complexes in visceral organs represent a basic pathogenic mechanism of the disease.[2] The potentially most severe clinical manifestation of SLE is lupus nephritis.[3] It is initiated through immune complex deposition in glomeruli which impose kidney dysfunction and finally renal failure.[4,5] However, progression of the disease varies in intensity. Some patients experience progression from the mild mesangial form into full-blown membrano-proliferative nephritis, while others remain with a benign mesangial pattern throughout life.[6,7]

In a recent study, we observed that lupus nephritis in female (NZBxNZW)F1 (BW) mice is a principally two-stepped organ disease.[8] The early phase correlated with deposition of complexes of chromatin fragments and IgG in the mesangial matrix. Progression of the disease, which was characterized by deposition of large chromatin fragments in glomerular basement membranes (GBM) and severe proteinuria, correlated with an acquired loss of renal DNaseI mRNA level and enzyme activity. Loss of DNaseI, as a dominant renal nuclease,[9] correlated with reduced chromatin degradation during regular apoptosis in kidneys.[10,11] In a case of impaired clearance of apoptotic cells,[12] this may explain increased exposure of chromatin fragments in membranes and matrices of affected glomeruli. Binding of such fragments to GBM are most probably facilitated by increased matrix metalloprotease 2 (MMP2) and MMP9 expression, observed in nephritic kidneys.[13]

Expression profiles of DNaseI in other organs in BW mice during kidney disease progression are unknown. In sera, DNaseI was shown to be reduced in mice with lupus nephritis.[14,15] The reason for this is, however, unclear. Either, DNaseI is down-regulated in organs responsible for secreted DNaseI, or serum DNaseI is reduced just because of

consumption during nephritis. Therefore, it became important to verify if silencing of the renal DNaseI gene is an organ specific feature or if it reflects a systemic error in BW mice with sever lupus nephritis.

The aim of the present study was, therefore, to determine *i.* whether loss of renal DNaseI is unique to the kidney, or occurs simultaneously in other organs as well, *ii.* whether silencing of renal DNaseI is a selective process not involving other nucleases in defined stages of the disease; and *iii.* whether there is any compensatory up-regulation of DNaseI or other nucleases in kidneys and other organs.

In this study we examined DNaseI expression in kidneys, livers and spleens of BW mice at different stages of lupus nephritis. This allowed us to analyze for deviations in DNaseI levels in one organ with strongly (liver) and another organ with marginally detectable DNaseI (spleen). Published data have demonstrated that liver is the main source for serum DNaseI[16] while in spleen, the level of the DNaseI is very low in a physiological context.[17,18] Both organs are also responsible for clearance of circulating immune-complexes (IC)[19,20] and participate in the pathogenesis of lupus nephritis in a case of defective chromatin degradation. Therefore, data described in this study are important to inform whether the processes observed in the kidney are unique for DNaseI and for the kidney, or represent systemic errors globally in the body.

Our results demonstrate that renal DNaseI is reduced strictly in membrano-proliferative (end-stage) nephritis, and is the only nuclease that is down-regulated among 7 analyzed nucleases (DNaseI, DNaseIl1-3, caspase activated deoxyribonuclease (CAD), Dnase2a, and endonuclease G (EndoG)). While dramatically reduced in the kidneys, DNaseI is not reduced in the spleen or in the liver. We observed no significant down- or up-regulation of any of the other 6 nucleases in the examined organs. These data and the background for

them may point at mechanisms responsible for silencing of the DNaseI gene as a future causal therapeutic target to treat and prevent progressive lupus nephritis.

Materials and Methods

Animals

Female BW and BALB/c mice were purchased from Harlan (Blackthorn, UK). Treatment and care of animals were in accordance with the guidelines of The Norwegian Ethical and Welfare Board for Research Animals, and the study was approved by the Institutional Review Board.

Collection of samples from groups of mice

Serum samples from BW mice were collected from pre-nephritic mice (Group 1, n=3), mice with mesangial nephritis (Group 2, n=3), and from mice with membrano-proliferative nephritis (Group 3, n=6). Sera were stored at –20°C. Degree of proteinuria was monitored weekly with sticks from Bayer Diagnostics (Bridgend, UK). Animals were euthanized by CO_2-suffocation. Kidneys, livers and spleens were extirpated, cut, and fixed in RNAlater (Qiagen Nordic, Norway) for further analysis of gene transcription, or fixed in 4% buffered depolymerized paraformaldehyde and embedded in paraffin for immunohistochemical analysis, or fixed in 8% buffered depolymerized paraformaldehyde for immune electron microscopy (kidneys and livers only).[21] The same samples were collected from sex- and age-matched BALB/c control mice.

Anti-dsDNA Enzyme-Linked Immunosorbent Assay

Murine anti-dsDNA antibodies were detected and titrated by standard indirect ELISA, as described in details.[22]

Immune Electron Microscopy

Immune electron microscopy (IEM) was performed on murine kidney and liver sections exactly as described previously.[21] In short, sections were incubated with rabbit anti-mouse

IgG (RaM IgG, Cappel, ICN Pharmaceuticals, Inc) followed by protein A conjugated with 5-nm gold particles (PAG-5 nm, University of Utrecht, The Netherlands) to detect in vivo-bound IgG autoantibodies. Micrographs were taken using a Jeol JEM-1010 Transmission Electron Microscope (Tokyo, Japan).

Immunohistochemistry

Endonucleases in kidneys and livers were detected using polink-2 plus HRP detection kit (Golden Bridge International, Inc, Mukilteo, WA, USA) as described in detail.[10] DNaseI was detected by anti-DNaseI antibody (Santa Crus Biotechnology, Inc), diluted 1:50 for kidney and 1:30 for liver sections. CAD and EndoG were detected by anti-CAD antibody (diluted 1:150 for kidney and 1:70 for liver) and anti-EndoG antibody (diluted 1:100 for kidney and 1:70 for liver) from BioSite (CA, USA). Secondary antibodies and diaminobenzidine chromogen solution were used according to instruction provided in the kit. Sections were counterstained with hematoxylin and Scott's solution and analysed by an Olympus BX51 microscope.

Immunoexpression score

To evaluate the degree of protein expression we blindly quantified the intensity of staining in 10 view fields per kidney and liver sections. Immunoexpression score (IEXP-SC) was adapted from Richardsen at. al.[23] (IEXP-SC) = AEX x ISI, where AEX = percentage cellular area of expression and ISI = immunostaining intensity, grade 1-3. The product gives a score from 0 to 300, which was expressed as: negative - IEXP-SC = 0; weakly positive - IEXP-SC = 1 – 100; moderately positive - IEXP-SC = 101 - 200; strongly positive - IEXP-SC = 201 – 300.

Gene Expression Analysis

Total RNA was isolated with EZ-1 RNA tissue mini kit (Qiagen). The quality of RNA was analyzed by Agilent Bioanalyzer using the RNA 6000 assay kit (Agilent Technologies, Inc, CA). The cDNA was transcribed from 2μg of RNA using the cDNA Archive kit (Applied Biosystems, CA). For real time PCR we used pre-designed TaqMan Gene Expression Assays (Applied Biosystems, CA): Dnase1 Mm01342389_g1; Dnase1l1 Mm00510102_m1; Dnase1l2 Mm00481868_g1; Dnase1l3 Mm00432865_m1; CAD Mm00432822_m1; Dnase2a Mm00438463_m1; EndoG Mm00468248_m1; endogenous control – Mouse actin β 4352933E, and TATA binding protein Mm00446973_m1. The assays were performed on ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Expression levels were calculated using the ΔΔCT method. Data are given as fold change compared with transcription in 12 weeks old mice. The protocol for analysis of DNaseI expression in spleens was modified due to low expression levels. To be able to detect mRNAs we had to use 2 times higher concentration of cDNA than we used for kidney or liver samples.

DNase radial diffusion assay to determine activity of neutral pH, Ca^{2+}, and/or Mg^{2+} dependent endonucleases.

To evaluate DNase activity in kidneys, livers, spleens and sera, a DNase radial diffusion assay was performed as described.[24] 10 mg of tissue was homogenized in DNase reaction buffer (40 mM Tris, pH 7.6, 2 mM $CaCl_2$ and 2 mM MgC_2) with 0.1% Triton. Cell debris were removed by centrifugation at 13000 x g, and supernatant was stored at -80 ^{0}C. Total protein amount was measured using BCA assay (Pierce Biotechnology, Rockford, IL, USA). For analysis, tissue samples were thawed on ice, protein concentration was normalized for every tissue to be equal in all samples and 2.5 μl aliquots of tissues lysates were loaded into 1mm wells in a 1% agarose gel containing 50 μg/ml calf thymus DNA (Sigma-Aldrich) (for livers and

spleens) or 100 μg/ml (for kidneys and sera) and 1 μg/ml ethidium bromide in DNase reaction buffer. Serum samples were loaded directly to the gel after defrosting on ice. Recombinant human DNaseI from Amersham Biosciences (Piscataway, NS, USA) was used as a standard. The gel was incubated in a humidified chamber at 37°C for 17 hours and photographed under UV illumination.

Denaturing SDS-PAGE zymography (DPZ).

DNaseI activity in the kidneys, livers and sera was determined after protein separation in a 10% SDS-polyacrylamide gel containing 40 μg/ml heat-denatured salmon sperm DNA (Invitrogen Corp, Carlsbad, CA) as described.[25]

Native PAGE zymography (NPZ)

In order to analyze for DNaseI in the spleens, we also performed the more sensitive native PAGE zymography instead of SDS-PAGE.[16] DNaseI activity was determined after protein separation in 7.5% polyacrylamide gels containing 30 μg/ml heat-denatured salmon sperm DNA (Invitrogen) as described in details elsewhere.[16,17] Liver samples were analyzed by SDS- and native PAGE zymographies, both techniques demonstrated similar results in liver tissue.

Statistical Analysis

One-way analysis of variance with Dunett post hoc test was used in this study. Differences were regarded significant at $p < 0.05$.

Results

Analysis of serum anti-dsDNA antibodies, renal and liver morphology in BW mice.

To investigate the DNaseI mRNA level and enzyme activity in different organs of BW mice, we divided female BW into 3 groups according to age, anti-DNA antibody titer and disease severity (Table 1). Group 1 represents young non-proteinuric, anti-dsDNA antibody negative BW mice. IEM analysis of corresponding kidney sections did not demonstrate any morphological changes (Figure 1 upper panels, Table 1). Group 2 is composed of non-proteinuric animals with variable titers of serum anti-dsDNA antibody and deposits of immune complexes, seen as electron dense structures (EDS) containing IgG in the glomerular mesangial matrix (Figure 1 upper panels, Table 1). Nephritic BW mice with severe proteinuria, circulating anti-dsDNA antibodies and EDS with IgG in both the mesangial matrix and in GBM represent Group 3 (Figure 1 upper panels and Table 1). Liver tissue of animals from each group was also analyzed by IEM. We did not find any immune deposits or electron dense structures in liver capillary and arterial walls in any of the mice included in this study (Table1, Figure 1 lower panels). Sex and age matched BALB/c mice were used as healthy control mice. They were all negative for anti-DNA antibodies and did not have any immune deposits in kidneys or livers (data not shown).

DNaseI expression in kidneys, livers, spleens and sera of BW mice.

Relative DNaseI gene expression levels in different organs from Group 1-3 mice was analyzed by real time PCR, corresponding enzyme activity and protein expression. Figure 2 A,B,C shows mRNA levels of DNaseI (columns) and enzyme activity (gels) in individual mice, as determined in kidneys (A), livers (B) and spleens (C). The average ($\pm$SD) of DNaseI mRNA levels in each group of mice is presented as insert for each organ. DNaseI zymography applied to serum samples is shown in (D).

DNaseI mRNA is normally expressed in pre-nephritic kidneys (Group 1, Figure 2A) and in kidneys from mice with mesangial nephritis (Group 2, Figure 2A). However, DNaseI mRNA is dramatically reduced in kidneys from Group 3 BW mice (Figure 2A). The low DNaseI mRNA level in Group 3 mice is also reflected by low enzyme activity as determined by zymography (Figure 2A) and weak protein expression in kidneys as determined by IHC (Figure 2E upper row). Low DNaseI expression was presented uniformly in glomeruli and tubuli in kidney sections from Group 3 mice.

To analyze if silencing of the DNaseI gene in nephritic BW mice is an organ specific feature, mRNA levels were determined in livers and spleens of the same BW mice. Data demonstrate that DNaseI mRNA expression is not reduced in livers (Figure 2B) or in spleens (Figure 2C) in all groups of mice. Those results compare well with assays on DNaseI enzyme activity as analyzed by gel zymography (Figure 2B and 2C for livers and spleens, respectively) and IHC analysis of livers for DNaseI protein expression in situ (Figure 2E lower panels). DNaseI mRNA level and enzyme activity in livers in Group 2 and in spleens in Group 2 and 3 tended to be higher when comparing with Group 1 results. (Figure 2B and 2C, for livers and spleens, respectively). However statistical significance reached only increased mRNA level of DNaseI in spleen in Group 2 mice. IHC analysis of DNaseI on liver sections reflects results of DNaseI mRNA levels (Figure 2E lower panels). Quantification of staining intensity of DNaseI on kidney and liver sections of BW mice is provided in Supplementary Figure 1A, C, and demonstrates loss of DNase I in kidneys, but not in livers.

Data in Figure 2D demonstrate no reduction of serum DNaseI enzyme activity, as determined in Group 3 compared with Group 1 and Group 2 mice. Interestingly, zymograms of serum samples exhibit a pattern similar to that seen in zymograms of liver lysates, in agreement with a report showing that liver is a main source of serum DNaseI.[16]

The mRNA level and enzyme activity of renal DNaseI in age and sex-matched BALB/c mice are presented in Supplementary Figure 2A. As demonstrated, DNaseI is expressed at stable levels in all groups of the control animals. Delta Ct values and calculated folds change of renal DNaseI mRNA levels in examined groups of BW and BALB/c mice are shown in Supplementary Table 1.

Total nuclease activity in kidneys, livers, spleens and sera of BW mice.

Results of the DNaseI zymography lead us to analyze if DNaseI levels influenced on total nuclease activity in kidneys, livers, spleens and sera of BW mice during progression of lupus nephritis. Figure 3 demonstrates nuclease activity, as determine by a single radial diffusion nuclease assay, in kidneys (A), livers (B), spleens (C) and sera (D) at different stages of the disease. Calculated group averages ($\pm$SD) are given as insert in each panel. The results demonstrate that there is no decrease in total nuclease activity between Group 1, 2 or 3 in livers or spleens of BW mice, while total nuclease activity in kidneys of animals with sever nephritis (Group 3) is markedly reduced in comparison with mice in Group 1 and Group 2. In serum samples (Figure 3D), total nuclease activity was not reduced during lupus nephritis progression, similar to the results of DNaseI zymography on serum samples shown in Figure 2D.

Expression profiles of other nucleases in kidneys, livers and spleens from Group 1-3 BW mice.

We measured gene expression levels of other endonucleases by real time PCR applied to kidneys, livers and spleens to analyze for two possible phenomena: *i.* selectivity of DNaseI down-regulation compared to the other nucleases, and *ii.* possible compensatory changes of other nucleases in all three organs in situations were the important renal DNaseI gene is shut

down. Figure 4 demonstrates mRNA levels of DNaseIl1, DNaseIl2, DNaseIl3, CAD, Dnase2a and EndoG in kidneys (A), livers (B) and spleens (C) of Group 1-3 BW mice. The average of each nuclease mRNA level in each group of mice is presented for each organ. There is no significant difference found in the transcriptional levels of any of the nucleases in kidneys, livers or spleens, except for those indicated by asterx in Figure 4B and 4C, when compared with the pre-nephritic (Group 1) mice. IHC analysis performed on kidney (Figure 5A) and liver (Figure 5B) sections confirmed the results of real time PCR on CAD (Figure 5 A, B upper panels) and EndoG (Figure 5A, B low panels), as the staining intensity was consistently similar in all groups of mice. Quantitative scoring analysis of staining intensity of CAD and EndoG on kidney and liver sections is provided in Supplementary Figure 1A, B, and showed stable expression throughout the observation period. Expression profiles of renal DNaseIl1, DNaseIl2, DNaseIl3, CAD, Dnase2a and EndoG in age and sex-matched BALB/c mice demonstrated in Supplementary Figure 2B, and showed largely stable values for all endonucleases studied throughout the observation period.

Discussion

The role of reduced DNaseI in the pathogenesis of SLE has been discussed for decades. DNaseI was seen as a promising target for substitutive therapy to prevent autoimmunity[26] but was fallen into oblivion for years after insufficient results of the trial treatment.[27] In one single reported study done by Macanovic et al., injection of DNaseI in pre-nephritic BW mice postponed the disease, while treating nephritic animals decreased level of proteinuria and serum creatinin.[26] Unfortunately, these observations were not followed by further penetrating studies that would provide insight into the therapeutic effect of DNaseI treatment. In another study performed in 1998, administration of DNaseI in BW mice did not affect development or progression of lupus nephritis.[28] Similarly, intravenous or subcutaneous administration of recombinant human DNaseI in patients with lupus nephritis class III-V did not show any impact on kidney function or activity of the disease.[27] This may signify that serum DNaseI has little or no influence on degradation of chromatin in dying renal cells.[29] Only intra-cellular renal DNaseI initiates and ensures a safe and effective degradation of chromatin in dying cells within the kidneys (see e.g.[29]).

Tubular cells dominate among renal cells that express DNaseI (Figure 2E),[9,15,30] but DNaseI is also expressed in mesangial cells ([15], unpublished data). Detection of apoptotic cells in kidney sections demonstrated that both tubular and glomerular cells undergo apoptosis.[10,11,21] Reduced DNaseI expression is furthermore observed uniformly in glomerular and tubular cells in BW mice with severe nephritis.[15] An acquired loss of renal DNaseI in lupus nephritis may therefore be the event that results in reduced chromatin fragmentation in a dominant proportion of renal cells, resulting in exposure of large chromatin fragments within the kidneys. Such fragments participate in the formation of immune complexes in GBM and thus accounts for progression of lupus nephritis into end-stage organ disease.

To better understand the role of DNaseI expression in progressive lupus nephritis, it became important to determine 3 aspects of this phenomenon: *i.* is shut-down of renal DNaseI unique for DNaseI, or do other renal nucleases behave in a similar manner; *ii.* is renal DNaseI shut-down unique to kidneys, or is DNaseI down-regulated in other organs as well; and *iii.* are other nucleases up-regulated as a compensation in situations where DNaseI is lost in the kidneys.

Data in the present study provide answers to all these aspects. For the first, data confirm that renal DNaseI is lost when lupus nephritis transform mild into severe membrano-proliferative organ disease.[8,15] Among expression profiles of 7 nucleases analyzed in kidneys, livers and spleens, reduced nuclease expression is restricted to DNaseI, in agreement with a recent preliminary observation.[15] Furthermore, the DNaseI shut-down was restricted to the kidneys of BW mice with severe nephritis, as expression appeared normal in livers and spleens of the same animals. Importantly, no clear tendency for compensatory up-regulation of other nucleases involved in the initial fragmentation (DNaseI and CAD) or in subsequent chromatin degradation (EndoG, Dnase2) was observed.[29,31]

Notably, serum DNaseI activity in the different groups of mice correlated with DNaseI activity in the liver and not in the kidney, in accordance with results provided by Ludwig et al.[16] There are several reports about reduced serum DNaseI activity in patients with SLE [32-35] and lupus-prone mice.[14,15] All of those analyses are based on measurement of nuclease activity in sera by radial diffusion assay which is a sensitive but not a DNaseI specific method. Published data indicating correlation of serum DNaseI activity with activity of SLE or with renal involvement in SLE remain, however, controversial. The first study done by Chitrabamrung on 36 SLE patients demonstrated that individuals with active lupus (including active lupus nephritis) had the lowest level of enzymatic activity compared with healthy volunteers, patients with rheumatoid arthritis and scleroderma.[32] This observation was

challenged by Sallai et al,[34] who demonstrated that serum DNaseI did not correlate with disease activity among 113 SLE patients with or without lupus nephritis. Moreover, activity of serum DNaseI in SLE patients was not reduced compared to enzymatic activity in patients with undifferentiated connective tissue disease.[34] Data in our study demonstrate that total serum nuclease activity, as well as selective serum DNaseI activity were not reduced in BW mice during progression of lupus nephritis. Thus, serum DNaseI may not be important for the kidneys in a situation were renal DNaseI is lost. This may also explain why injection of DNaseI does not affect the activity of lupus nephritis in patients..[27] This leaves us with the perception that renal, intra-cellular DNaseI is required for safe degradation and elimination of chromatin from dying renal cells. Without this enzyme, chromatin degradation is impaired, and large chromatin fragments accumulate in situ, where they are released from dying cells. This may indeed be one of the factors that result in impaired clearance of apoptotic, secondary necrotic cell debris.[36-39]

Interestingly, we found increased DNaseI mRNA level and enzyme activity in liver and in spleen in early and late stages of nephritis. The observed increase of DNaseI expression in those organs was associated with high titers of serum anti-dsDNA antibodies. Since one common function of liver and spleen is uptake of circulating immune complexes we assume that DNaseI may participate in chromatin degradation of cleared IC. This may eventually explain increased DNaseI expression in these organs.

Selective loss of renal DNaseI creates a basis for in situ deposition of chromatin, a phenomenon independently demonstrated in different experimental nuclease deficiencies. In several of such studies, it is demonstrated that chromatin fragments accumulates in situ where it is released from dead cells (reviewed in[40]) instead of entering circulation, which would eventually promote deposition in distant organs as well.

The present results add weight to the assumption that DNaseI shut-down is secondary to early anti-dsDNA antibody-mediated deposition of immune complexes in the mesangial matrix.[8] This may mean that inflammation linked to (silent or mild) mesangial nephritis in fact impose the loss of renal DNaseI with the consequences for renal function as described here. This proposed mechanism is in agreement with results of high resolution techniques to determine the nature of target structures within the glomeruli. These structures appear as electron-dense structures (EDS) by transmission electron microscopy (TEM), and have been shown to be composed of chromatin fragments and IgG molecules by different forms of immune electron microscopy and by co-localization terminal deoxy-nucleotidyl-transferase (TdT) biotin-dUTP nicked end-labeled (TUNEL) IEM assay.[41-43]

Comparative studies of components and localization of EDS in skin and glomeruli in SLE demonstrated that they are composed of similar chromatin-related structures.[41,42] Deposition of ICs in glomeruli did not, however, predict deposition in skin.[41,42] Examination of DNaseI expression in skin of MRL-lpr/lpr and BW mice has demonstrated stable DNaseI activity during development of lupus nephritis.[42] Those results indicate that chromatin-IgG complexes that deposits in skin have another origin than from skin itself. In harmony with this published data demonstrate presence of polyomovirus large T antigen in extra-cellular chromatin associated with glomerular and skin membranes,[41] suggesting that chromatin fragments in both kidneys and skin might derive from polyomovirus-infected renal cells since renal polyomovirus infection is a frequent finding in patients with lupus nephritis.[22] Further investigations are needed to confirm if chromatin fragments found in skin EDS of SLE patients have a renal origin.

The precise mechanism by which renal DNaseI is shutting down is unknown, but is currently analyzed in our laboratory. Contemporarily, we follow three lines of analyses: transcriptional interference with a convergently encoded gene (Trap1) which overlaps with

the DNaseI gene in their 3' untranslated region; regulation by microRNAs; or by DNA methylation. The initial event, accounting for DNaseI shut-down may, however, represent a response to inflammatory signals provided by the early mesangial nephritis. This would be in favor of transcriptional interference with the Trap1 gene, since Trap1 is upregulated during stress and inflammation ([43,44], manuscript in preparation). Therefore, we currently perform in-vitro studies using cultures of primary mesangial and tubular cells exposed to autoantibodies, nucleosomes, immune complexes and pro-inflammatory cytokines to determine impact of the inflammatory mesangial nephritis environment on DNaseI expression in renal cells. We also analyze the impact of albumin overload on ability of tubular cells to express DNaseI since its contribution to renal proximal cell apoptosis has been reported.[45,46] Furthermore, microRNA targeting DNaseI mRNA has been identified in the mesangial nephritis and in end-stage organ disease in our laboratory (studies in progress).

From data presented here, adding to previous results,[8,10,11,15] we may conclude that the processer accounting for transformation of mild mesangial nephritis into severe end-stage organ disease is linked to events within the kidneys, and is selective for the kidneys. This event is regulated by renal DNaseI shut-down.

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Table 1. Laboratory parameters and morphological characteristics of BW mice included in this study.

Mouse Group	Mouse number	Age w.o.	Anti-DNA antibody titer	Proteinuria*	EDS MM**	EDS GBM	EDS liver
1	1	12	0	-	-	-	-
	2	12	0	-	-	-	-
	3	12	0	-	-	-	-
2	4	30	100	1+	+	-	-
	5	30	1400	-	+	-	-
	6	30	3000	-	+	-	-
3	7	32	800	4+	+	+	-
	8	26	200	4+	+	+	-
	9	32	200	4+	+	+	-
	10	27	500	4+	+	+	-
	11	24	400	4+	+	+	-
	12	24	400	4+	+	+	-

* Proteinuria was determined by urine stix: 0–1+ (≤0.3 g/L, regarded as physiological proteinuria); 2+, (≥ 1 g/L); 3+, (≥ 3 g/L); and 4+, (≥ 20 g/L).

** MM – mesangial matrix

Figure legends.

Figure 1. Immune electron microscopy analyses of electron-dense structures (EDS) in kidneys and livers from BW mice at different stages of lupus nephritis. BW mice were divided into 3 groups according to kidney morphology (upper panels): Group 1 – mice without EDS in glomeruli; Group 2 – animals with immune deposits in mesangium; Group 3 – mice with deposition in both mesangial matrix and glomerular basement membranes. In-vivo bound IgG are traced by 5 nm gold particles. No EDS was, however, found in arterial or capillary walls in corresponding liver sections in Group 1-3 BW mice (lower panels).

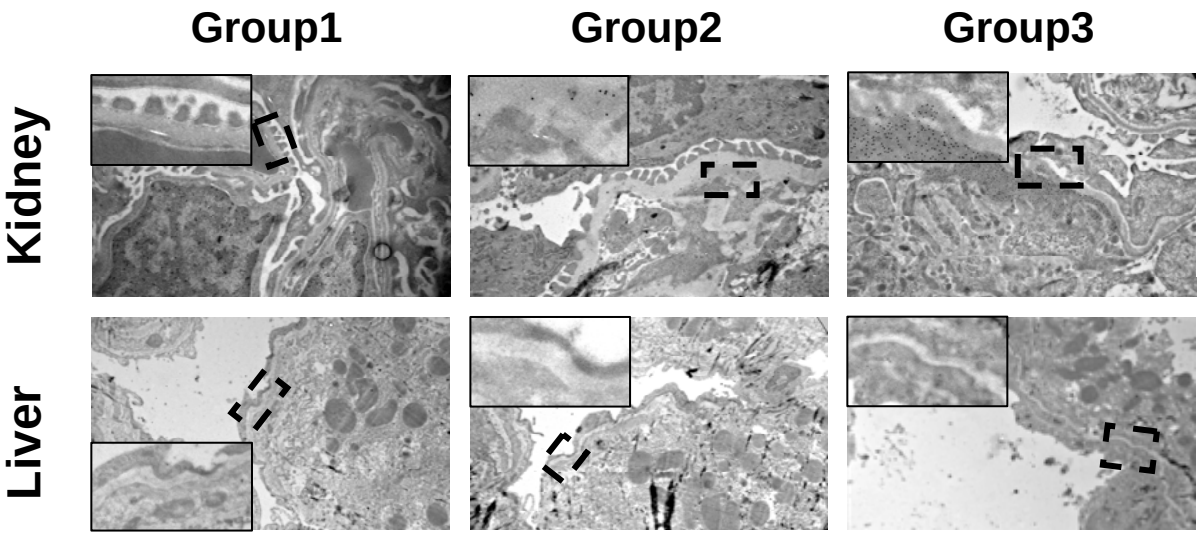
Figure 2. DNaseI expression in organs and sera of BW mice at different stages of lupus nephritis. The mRNA levels (columns) and enzyme activity (gels) of DNaseI are determined in kidneys (A), livers (B) and spleens (D) of individual BW mice in pre-nephritic Group 1 mice, and in mice with mesangial nephritis (Group 2) or with membrane-proliferative nephritis (Group 3). Insert in each panel demonstrates mean ($\pm$ SD) of DNaseI mRNA levels in each group for each organ. Asterix indicates a significant difference compared to Group 1 ($p<0.05$). The mRNA levels of DNaseI in livers and spleens of BW mice with sever nephritis (Group 3) is not reduced while it is dramatically low in kidneys of the same animals. Enzyme activity of DNaseI measured by SDS-PAGE zymography for kidneys and livers, and by Native PAGE zymography for spleen samples correlates well with results of mRNA levels. DNaseI activity in sera of individual BW mice (D) in the different groups of lupus nephritis, as measured by SDS-PAGE zymography, is similar to the expression patterns of the DNaseI activity in livers and is not reduced in Group 3 BW mice. IHC analysis of DNaseI expression in kidney (E upper panels) and liver (E lower panels) sections in Group 2 and 3 BW mice is demonstrated in comparison to expression in age-matched healthy controls. Expression of

DNaseI in tubular cells of BW mice with sever nephritis (Group 3) is nearby undetectable while it is normally present in liver sections in the same animals. Magnification x 200.

Figure 3. Total nuclease activity in organs and sera of BW mice at different stages of lupus nephritis. Nuclease activity in kidney (A), liver (B), spleen (C) and serum samples (D) of individual BW mice is measured by a single radial nuclease diffusion assay. Insert in each panel represents mean (± SD) of total nuclease activity in each group for each organ. The asterix indicates a significant difference compared to Group 1 ($p<0.05$). Only in kidneys nuclease activity is markedly reduced in Group 3 BW mice.

Figure 4. Expression profiles of DNaseIl1-3, caspase-activated Dnase, Dnase2a and endonuclease G in BW mice during progression of lupus nephritis. The mRNA levels of different nucleases are determined in kidneys (A), livers (B) and spleens (C) in pre-nephritic Group 1 mice, in mice with mesangial nephritis (Group 2) and in mice with membrano-proliferative lupus nephritis (Group 3). Values are given as mean (± SD). Asterix indicates a significant difference compared to Group 1 ($p<0.05$). No significant changes in transcription levels of DNaseIl1-3, CAD, Dnase2a and EndoG was observed in kidneys in BW mice at different stages of lupus nephritis.

Figure 5. IHC analysis of caspase-activated Dnase (CAD) and endonuclease G (endoG) in kidneys and livers from BW mice. Expression of CAD (A,B upper panels) and EndoG (A,B lower panels) proteins was determined in kidney (A) and liver (B) sections of BW mice in Group 2 and 3 and compared with data obtained in healthy BALB/C control mice. No difference in nuclease expression was observed in organs of BW mice with severe nephritis when compared with data from control animals. Magnification x 200.



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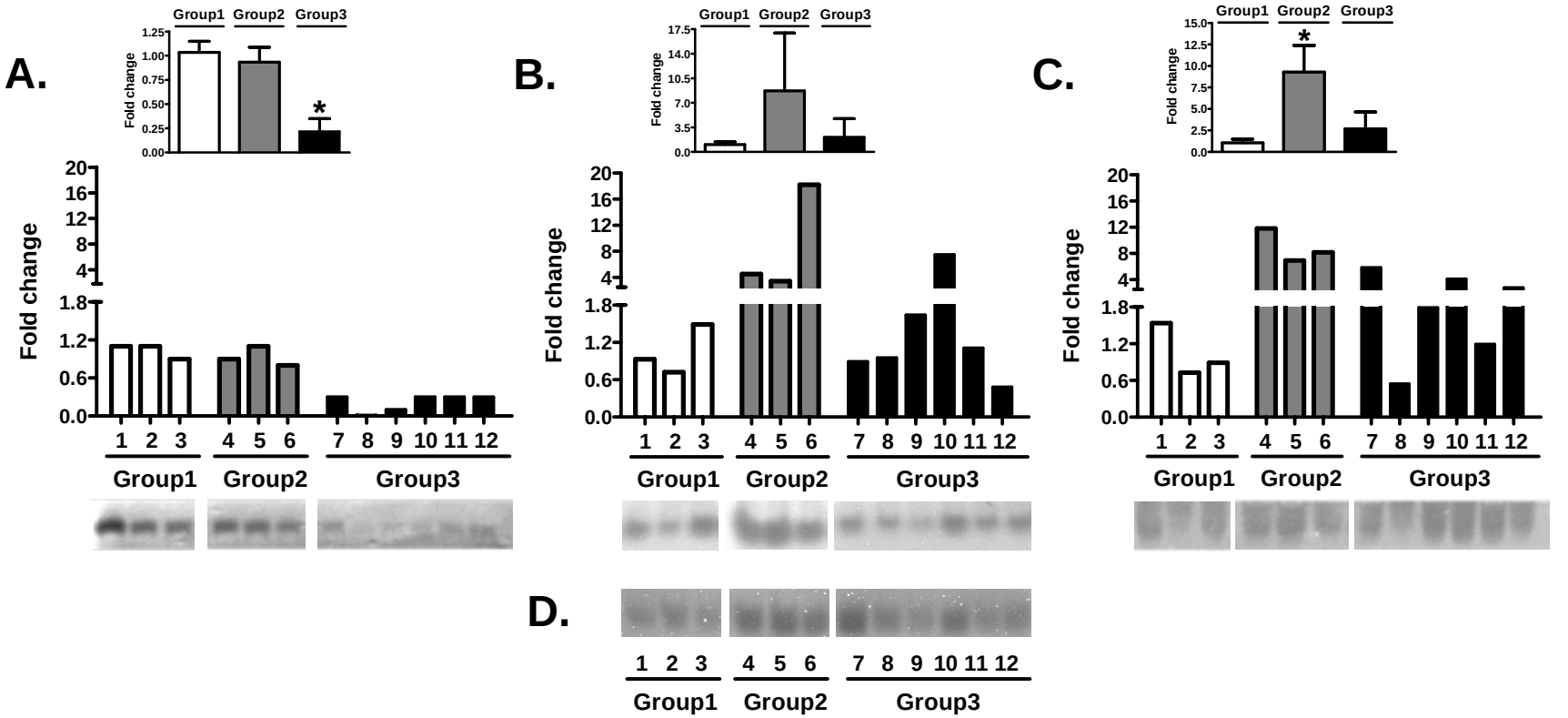

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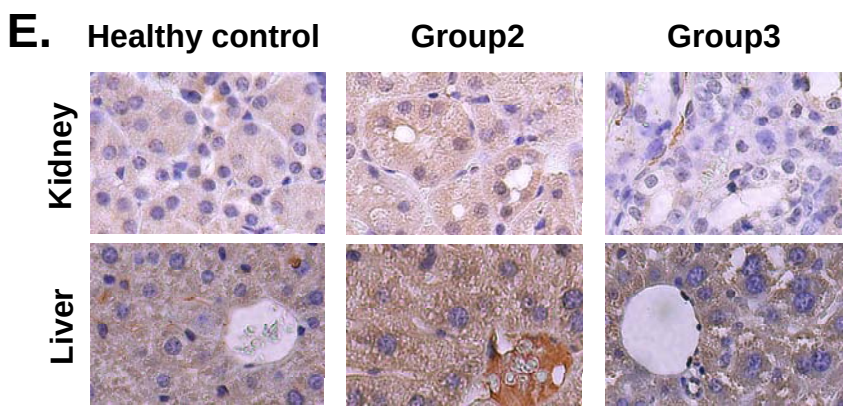

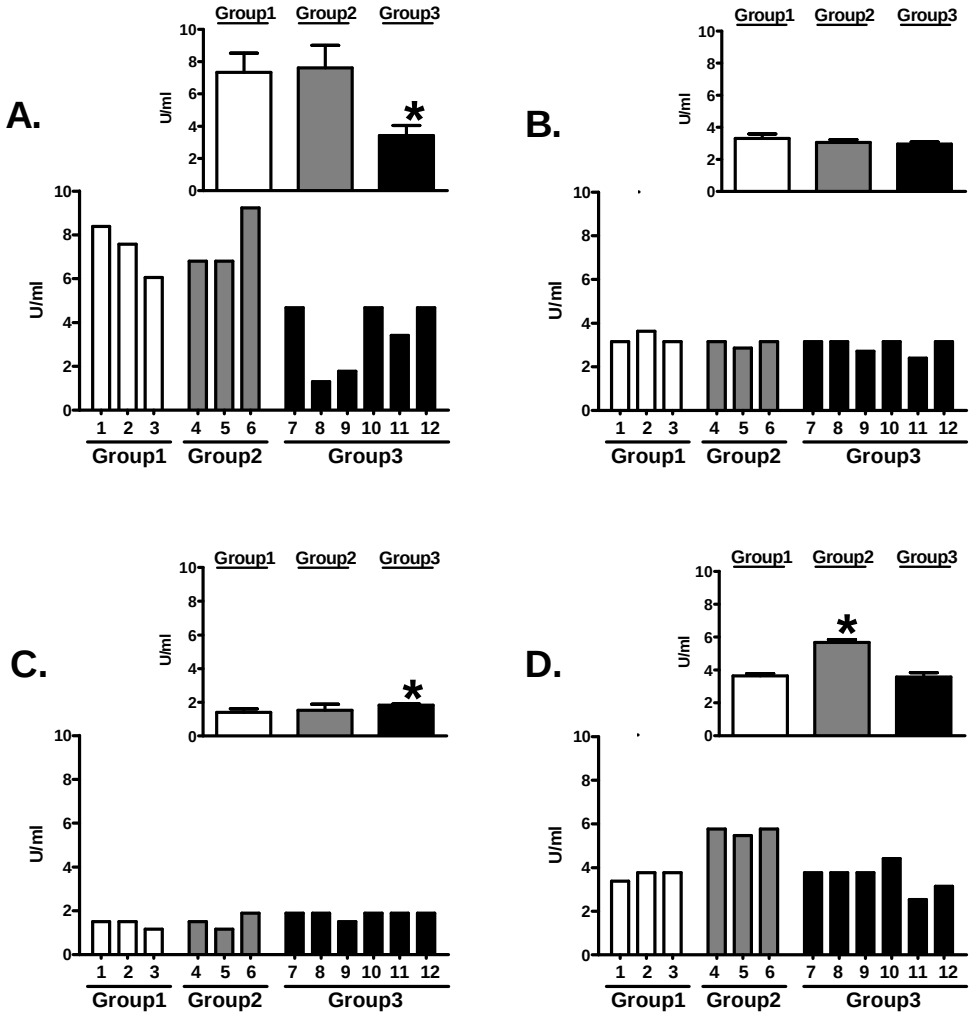

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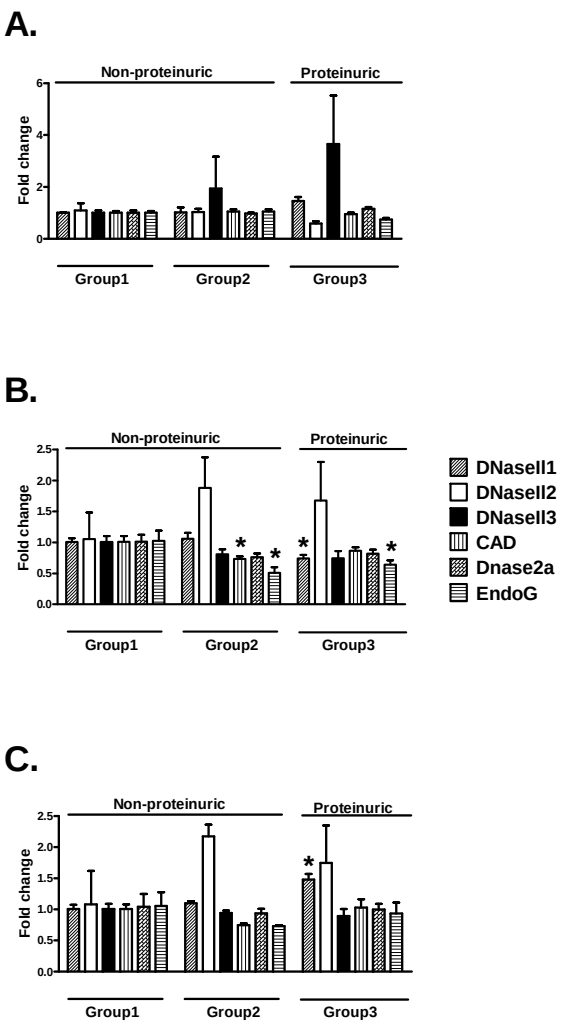

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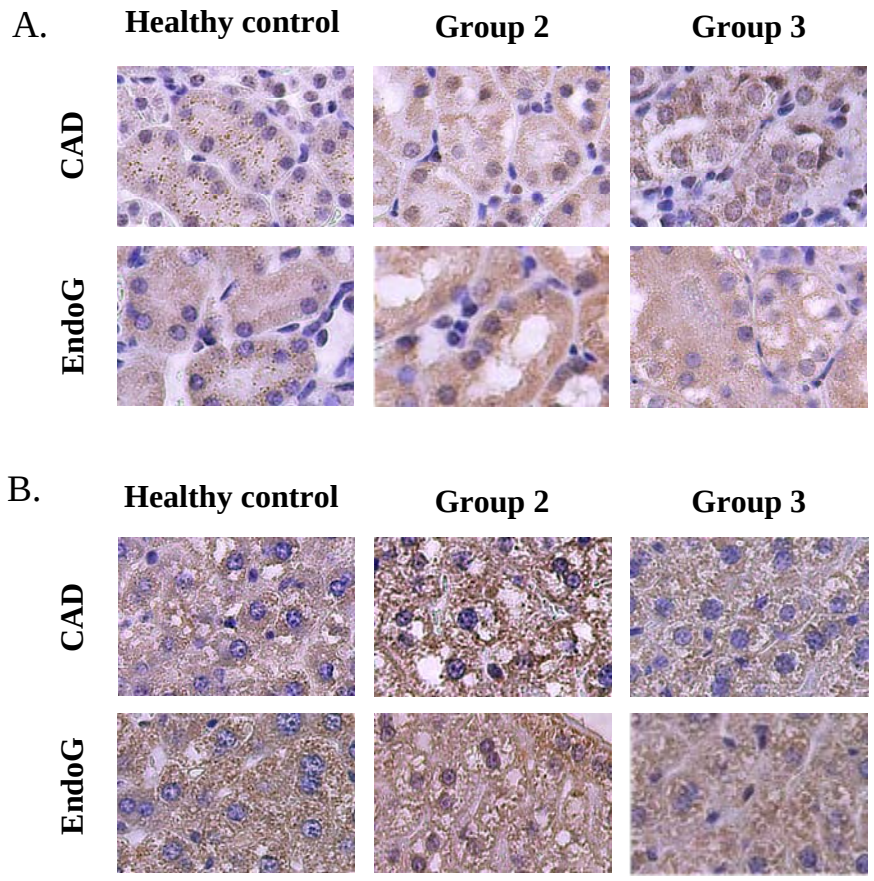

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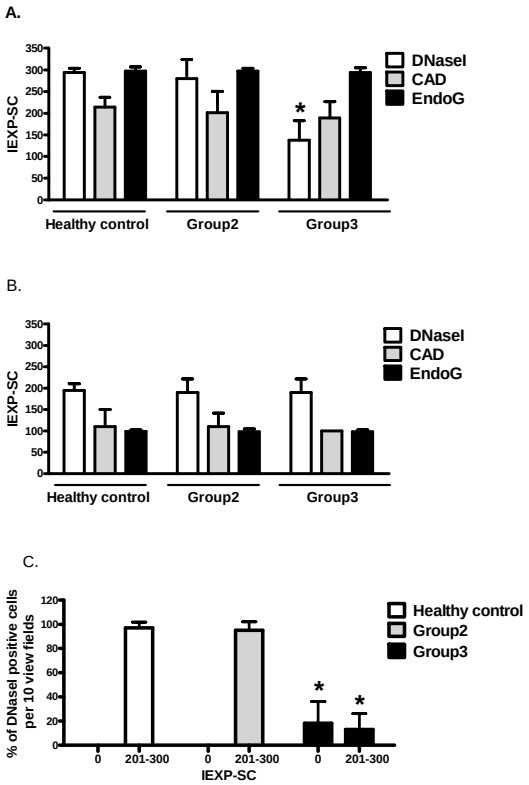

Figure 1S. Quantitative analysis of DNaseI, CAD and EndoG expressions on kidney and liver sections of nephritic BW mice. Intensity of cells staining on kidney (A) and liver (B) sections with anti-DNaseI, CAD and EndoG antibodies were quantified by immunoexpression score (IEXP-SC) = AEX x ISI, where AEX = percentage cellular area of expression and ISI = immunostaining intensity. Given numbers are the averages of IEXP-SC in 10 view fields (±SD). Where is 0 represent negative-IEXP-SC; 1-100 is weakly positive-IEXP-SC; 101-200 is moderately positive-IEXP-SC and 201-300 is strongly positive-IEXP-SC. Expression of DNaseI is markedly reduced in Group 3 of BW mouse with membrano-proliferative nephritis while it is strongly expressed in healthy control and BW mouse with mild mesangial nephritis in Group 2 (A). No significant differences were observed in nucleases expression in livers between healthy control and BW mice in Group 2 and 3 (B). The percentage of tubular cells with expression of DNaseI quantified by IEXP-SC as strongly positive and tubular cells with totally absent DNaseI expression quantified by IEXP-SC as negative were determined on kidney sections from healthy control and BW mice Group 2 and 3 (C). Renal cells that did not express DNaseI were present only on kidney section from BW mouse with severe nephritis Group3. Amount of cells that had DNaseI expression equivalent to that observed on section from healthy control was dramatically reduced on kidney section of BW mouse Group 3. Asterix indicates a significant difference compared to Healthy control and Group 2 ($p<0.05$).

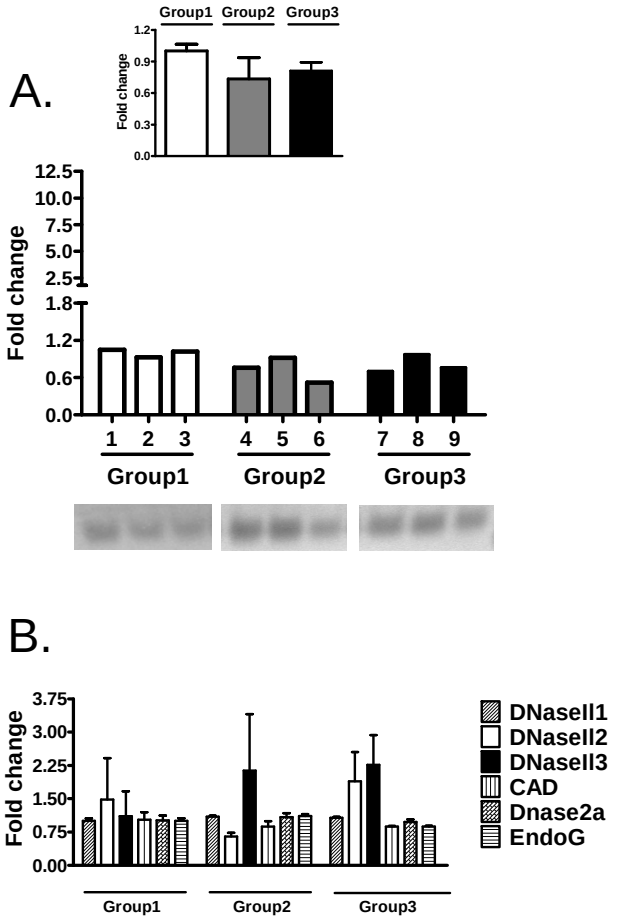

Figure 2S. Expression profiles of nucleases in kidneys of BALB/c mice. The mRNA levels (columns) and enzyme activity (gels) of DNaseI are determined in kidneys of female BALB/c mice divided into three groups accordingly to age of BW mice in Group 1-3 (A). Insert in panel demonstrates mean (±SD) of renal DNaseI mRNA levels. Expression and enzyme activity of DNaseI is stable in kidneys of BALB/c mice at different age groups. The mRNA levels of DNaseII1-3, CAD, Dnase2a and EndoG are not significantly different in kidneys of BALB/c mice in Group 2 and 3 compared to transcription levels in Group 1 (B). Values are given as mean (±SD).

Table 1S. Transcription level of renal DNaseI in BW and BALB/c mice*

	Group 1	Group 2	Group 3
		Delta Ct values	
BW	0.86±0.16	0.99±0.19	3.43±1.49†
BALB/c	1,79±0.1	2,27±0.42	2,10±0.45
		Fold change	
Normalization to Group1 BW			
BW	1.00±0.11	0.90±0.13	0.20±0.13‡,§
BALB/c	0.67±0.04‡	0.49±0.13‡	0.54±0.10‡
Normalization to Group1 BALB/c			
BALB/c	1.00±0.07	0.73±0.20	0.81±0.14‡
BW	1.90±0.20§	1.75±0.24§	0.43±0.25‡,§

* Data are given as mean ± SD

† Means statistically significant $P < 0.007$ in delta Ct values versus Group1 BW mice

‡ Means statistically significant at $P < 0.05$ in fold change versus Group1 BW mice

§ Means statistically significant at $P < 0.05$ in fold change versus Group1 BALB/c mice