Targeted disruption of \textit{Cd40} in a genetically hypertensive rat model attenuates renal fibrosis and proteinuria, independent of blood pressure

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Abstract

High blood pressure is a common cause of chronic kidney disease. Since CD40, a member of the tumor necrosis factor receptor family, has been linked to the progression of kidney disease in ischemic nephropathy, we studied the role of \textit{Cd40} in the development of hypertensive renal disease. The \textit{Cd40} gene was mutated in the Dahl S genetically hypertensive rat with renal disease by targeted-gene disruption using zinc-finger nuclease technology. These rats were then given low (0.3\%) and high (2\%) salt diets and compared. The resultant \textit{Cd40} mutants had significantly reduced levels of both urinary protein excretion (41.8 ± 3.1 mg/24hours vs. 103.7 ± 4.3 mg/24hours) and plasma creatinine (0.36 ± 0.05 mg/dL vs. 1.15 ± 0.19 mg/dL), with significantly...
higher creatinine clearance compared to the control S rats (3.04 ± 0.48 ml/minute vs. 0.93 ± 0.15 ml/minute) indicating renoprotection was conferred by mutation of the Cd40 locus. Furthermore, the Cd40 mutants had a significant attenuation in renal fibrosis, which persisted on the high salt diet. However, there was no difference in systolic blood pressure between the control and Cd40 mutant rats. Thus, these data serve as the first evidence for a direct link between Cd40 and hypertensive nephropathy. Hence, renal fibrosis is one of the underlying mechanisms by which Cd40 plays a crucial role in the development of hypertensive renal disease.

Keywords
CD40; Hypertension; Hypertensive Renal Disease; Renal Fibrosis; Renal Function

Introduction
Hypertension may account for as much as 30% of the new cases of end-stage renal disease in the United States (1). Strategies to improve clinical outcomes include blood pressure control and the use of renin-angiotensin inhibiting drugs (2). We previously demonstrated that circulating CD40 levels predict changes in renal function in people with renal ischemia and hypertension (3). CD40, a type-I transmembrane receptor and a member of the tumor necrosis factor (TNF) receptor superfamily, is expressed on a variety of cells and is a vital mediator of thrombosis and inflammation (4). The immunological function of CD40 has been extensively characterized (5), however the role of CD40 in the development of hypertension-related renal fibrosis has yet to be elucidated. CD40 is expressed by renal proximal tubule epithelial cells, where activation of CD40 has been proposed to mediate inflammation and renal fibrosis. Specifically, proximal tubule expression of CD40 is up-regulated following chronic renal allograft rejection (6). Activation of CD40 increases monocyte chemoattractant protein-1 (MCP-1), intracellular adhesion molecule-1 (ICAM-1), and plasminogen activator inhibitor type 1 (PAI-1) expression leading to interstitial inflammation and fibrosis (7–10). Importantly, renal ischemia increases Angiotensin II release, which has been shown to induce transforming growth factor beta (TGF-β) that in turn stimulates proximal tubule CD40 expression leading to a cytotoxic CD8+ T cell response resulting in damage to tubular epithelial cells (9, 11). Furthermore, inhibition of CD40 signaling reduced the severity of renal injury in chronic proteinuric renal disease (12).

The above correlative studies raise the question of whether CD40 is functionally or consequentially associated with hypertensive renal disease, a question that cannot be addressed with association studies in humans. In order to determine the role of CD40 in the development of hypertensive renal disease, we took the approach of generating a zinc-finger mediated targeted mutation of the Cd40 locus in a genetically susceptible model of hypertensive renal disease and assessing the effects of disrupting the functionality of the Cd40 protein on the extent of renal disease and hypertension. The model of choice was the Dahl S rat, which is a well-characterized model of hypertension (13, 14) prone to the development of renal disease as demonstrated by progressive proteinuria, glomerular sclerosis, and renal fibrosis (15, 16) as well as a strain with proven success of the ZFN-mediated targeted gene disruption strategy (17–22). The results of our study lend support to

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the view that the correlative observation of high levels of CD40 in patients with renal dysfunction is not dismissible as a mere association, but rather that CD40 is a previously unrecognized, important contributor to the development of hypertensive renal dysfunction.

Results

Targeted editing of the Cd40 locus using Zinc-finger nucleases

To investigate the role of Cd40 in hypertensive renal disease using a mutant rat model of Cd40, ZFNs were designed to target the third exon of the Cd40 rat gene (Figure 1A). Injection of these ZFNs into single-cell S embryos resulted in the generation of a founder rat with an 11-bp deletion mutation in the genomic sequence of Cd40. The targeted deletion within the coding sequence of Cd40 is predicted to cause the translation of 6 nonsense amino acids followed by the introduction of a premature stop codon at position 87, thus yielding a truncated protein (Figure 1B). Compared to the wild-type Cd40, which consists of 289 amino acids, the mutant Cd40 lacked 208 amino acids from the c-terminal end (Figure 1B). Western blot analysis confirmed the mutant status of Cd40. Cd40 was not immunologically detectable in kidney tissue derived from the Cd40 mutant rats and was detectable in S rats (Figure 1C). The Cd40 mutant rats appeared healthy and gained weight similar to the S rats (356 ± 4 g vs. 348 ± 6 g).

Targeted disruption of Cd40 prevents Cd40-dependent B-cell activation

In order to determine that the Cd40 mutant receptor is nonfunctional, we isolated peripheral blood mononuclear cells (PBMCs) from Cd40 mutants and S rats followed by treatment with soluble CD40 ligand (sCD40L) to induce Cd40 dependent B-cell activation. PBMCs were labeled with a B-cell specific marker (CD45RA) followed by CD86 (a well-known marker of B-cell activation) (23). As shown in Supplemental Figure 1, sCD40L treatment substantially increased CD86 % positive B-cells in S rats compared to Cd40 mutants which were unresponsive to treatment as assessed by flow cytometry. These data indicate that targeted disruption of the Cd40 receptor results in a loss of Cd40 function in the Cd40 mutant rats.

Targeted mutation of Cd40 improves renal function and significantly reduces renal fibrosis

Renal function was assessed by 24-hour urinary protein excretion (UPE), plasma creatinine (Cr), and creatinine clearance (CrCl) of Cd40 mutant rats (n=10) and S rats (n=10). The Cd40 mutant rats demonstrated a 40% decrease in UPE compared to S rats (41.8 ± 3.1 mg/24hrs vs. 103.7 ± 4.3 mg/24hrs, p<0.01) (Figure 2A). The Cd40 mutant rats had significantly lower plasma creatinine compared to the S rats (0.36 ± 0.05 mg/dL vs. 1.15 ± 0.19 mg/dL, p<0.01) (Figure 2B), while the Cd40 mutant rats demonstrated a threefold higher creatinine clearance compared to S rats (3.04 ± 0.48 ml/min vs. 0.93 ± 0.15 ml/min, p<0.01) (Figure 2C). Renal fibrosis as measured by Mason’s trichrome staining demonstrated a fivefold decrease in the Cd40 mutant rats compared to S rats (Figure 2D, p<0.01). In support of the renal fibrosis data, renal collagen-1 protein expression was also significantly reduced in the Cd40 mutant rats compared to S rats (Figure 2E, p<0.01). Collectively, these results indicated that the renal function of Cd40 mutant rats was superior to that of the S rats, and demonstrated that a targeted disruption of Cd40 has a significant impact on the development of renal
fibrosis. The S rats and Cd40\textsuperscript{mutant} rats demonstrated a similar degree of glomerular injury (Figure 3A.). However, the Cd40\textsuperscript{mutant} rats exhibited a significant decrease in renal tubular injury where CD40 is predominantly expressed within the kidney (Figure 3B, p<0.05).

**Targeted mutation of Cd40 does not alter BP**

In order to determine if improved renal function and decreased fibrosis was attributable to BP differences in the Cd40\textsuperscript{mutant} rats, blood pressure was first measured by the tail-cuff method. There was no change in systolic BP in the Cd40\textsuperscript{mutant} rats compared to the S rats (181 ± 3.8 vs. 183 ± 4.8 mmHg, p=ns) (Figure 4A). To confirm these results, radiotransmitters were surgically implanted, and BP was continuously recorded by telemetry. At all the time points of the three days of continuous monitoring, both systolic and diastolic blood pressures were similar in the Cd40\textsuperscript{mutant} rats compared to S rats (Figure 4B). Further, there were no differences in heart rate or pulse pressure between the two test groups (Figure 4C and 4D). Overall, these data point to an improvement in renal function and a reduction in renal fibrosis in the Cd40\textsuperscript{mutant} rats independent of blood pressure.

**Targeted disruption of Cd40 improves renal function and decreases renal fibrosis under the influence of a high salt diet while maintaining high blood pressure**

Twenty four-hour urinary protein excretion (UPE), plasma creatinine (Cr), and creatinine clearance (CrCl) was assessed in Cd40\textsuperscript{mutant} rats (n=10) and S rats (n=10) after 24 days on a high salt diet (Cd40\textsuperscript{mutant} HS, and S rat HS) in order to determine the effects of high salt on Cd40 and renal function. The Cd40\textsuperscript{mutant} HS rats had lower levels of UPE compared to S rats on high salt, however this trend was not significant (128.3 ± 5.9 mg/24hrs vs. 145.4 ± 7.0 mg/24hrs, p=ns) (Figure 5A). The Cd40\textsuperscript{mutant} HS rats demonstrated significantly less plasma Cr compared to S rats on high salt (0.71 ± 0.07 mg/dL vs. 1.06 ± 0.05 mg/dL, p<0.01) (Figure 5B) as well as significantly elevated creatinine clearance (1.45 ± 0.48 vs. 0.96 ± 0.15, p<0.01) (Figure 5C). The Cd40\textsuperscript{mutant} HS rats demonstrated a significant decrease in renal fibrosis compared to S rats on high salt as assessed by Mason’s trichrome staining (p<0.01, Figure 5D), which was confirmed by collagen-1 protein expression (p<0.01, Figure 5E). There was no significant difference in systolic or diastolic blood pressure between Cd40\textsuperscript{mutant} rats and S rats on a high salt diet following three continuous days of assessment by radiotelemetry (Figure 6). Taken together, these data indicate that mutation of Cd40 lessens the development of renal dysfunction and significantly decreases renal fibrosis even under the influence of a high salt diet independent of blood pressure.

**High salt induces T-cell infiltration in the kidneys**

Infiltrating T-cells have been shown to significantly contribute to the development of hypertension in Dahl S rats following a high salt diet (24, 25), and B-cells stimulate T-cell activation via CD40/CD40L signaling (26). Therefor, we performed immunohistochemistry in kidney tissue sections to determine the extent of T-cell infiltration in S rats compared to Cd40\textsuperscript{mutant} rats. In agreement with previous reports (24, 25), S rats maintained on a high salt diet exhibited a significant increase in CD3+ T-cell infiltration compared to S rats on a low salt diet (p<0.01), as well as compared to Cd40\textsuperscript{mutant} rats on a low salt diet (p<0.01, Supplemental Figure 8A and B). The Cd40\textsuperscript{mutant} rats maintained on a high salt diet also demonstrated a significant increase in CD3+ T-cell infiltration compared to S rats (p<0.05).
and Cd40mutant rats (p<0.01) on low salt (Supplemental Figure 8A and B). We also observed a significant increase in cytotoxic CD8+ T-cell infiltration in both S rats and The Cd40mutant rats maintained on a high salt diet compared to Cd40mutant rats and S rats on a low salt diet (p<0.01 for both, Supplemental Figure 9A and B). These data indicate that infiltrating T-cells may contribute to the sustained hypertension we observed in both the S and Cd40mutant rats following a high salt diet.

Cd40mutant rats demonstrate a significant reduction in proximal tubule PAI-1 and phospho-lyn kinase expression

Activation of proximal tubular CD40 has been shown to stimulate the pro-fibrotic mediator PAI-1 acting through a signaling pathway mediated by the phosphorylation of the lyn kinase (a src family kinase) (27). Therefore, we evaluated phospho-lyn kinase and PAI-1 as potential signaling mechanisms in the development of renal fibrosis. Protein expression of PAI-1 and phospho-lyn kinase were measured in proximal tubules isolated from Cd40mutant rats and S rats maintained on a low salt diet. The Cd40mutant rats demonstrated a significant reduction in proximal tubule phospho-lyn kinase (p<0.05, Figure 7A) and PAI-1 expression (p<0.01, Figure 7B) implicating the phospho-lyn/PAI-1 signaling axis as a potential mediator of renal fibrosis in experimental hypertensive nephropathy.

Discussion

There are obvious questions that arise as a result of our definitive report which relate to the pathways that are potentially perturbed as a result of the functional disruption of Cd40. Previous work in renal disease and renal transplant models has implicated activation of the CD40/CD40L signaling cascade as a key mediator of inflammation and fibrosis within the kidney (27, 28). Specifically, stimulation of the CD40 receptor causes T-cell activation and increased expression of numerous pro-inflammatory mediators including IL-6, IL-8, RANTES, and MCP-1 (10, 27). CD40 activation in human proximal tubule cells has been shown to activate the pro-fibrotic mediator PAI-1 in a pathway mediated by the activation of phospho-lyn kinase implicating proximal tubular activation of CD40 in the generation of renal fibrosis (27). Furthermore, inhibition of CD40/CD40L signaling decreased the severity of renal injury in an animal model of chronic proteinuric renal disease (12).

In our novel Cd40mutant model created on a background prone to the development of renal disease we report a significant reduction in renal fibrosis and improvement in renal function, which persists even under the influence of a high salt diet. The Cd40mutant rats also demonstrated a significant reduction in tubular injury with no significant change in glomerular injury compared to S rats indicating that proximal tubule-specific Cd40 is an important mediator of renal injury. Importantly, the Cd40mutant rats demonstrate a significant reduction in proximal tubule phospho-lyn kinase and PAI-1 providing evidence for this signaling pathway in the development of renal fibrosis. In human proximal tubule epithelial cells Pontrelli et al. demonstrated that activation of CD40 induced a significant increase in the phosphorylation of lyn and PAI-1 expression, and inhibition of lyn attenuated PAI-1 expression (27). PAI-1 has been implicated in the development of renal fibrosis in many different forms of both clinical and experimental renal disease. Clinically, PAI-1 has been
implicated in the development of renal fibrosis in chronic allograft nephropathy, crescentic glomerulonephritis, and proliferative glomerulonephritis (29). In experimental models of crescentic glomerulonephritis and unilateral ureteral obstruction PAI-1 knockout mice exhibited a significant reduction in renal fibrosis, while overexpression of PAI-1 significantly increased renal fibrosis (30–32). Future work will need to address whether the Cd40 stimulated phospho-lyn/PAI-1 signaling axis is a key mediator in the development of renal fibrosis.

Surprisingly, the Cd40mutant rats remained hypertensive despite an improvement in renal function and reduced renal fibrosis. Others have reported a reduction in renal fibrosis following NADPH oxidase inhibition in an animal model that remains hypertensive (33). These results complement our current work in regard to improved renal function despite hypertension, as CD40 appears to be activated either down-stream or independent of signaling events triggered by hypertension. Others have demonstrated that T-cell infiltration within the kidney contributes to amplification of Dahl-S high salt-induced hypertension (24, 25). We observed a significant increase in both CD3+ and CD8+ infiltrating T-cells following high salt treatment in both S and Cd40mutant rats, suggesting that this may be a potential mechanism contributing to the persistent hypertension observed in our model as well. Further work will be necessary to determine the extent to which CD40 may mediate the inflammatory response in this model. While the global deletion of Cd40 in our model does not allow us to directly address the specific cell types involved in potentiating renal fibrosis, it does suggest that Cd40 plays and important role in mediating renal fibrosis and renal injury in a hypertensive model of renal disease. The S and Cd40mutant rats have similar levels of T-cell infiltration in both low and high salt conditions, which suggests that Cd40 on cell types other than inflammatory cells in the kidney are mediating the pro-fibrotic response.

We have created a novel Cd40mutant model, which demonstrates a renoprotective phenotype in a background prone to the development of renal dysfunction as exemplified by significant attenuation of renal fibrosis and improved renal function. Notably, our data suggest that Cd40 mediated phospho-lyn/PAI-1 signaling may be an important contributor to the development of renal fibrosis in hypertensive nephropathy. The availability of the model described in our study moves the field forward from mere association studies by providing direct evidence that CD40 is a key mediator of hypertensive renal disease. Treatments aimed at inhibiting CD40L have been complicated by the development of thrombotic events in human trials and in animal models (34, 35). However, treatment strategies aimed at inhibiting CD40 receptor signaling as a form of cancer immunotherapy have shown promising results in early-phase clinical trials (36). Our results indicate that inhibition of the CD40 receptor could offer a promising therapeutic option for the treatment of renal fibrosis in hypertensive renal disease.

Materials and Methods

Animals

All animal experimentation described in this article was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.
under protocols approved by the University of Toledo Institutional Animal Care and Use Committee. The Dahl Salt-sensitive rats were from our colony inbred at the University of Toledo College of Medicine and Life Sciences. Each set of homozygous \( Cd40^{\text{mutant}} \), and parental strain S rats were bred, housed, and studied concomitantly to minimize environmental effects.

**Generation of a ZFN-mediated \( Cd40^{\text{mutant}} \) rat**

ZFN construct pairs specific for the rat \( Cd40 \) gene were designed, assembled, and validated by Sigma-Aldrich to target the third exon of \( Cd40 \) (target sequence

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\text{CAGCACCACACACCTGCgaactcAGTGC GGCTGCCGGG} \quad \text{ZFN binds to each sequence shown in bold on opposite strands). mRNA encoding the} \quad Cd40 \text{ZFN pairs were diluted in RNAse-free tris-EDTA buffer, pH 7.4, at a concentration of 2ng/μL and injected into fertilized Dahl S rat eggs as described previously (37). Thirty six rat egg donors produced 484 eggs, 364 were fertilized and microinjected, 328 eggs survived injection, and 328 eggs were transferred to pseudopregnant Sprague-Dawley females (Charles River Laboratory SAS SD rats), which gave birth to 75 rat pups. DNA was extracted (Wizard SV 96 Genomic DNA purification system, Promega) from tail tissue and was amplified using \( Cd40 \) ZFN Forward (5′ CCACACCTGCCTCATATTCT 3′) and \( Cd40 \) ZFN Reverse (5′ AAGGACCTCCCTCACAT 3′) primers. PCR products were analyzed through 2% (wt/vol) agarose gel followed by DNA sequencing using MWG operon sequencing service. The sequencing data were analyzed using Sequencher 4.10.1. Among the 75 pups born, 19 positive heterozygous founder males were identified. The founder population was generated at the University of Michigan transgenic animal model core facility (Ann Arbor, MI.) The founder male rats were backcrossed to the same littermate of nonfounder females. Multiple separate pairs of mutation-carrying progeny were then intercrossed to generate an F2 population that was used for phenotyping and breeding to homozygosity. The DNA sequencing of the homozygous \( Cd40^{\text{mutant}} \) rats showed an 11 bp deletion of the sequence “CTCGAACLCTCA” in exon 3 of the \( Cd40 \) gene.

**Urinary protein excretion (UPE)**

Twenty-four hour urinary protein excretion was performed as previously described (38).

**Creatinine (Cr) and creatinine clearance (CrCl)**

At the conclusion of the study, 24-hour urine samples were collected. Following the urine collection the study animals were euthanized and blood samples were obtained from the abdominal aorta. Plasma and urine creatinine were evaluated with a colorimetric method using a commercial kit from Teco Diagnostics (Anaheim, CA). Creatinine and creatinine clearance was calculated as previously described (39).

**Histology**

Trichrome staining was performed on kidney tissues and tissue fibrosis was quantified as previously reported (40). Kidney sections were immediately fixed in 4% formalin buffer solution (pH 7.2) for 18 h, dehydrated in 70% ethanol, and then embedded in paraffin and cut with a microtome. Trichrome staining was then performed and fibrosis was quantified.
using ImageJ software. For quantitative morphometric analysis, five random images of trichrome slides were taken at 40x magnification and electronically scanned into an RGB image which was subsequently analyzed using ImageJ (version 1.48) software. The amount of fibrosis was then estimated from the RGB images with a macro written by the authors (J.I.S.) by converting pixels of the image with substantially greater (>120%) blue than red intensity to have the new grey scale amplitude = 1, leaving other pixels as with amplitude = 0 (40).

**Glomerular and Tubular Injury**

Kidney sections were cut into 4 μm sections and were subjected to trichrome and hematoxylin and eosin staining. Tubules and glomeruli were graded in blinded fashion on an arbitrary semiquantitative scale from 0 (normal), 1 (mild), 2 (moderate), and 3 (severe) by an experienced nephrologist (Joseph I. Shapiro, MD) with the overall average represented for each group. Glomeruli were assessed for the presence of hypercellularity, cellular crescents, necrosis, and sclerosis. Tubules were assessed for degeneration and atrophy, and interstitial inflammation and fibrosis.

**Isolation of renal proximal tubules**

Renal proximal tubules were isolated from the outer cortices as previously described (41, 42). Briefly, harvested kidneys were decapsulated and rinsed with ice-cold oxygenated PBS. Cortices were dissected, minced, and digested in digesting solution (oxygenized DMEM with 1 mg/ml collagenase Type 2, 0.5% filtered BSA fraction V) four times at 37 °C, 15 min each. Pooled tubular segments were further separated with 42% Percoll gradient (pH 7.4), and the renal proximal tubule segments were collected from the lowest two bands.

**Immunoblot analysis of Cd40, Collagen-1, phospho-lyn kinase, and PAI-1**

Western blot analyses were performed on proteins from tissue homogenates as previously reported (39, 43). Briefly, kidney tissue was homogenized in ice-cold RIPA lysis buffer (pH 7.0; Santa Cruz Biotechnology, Santa Cruz, CA; sc-24948). The homogenate was centrifuged at 1,400g for 30s at 4°C. The supernatant was separated and used for Cd40, phospho-lyn kinase, and PAI-1 analysis, and the pellet fraction (used for the detection of collagen-1) was resuspended in 5% sodium dodecyl sulfate (SDS) in 50mmol/L Tris-HCl (pH 7.4). The protein was quantified in the supernatant and the resuspended pellet fraction. Protein was solubilized at a concentration of 60μg for detection of Cd40, 40μg for detection of phospho-lyn kinase and PAI-1, and 10–20μg of protein per well for detection of collagen-1 in sample buffer (2% SDS, 5% β-Mercaptoethanol, 20% glycerol, 0.005% bromophenol blue, and 50mmol/L Tris-HCl; pH 7.0).

The proteins were then resolved via SDS-polyacrilamide gel electrophoresis (PAGE) using Precast Ready Gels 4–15% Tris-HCl, purchased from Bio-Rad (Hercules, CA). Following PAGE the proteins were electrotransferred from the gel onto nitrocellulose membranes. The membrane was blocked with 5% nonfat dry milk in 20mmol/L Tris-HCl (pH 7.5, 150 mmol/L NaCl, and 0.1% Tween-20). Goat anti-type 1 collagen antibody (Southern Biotech, Birmingham, AL) was used to probe for collagen-1 and secondary anti-goat horseradish peroxidase conjugated antibody was purchased from Santa Cruz Biotechnology (sc-2020).
Mouse anti-CD40 antibody was used to probe for Cd40 (ab-50849, Abcam Inc, Cambridge, MA). Rabbit anti-phospho-lyn HRP conjugated antibody was used to probe for phospho-lyn kinases (Bioss, Woburn, MA). Rabbit anti-PAI-1 antibody was used to probe for PAI-1 (Cell Signaling Technology, Danvers, MA). Secondary anti-mouse horseradish peroxidase conjugated antibody was purchased from Invitrogen (626520). Secondary anti-rabbit horseradish peroxidase conjugated antibody was purchased from Santa Cruz Biotechnology (sc-2301). For chemiluminescent detection and quantification, ECL and ECL plus were employed (Bio-Rad, Hercules, CA). Loading conditions were controlled by using anti-actin goat polyclonal antibody, and anti-lyn monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (39, 43).

BP measurements by tail-cuff and radiotelemetry

_Cd40mutant_ rats (n=20) and S rats (n=20) were weaned on a low salt diet (0.3% NaCl, Harlen Teklad) at the age of 30 days. Rats were divided into two groups at the age of 40 days. One group of rats were continuously fed a low salt diet (n=10 _Cd40mutant_ rats and n=10 S rats), while the remaining _Cd40mutant_ rats (n=10) and S rats (n=10) were fed with a high salt diet (2% NaCl, Harlen Teklad), referred to as _Cd40mutant_ HS, and S rat HS. Systolic BP measurements were obtained on rats 24 days following low salt diet treatment by the tail-cuff method as previously described (44). One week after the tail-cuff BP measurements, radiotelemetry experiments and statistical analysis were conducted as previously reported (44). For rats maintained on a high salt diet, blood pressure was assessed by radiotelemetry 24 days following high salt diet treatment. Rats were euthanized after obtaining BP measurements, and total body weights and organ weights were collected.

Statistical Analysis

Data are presented as the mean ± s.e.m. Data were analyzed by 2-tailed Student’s t-test. A P-value of less the 0.05 was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism software (San Diego, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Figure 1. Screening animals for ZFN-targeted mutation at the Cd40 locus
(A) ZFNs were designed to target the sequence labeled as the ZFN cutting site, which is in exon 3 of the rat Cd40 gene (http://useast.ensemble.org, Version 5.0). To determine whether a site-specific editing had occurred in the Cd40 locus, tail DNA samples from pups born post-microinjection of custom ZFNs targeting exon 3 of the Cd40 locus were screened by PCR amplification with primers designed to amplify over the target site. The gel image is that of PCR products obtained. There was a shift in band size between the S (347bp) and Cd40mutant (336bp) rats. Representative sequencing results from the PCR products shown in panel A detected an 11 bp deletion in the Cd40 gene. (B) The mutation created within the Cd40 gene introduced missense mutations shown in black followed by a premature stop.
codon. The predicted domain features of the wild-type and mutant forms of Cd40 are shown below the amino acid sequences; TNFR (tumor necrosis factor receptor). To the right of this representation, the 3D protein structure for wild-type and Cd40 mutant are shown as predicted using Phyre, a web-based server for predicting 3D protein structure (http://www.sbg.ic.ac.uk/phyre2/html/page.cgi?id=index). (C) Immunoblot analysis of Cd40 protein expression in Cd40 mutant and S rats. The anti-Cd40 antibody was purchased from Abcam (ab50849), and raised against a synthetic peptide directed towards amino acids 21–193.
Figure 2. Renal function and renal fibrosis analysis

(A) Twenty-four hour urinary protein excretion (UPE), (B) plasma creatinine (Cr), and (C) creatinine clearance (CrCl) in Cd40\textsuperscript{mutant} rats vs. S rats. (D) Representative (upper panel) and quantitative analysis (lower panel), mean ± s.e.m. of trichrome-stained photomicrographs obtained from kidney tissue from Cd40\textsuperscript{mutant} rats and S rats. Scale bar = 50 μm. (E) Representative (upper panel) and quantitative analysis (lower panel), mean ± s.e.m. of Collagen-1 immunoblot performed using kidney tissue homogenates from Cd40\textsuperscript{mutant} rats and S rats. Actin was used as a loading control. *p<0.01
Figure 3. Glomerular and tubular injury
Representative (upper panel) and quantitative analysis (lower panel), mean ± s.e.m. of hematoxylin and eosin and trichrome-stained photomicrographs obtained from (A) glomeruli and (B) tubule tissue from Cd40mutant rats and S rats. Scale bar = 50 μm. *p<0.05
Figure 4. Blood pressure measurement

(A) Mean systolic BP effect ± s.e.m. by the tail-cuff method. (B) Systolic and diastolic BP, (C) heart rate, and (D) pulse pressure measurements after surgical implantation of radiotelemetry transmitters. Data plotted are the recordings obtained once every 5 min continuously for 24 hours and averaged for 4 hour intervals over 3 days. Levels of statistical significance for all data were analyzed by independent sample t-test.
Figure 5. Renal function and renal fibrosis analysis from \textit{Cd40}\textsuperscript{mutant} rats and \textit{S} rats maintained on a high salt diet

(A) Twenty-four hour urinary protein excretion (UPE), (B) plasma creatinine (Cr), and (C) creatinine clearance (CrCl) in \textit{Cd40}\textsuperscript{mutant} rats vs. \textit{S} rats following 24 days on a high salt diet (HS). (D) Representative (upper panel) and quantitative analysis (lower panel), mean ± s.e.m. of trichrome-stained photomicrographs obtained from kidney tissue from \textit{Cd40}\textsuperscript{mutant} rats and \textit{S} rats following 24 days on a high salt diet (HS). Scale bar = 50 μm. (E) Representative (upper panel) and quantitative analysis (lower panel), mean ± s.e.m. of Collagen-1 immunoblot performed using kidney tissue homogenates from \textit{Cd40}\textsuperscript{mutant} rats and \textit{S} rats following 24 days on a high salt diet (HS). Actin was used as a loading control. *p<0.01
Figure 6. Blood pressure measurements from $Cd40^{mutant}$ rats and S rats maintained on a high salt diet
Systolic and diastolic BP measurements after surgical implantation of radiotelemetry transmitters in rats following 24 days on a high salt diet (HS). Data plotted are the recordings obtained once every 5 min continuously for 24 hours and averaged for 4 hour intervals over 3 days. Levels of statistical significance for all data were analyzed by independent sample t-test.
Figure 7. Phospho-lyn kinase and PAI-1 expression

Representative (upper panel) and quantitative analysis (lower panel), mean ± s.e.m. of (A) phospho-lyn kinase (p-Lyn) and (B) PAI-1 immunoblots performed using proximal tubule tissue homogenates from Cd40mutant rats and S rats. Actin and total lyn were used as loading controls. *p<0.05, #p<0.01