A Sensitized Screen of N-ethyl-N-nitrosourea–Mutagenized Mice Identifies Dominant Mutants Predisposed to Diabetic Nephropathy

Elena E. Tchekneva,* Eugene M. Rinchik,† Dina Polosukhina,* Linda S. Davis,* Veronika Kadkina,* Yassir Mohamed,* Steve R. Dunn,‡ Kumar Sharma,‡ Zhonghua Qi,* Agnes B. Fogo,*§ and Matthew D. Breyer*¶

*Division of Nephrology, Department of Medicine, §Department of Pathology, and ¶Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, Tennessee; †Life Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Department of Biochemistry, Cellular and Molecular Biology, University of Tennessee, Knoxville, Tennessee, and Taconic Farms, Inc., Hudson, New York; and ‡Dorrance Hamilton Research Laboratories, Division of Nephrology, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania

Diabetic nephropathy (DN) is a late diabetic complication that comprises progressively increasing albuminuria, declining GFR, and increased cardiovascular risk. Only a minority of patients with diabetes (25 to 40%) develop nephropathy, and there is evidence that heritable genetic factors predispose these “at-risk” individuals to DN. Comparing variability among inbred mouse strains with respect to a specific phenotype can model interhuman variability, and each strain represents a genetically homogeneous system with a defined risk for nephropathy. C57BL/6 mice, which are relatively resistant to DN, were mutagenized using N-ethyl-N-nitrosourea and screened for mutants that developed excess albuminuria on a sensitizing type 1 diabetic background contributed by the dominant Akita mutation in insulin-2 gene \( (\text{Ins}2^{\text{Aki}}) \). Two of 375 diabetic G1 founders were found to exhibit albumin excretion rates persistently 10-fold greater than albumin excretion rates in non-mutagenized \( \text{Ins}2^{\text{Aki}} \) controls. This albuminuria trait was heritable and transmitted to approximately 50% of G2 and G3 progeny, consistent with a simple, dominantly inherited trait, but was never observed in nondiabetic offspring. During the course of 1 yr, albuminuric \( \text{Ins}2^{\text{Aki}} \) G2 and G3 progeny developed reduced inulin clearance with elevated blood urea nitrogen and plasma creatinine. Glomerular histology revealed mesangial expansion, and glomerular basement membrane thickening as determined by electron microscopy was enhanced in diabetic mutant kidneys. Hereditary albuminuric N-ethyl-N-nitrosourea–induced mutants were redesignated as Nphrp1 (nephropathy1) and Nphrp2 (nephropathy2) mice for two generated lines. These novel mutants provide new, robust mouse models of DN and should help to elucidate the underlying genetic basis of predisposition to DN.


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several studies have examined the impact of deletion of candidate genes (e.g., bradykinin B2 receptor, apolipoprotein E) on the progression of DN in mice (12,13), unbiased genome-wide approaches to identify genes that predispose to DN in mice have not been undertaken.

The use of phenotype-driven whole-genome mutagenesis to recover new N-ethyl-N-nitrosourea (ENU)-induced (14) heritable mutations in mice, together with the extensive homology between the mouse and human genomes, makes mutagenesis an attractive approach to discover new genes that predispose to DN. Mutagenesis offers significant advantages for the analysis of the complex traits (15). ENU is a supermutagen of spermatogonial stem cells that induces a mutation rate of approximately $10^{-3}$ per locus per gamete (16). Therefore, one in every 1000 gametes from a mutagenized male might be expected to carry a mutation in a particular gene of interest. Recent studies indicate that up to 2% of ENU-induced progeny carry a heritable mutant phenotype (17,18). This high frequency of induced mutations potentially should affect every gene that might contribute to any given trait.

In this study, we performed a sensitized screen of G1 progeny for mutants that exhibit renal dysfunction only in the presence of diabetes as the sensitizing condition. ENU-induced mutations in the nephropathy-resistant C57BL/6 strain were bred into a diabetic environment by crossing mutagenized male mice with diabetic C57BL/6$^{ins2Akita}$ (hereafter referred to as $Ins2^{Akita}$) female mice (19), a model of type 1 diabetes. We report the identification and phenotypic characterization of two heritable mutations that result in dominantly inherited nephropathy that is evident only in a diabetic background.

**Materials and Methods**

**Experimental Animals**

All experimental procedures were in compliance with the Vanderbilt University Guide for Care and Use of Laboratory Animals. Mice were housed in a pathogen-free veterinary facility that is accredited by the American Association for the Accreditation of Laboratory Animal Care. Mice were maintained under a controlled 12-h light/dark cycle at a constant temperature of 21 ± 2°C and humidity of 55 ± 10%. Male C57BL/6 and C57BL/6$^{ins2Akita}$ female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Carriers of the Akita mutation were identified by genotyping using a PCR reaction with sense 5′-H11032/H11006 primer at 94°C for 30 s, 61°C for 30 s, and 72°C for 60 s. 

**ENU Mutagenesis**

Sixty male C57BL/6 mice (G0) received an intraperitoneal injection of 85 mg/kg ENU (Sigma) each week for 3 wk beginning at 8 to 12 wk of age to produce high mutation rates in this strain (20). After recovery of fertility, G0 mice then were bred with diabetic C57BL/6$^{ins2Akita}$ female mice to generate G1 offspring for primary renal phenotype screening.

**Fasting Blood Glucose Detection**

The National Institutes of Health has established mouse metabolic phenotyping centers (http://www.mmpc.org) that have developed a standard protocol that includes a fast between 7 a.m. and 1 p.m. and blood drawing at 1 p.m. This protocol has been adopted by the Animal Models of Diabetic Complications Consortium (http://www.amdcc.org). Blood glucose was determined using 4 μl of whole blood, freshly collected from saphenous vein (21), using B-glucose analyzer (HemoCueAB, Angelholm, Sweden) after a 6-h fast.

**Urine Protein Assays**

For detection of albumin/creatinine ratio (ACR; expressed as μg/mg), a 20- to 200-μl volume of spot urine was collected from each mouse that was transferred to the urine collection station that was designed as a corral for individually caged animals. Urinary albumin was detected using Albuwell M kit, and urinary creatinine was measured using the Creatinine Companion murine ELISA kit (Exocell, Philadelphia, PA). For determination of 24-h urinary albumin excretion rate, urine was collected in metabolic cages, and 24 h urinary excretion levels in urine, expressed as μg/24 h, also were assayed with Albuwell M assay kit.

**Inulin Clearance**

Renal inulin clearance was measured using previously described methods (22,23). Briefly, mice were anesthetized by isoflurane (Baxter Pharmaceutical Products, Deerfield, IL) for 60 s, and sterile FITC-inulin (Sigma) solution was injected retro-orbitally (3.74 μl/g body wt). Plasma (approximately 10 μl) was obtained from blood that was collected via the saphenous vein at 3, 7, 10, 15, 35, 55, and 75 min after bolus FITC-inulin injection. FITC concentration was determined by fluorescence of tritiated plasma samples that were loaded onto a 96-well plate using a Fluoroscan Ascent FL (FIN-00811; Labsystems, Helsinki, Finland). GFR was calculated using two-compartment clearance analysis (23).

**Plasma Creatinine and Blood Urea Nitrogen**

Blood urea nitrogen (BUN) was measured by an iSTAT analyzer (Heska Corp., Waukesha, WI) in 75 μl of whole mouse blood. Plasma creatinine was measured as described previously (24). Briefly, plasma was obtained from whole blood, and plasma proteins were precipitated with cold acetonitrile acidified with glacial acetic acid. After evaporation of acetonitrile and any of the residual aqueous phase in a SpeedVac (Farmingdale, NY), the residue that contained creatinine was resuspended in 25 μl of 5 mM sodium acetate (pH 4.2). Samples were centrifuged at 3000 rpm for 5 min (microcentrifuge 5415D; Eppendorf, Hamburg, Germany), and supernatants were loaded into the autosampler of an HPLC system (Perkin-Elmer, Norwalk, CT). Creatinine peak elution was detected at 225 nm at 3.65 ± 0.02 min. The concentration of creatinine was determined from a weighted regression formula that was created using an external standard regression line (Perkin-Elmer).

**Renal Histopathology and Electron Microscopy**

Kidneys were perfused at 140 mmHg with PBS (pH 7.0) followed by 4% paraformaldehyde solution as described previously (10), dissected, and embedded in paraffin, and cross-sections of 4 μm were cut and stained with periodic acid-Schiff. The mesangial expansion score was determined as described previously (25). All glomeruli on single cross-section were examined in each kidney from three animals per group. Mesangial expansion was scored from 0 to 4 according to the proportion of glomerular involvement: score 0, a normal glomerulus; score 1, increased mesangial matrix of up to 25% of glomerular tuft; score 2, mesangial expansion of 25 to 50% of glomerular tuft; score 3, mesangial matrix of up to 50% of glomerular tuft; score 4, mesangial matrix of up to 75% of glomerular tuft; score 5, mesangial matrix of up to 100% of glomerular tuft. Mesangial expansion was scored in each kidney from three animals per group.

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expansion of 50 to 75%; and score 4, mesangial expansion of >75% of glomerular tuft. Average tuft score then was obtained for each animal.

Portions of cortex were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), processed, and embedded in Spurr resin. Thin sections were examined using a FEI/Phillips CM12 transmission electron microscope. Glomerular basement membrane (GBM) was measured in areas of the cross-section of the GBM as evidenced by endothelial cell appearance. At least four measures were made in each glomerulus, and an average was calculated. Foot process effacement was assessed semiquantitatively by estimation of proportion of capillary loops with overlying effacement. All morphologic assessments were done without knowledge of the animal group.

Systolic BP Measurements in Conscious Mice

Systolic BP was determined simultaneously in conscious mice by using a computerized tail-cuff system (IITC, Life Science, Woodland Hills, CA) at the Vanderbilt Mouse Metabolic Phenotyping Center. Adequate training of mice during at least 4 d minimized physiologically apparent stress. At least 10 consecutive readings were averaged after stabilization of blood.

Statistical Analyses

All data are expressed as mean ± SEM. Chance differences probabilities (P) were calculated using ANOVA test. Statistical analysis for expected inheritance of Ins2Akita mutation in ENU-induced G1 progeny was performed by χ² test. P < 0.05 was considered to be statistically different.

Results

Generation of a Population of Diabetic Progeny Carrying ENU Mutations

Sixty male C57BL/6 G0 mice were treated with three intra-peritoneal injections of 85 mg/kg ENU at weekly intervals. The dosage resulted in the expected sterility (20) in all injected male C57BL/6 mice. Approximately 70% of ENU-injected mice regained their fertility by week 15 after the final ENU injection. ENU-mutagenized male mice were mated with C57BL/6 Ins2Akita heterozygous female mice to produce 429 first-generation mice (G1 progeny) during a 2-yr period (Figure 1A). A number of G1 progeny exhibited visible defects such as microphthalmia, as well as craniofacial and skeletal dysmorphology and died in the perinatal period. The remaining 375 G1 survivors were serially screened over 1 yr for a renal phenotype.

Inheritance of Ins2Akita Mutation in G1 Progeny

PCR genotyping showed that 55% of offspring of non–ENU-treated parents carried the Ins2Akita mutation, as expected for inheritance of a dominant trait. In contrast, genotyping of G1 progeny from ENU-mutagenized mice (Figure 1B) demonstrated that the inheritance of the Ins2Akita mutation significantly deviated from the expected 50% (P < 0.0001, χ² test).

Only 29% of G1 progeny (9% female and 20% male) were Ins2Akita mutation carriers, whereas 71% of mice (32% female and 39% male) carried wild-type Ins2+/− allele. This decreased survival of Akita mice was observed only in G1 progeny and was not observed in subsequent generations of Ins2Akita G2 and G3 progeny. This is consistent with a decreased survival of several ENU mutants in the context of the diabetic Ins2Akita mutation.

At 8 wk of age, Ins2Akita G1 progeny exhibited hyperglycemia with fasting blood glucose of 415 ± 144 mg/dl in female mice (n = 34) and 615 ± 116 mg/dl in male mice (n = 74). Fasting blood glucose in age-matched, nonmutagenized Ins2Akita heterozygous female mice was 293 ± 78 mg/dl (n = 9) versus 725 ± 85 (n = 7) in male mice. As expected, Ins2+/− (Akita negative) G1 progeny exhibited lower fasting blood glucose levels of 189 ± 28 mg/dl in female mice (n = 120) and 200 ± 24 mg/dl in male mice (n = 146).

Identification of Putative Primary Mutants with Albuminuria

Starting at 8 wk of age, all Ins2Akita G1 mice were screened for microalbuminuria every 2 wk for at least 2 mo. Frequency histograms of these averaged urine ACR values for each animal were generated (Figure 2; three or more determinations were averaged). ACR values were transformed to Log₁₀ACR to “normalize” the ratiometric data. The average ACR of multiple spot urine samples that were obtained from 23 control diabetic Ins2Akita male and female mice was 22 ± 10 μg/mg, corresponding to a median Log₁₀ACR value of 1.30. In contrast, the median for the Log₁₀ACR for mutagenized Ins2Akita G1 progeny was 1.64. Ten G1 outliers that exhibited an ACR ≥100 μg/mg (Log₁₀ACR ≥2) were identified, exceeding this empiric upper limit for ACR values in nonmutagenized Ins2Akita mice.

Urinary Albumin Excretion in ENU-Induced G1 Phenotyping Variants

The progression of albuminuria in the outliers was assessed by sequential monitoring every 2 wk during the first 4 mo of life and then monthly for up to 1 yr to confirm the phenotype. ACR in urine from the remainder of the G1 mice (i.e., nonoutliers) were examined at least six times in a 12-mo period. Among the 10 outliers initially identified, albuminuria persisted only in six Ins2Akita G1 phenotypic variants that were derived from different ENU-mutagenized C57BL/6 male mice. These phenotypic variants, identified as ENU10, ENU18, ENU20, ENU57, ENU76, and ENU161, showed significantly
greater urinary ACR than control Ins2Akita mice from non-mutagenized sires (223 ± 141, 199 ± 150, 177 ± 136, 214 ± 134, 302 ± 144, and 107 ± 129, respectively, versus 18 ± 15 μg/mg; P < 0.005; Figure 3A). Importantly, urine ACR in Ins2+/− (Akita negative) progeny from mutagenized G0 sires was not different from control Ins2Akita mice, and increased ACR was observed in ENU-induced phenotyping variants in only the setting of diabetes.

Inheritance of ENU-Induced Mutations in G2 and G3 Progeny from G1 Founders

To confirm the genetic transmission of ENU-induced mutations and determine whether these diabetic albuminuria phenotypes were heritable, these six G1 Ins2Akita phenotypic variants were bred with C57BL/6 female mice. For a simple dominant mutation that is responsible for diabetic albuminuria that unlinked to the Ins2Akita mutation, approximately 50% of G2 Ins2Akita diabetic heterozygotes should exhibit albuminuria. Two of G1 phenotypic variants (ENU18 and ENU57) failed to produce any albuminuric offspring in G2 progeny. However, four G1 phenotypic variants (ENU10, ENU20, ENU76, and ENU161) transmitted the albuminuric trait to diabetic Ins2Akita G2 offspring. The ENU10 founder produced only one litter of six pups, and of the four diabetic Ins2Akita G2 offspring, two were albuminuric and sterile and exhibited low sperm count (sperm was collected for cryopreservation). ENU20 and ENU76 founders produced five litters each, with an average of approximately four and six pups per litter, respectively. As was expected for a dominant trait, six of (46.1%) 13 Ins2Akita ENU76 G2 progeny and five (45.5%) of 11 Ins2Akita ENU20 G2 progeny exhibited increased albuminuria. Breeding of albuminuric G2 mice from both mutant lines to generate G3 progeny confirmed the transmission of albuminuria in 46.7% of ENU76 (seven of 15 Ins2Akita mice) and 47.6% ENU20 (10 of 21 Ins2Akita mice) diabetic offspring, respectively. Importantly, none of the nondiabetic Ins2+/− G3 progeny of the ENU76 line (20 of 35 mice) or the ENU20 line (22 of 43 mice) exhibited albuminuria. In contrast, approximately one third of nondiabetic Ins2+/− G3 mice in the ENU161 line exhibited albuminuria, indicating that the ENU-induced renal phenotype in ENU161 is distinct from that in ENU20 and ENU76 and not dependent on diabetes as a sensitizing condition.

Hereditary albuminuric ENU-induced mutants were reidentified as Nphrp1 (nephropathy1 for ENU20 line) and Nphrp2 (nephropathy2 for ENU76 line) mice. Progeny of Nphrp1 and Nphrp2 mutants exhibited significantly increased ACR in diabetic Ins2Akita mice but not in nondiabetic Ins2+/− mice. Half of the diabetic Ins2Akita mice from both Nphrp1 and Nphrp2 mutant lines exhibited albuminuria (averaging 153 ± 93 and 140 ± 106 μg/mg, respectively) versus 22 ± 10 μg/mg ACR in control Ins2Akita mice, again consistent with the presence of dominant mutations that cause diabetic albuminuria. Albuminuria was confirmed by measurement of 24-h urinary albumin excretion rates (AER) in progeny from Nphrp1 and Nphrp2 founders. AER was significantly greater in Nphrp1/Ins2Akita (227 ± 139 μg/24 h; n = 10) and Nphrp2/Ins2Akita mice (116 ± 12 μg/24 h; n = 4) versus control Ins2Akita mice (36 ± 14 μg/24 h; n = 8; Figure 3B) at the average age of 9 mo. Progressively increasing albuminuria was observed in both mutant lines during a period of 19 mo (Figure 4). By 3 mo of age, mutant mice from both lines exhibited elevated ACR (93 ± 19 μg/mg for Nphrp1, n = 26) and 81 ± 14 μg/mg for Nphrp2 (n = 30). ACR continued to

Figure 2. Identification of albumin/creatinine ratio (ACR) outliers in diabetic G1 progeny. C57BL/6 (Akita−) and control Ins2Akita mice (Akita+; top) and G1 Ins2+/− mice (ENU+Akita−; bottom left) follow a normal distribution in Log10 ACR values. This is in contrast to the bimodal distribution that was observed in G1 Ins2Akita heterozygotes that inherited ENU mutation (ENU+Akita+; bottom right). Among 10 identified outliers during primary screening (circled bars), six phenotypic variants showed persistent ACR (see Figure 3) during 1 yr.

Figure 3. (A) ACR in ENU-induced albuminuric variants. Each column represents the mean ± SEM for at least six measurements during 15 mo for each phenotypic variant. *P < 0.005 versus control mice. (B) Twenty-four-hour albumin excretion rates in G2 and G3 progeny from two fertile ENU-induced albuminuric founders. □, control Ins2Akita mice (n = 8); ■, Nphrp1/Ins2Akita mice (ENU20 diabetic progeny; n = 10); □, Nphrp2/Ins2Akita mice (ENU76 diabetic progeny; n = 4). **P < 0.05 for ENU founders' Ins2Akita progeny versus control Ins2Akita mice. Data are means ± SEM.
increase with age, with ACR of 187 ± 47 and 376 ± 115 µg/mg in Ins2Akita (n = 8; P < 0.05) and Nphrp2 (n = 7; P < 0.05) mice, respectively. These values were four- and eight-fold higher than control Ins2Akita mice (45 ± 4 µg/mg; n = 6) at 19 mo of age.

Renal Histopathology in Diabetic Nphrp1/Ins2Akita and Nphrp2/Ins2Akita Mutants

Renal histopathology was examined in albuminuric Ins2Akita G3 progeny of both Nphrp1 and Nphrp2 mutant lines after 9 to 12 mo of sustained hyperglycemia (Figure 5, A through C). Ins2Akita G3 progeny from Nphrp1 and Nphrp2 founders exhibited significantly greater mesangial expansion with scores of 1.49 ± 0.38 (n = 4) and 1.47 ± 0.28 (n = 3), respectively, with increased mesangial periodic acid–Schiff–positive staining versus only mild mesangial expansion in glomeruli from nonmutagenized Ins2Akita C57BL/6 mice (score 0.71 ± 0.16; n = 4; Figure 5G). Electron microscopy (Figure 5, D through F) confirmed increased mesangial matrix and also demonstrated greater GBM thickening of 406 ± 32 (P < 0.05) and 358 ± 19 nm (P < 0.05) in Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mutants versus 244 ± 25 nm in control diabetic Ins2Akita mice (Figure 5H) despite that HbA1c values were not different among these three groups. In addition, foot process effacement was greater (5 to 30%) in both mutant lines. These morphologic changes are consistent with DN in the ENU-induced mutants.

Renal Function in Ins2Akita G2 and G3 Progeny from Nphrp1 and Nphrp2 Founders

Renal function was assessed in diabetic Nphrp1/Ins2Akita (n = 11) and Nphrp2/Ins2Akita (n = 6) mice and compared withagematched control Ins2Akita mice (n = 9). Both the Nphrp1 and Nphrp2 lines exhibited renal enlargement with increased absolute kidney weight and kidney:body weight ratios of 10.92 ± 0.72 and 11.73 ± 0.84 versus 7.83 ± 1.67 mg/g, than in control Ins2Akita mice (P < 0.005 for both mutant lines; Table 1). Importantly, Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mice also exhibited significantly increased BUN and plasma creatinine at 30 to 40 wk of age compared with diabetic control Ins2Akita mice of the same age (Table 1). Furthermore, FITC-inulin clearance was reduced in progeny from both Nphrp1/Ins2Akita (330 ± 90 µl/min per mouse; P < 0.05) and Nphrp2/Ins2Akita mutants (320 ± 50 µl/min per mouse; P = 0.005) compared with control Ins2Akita mice (520 ± 120 µl/min per mouse; Figure 6), confirming decreased renal function in these mice.

Although blood glucose and HbA1c levels were not different between mutants and control Ins2Akita mice, plasma lipids (triglycerides and cholesterol) were increased significantly in both mutant lines compared with diabetic control Ins2Akita mice (Table 1). No significant differences in the white blood cell (neutrophils, lymphocytes, monocytes, eosinophils, and basophils) or platelet count, mean corpuscular volume, and mean corpuscular hemoglobin were observed in the complete blood count in

Figure 4. Progression of albuminuria in G2 and G3 progeny from Nphrp1 and Nphrp2 founders during 19 mo of observation. Each point presents the mean ± SEM ACR (n = 6 or more for each time point) in progeny from ENU founders. *P < 0.005 versus control Ins2Akita mice.

Figure 5. (A through C) Light photomicrographs (top) of glomeruli from control Ins2Akita (A), Nphrp1/Ins2Akita (B), and Nphrp2/Ins2Akita (C) G3 mice. The glomerular injury (G) for all glomeruli on a single section of kidney was scored in three mice per group: control Ins2Akita ( ), Nphrp1/Ins2Akita ( ), and Nphrp2/Ins2Akita ( ) mutants on a scale of 0 to 4. (D through F) Representative electron micrographs (middle) of glomeruli from control Ins2Akita (D), Nphrp1/Ins2Akita (E), and Nphrp2/Ins2Akita (F) mice. Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mice exhibit prominent expansion of mesangial matrix and thickening of glomerular basement membrane (GBM, arrow). (H) GBM thickening was significantly higher in Ins2Akita mice from Nphrp1 ( ) and Nphrp2 lines ( ) than in control Ins2Akita mice ( ). At least four measurements per glomerulus. Data are means ± SEM. *P < 0.05, **P < 0.005. Magnifications: ×400 in A through C (periodic acid–Schiff); ×8000 in D through F.
GFR was measured by FITC-inulin clearance in conscious mice. Nphrp1/Ins2Akita BP was slightly elevated but not significantly different in betic control -ethyl-N-nitrosourea; HbA1c, glycosylated hemoglobin. Most recent studies have identifiers that predispose to DN was supported by studies of dia-

between the incidence of nephropathy in diabetic siblings of 

Figure 6. GFR in progeny from Nphrp1 and Nphrp2 founders. GFR was measured by FITC-inulin clearance in conscious mice. ☐, control Ins2Akita mice (n = 8); □, Nphrp1/Ins2Akita mutants (n = 10); △, Nphrp2/Ins2Akita mutants (n = 11). *P < 0.05, **P = 0.005 for Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mice versus control Ins2Akita mice. Data are mean ± SEM.

Table 1. Anatomic and metabolic profiles of ENU-induced mutant mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Ins2Akita (n = 9)</th>
<th>Nphrp1/Ins2Akita (n = 11)</th>
<th>Nphrp2/Ins2Akita (n = 6)</th>
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<tr>
<td>Body weight (g)</td>
<td>26.40 ± 2.28</td>
<td>23.56 ± 1.36</td>
<td>23.38 ± 2.30</td>
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<td>Kidney weight (left; mg)</td>
<td>197.86 ± 30.65</td>
<td>247.86 ± 19.30</td>
<td>249.33 ± 20.89</td>
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<tr>
<td>Kidney weight:body weight (mg/g)</td>
<td>7.83 ± 1.67</td>
<td>10.92 ± 0.72</td>
<td>11.73 ± 0.84</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>30.14 ± 2.16</td>
<td>40.30 ± 4.68</td>
<td>39.10 ± 1.80</td>
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<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.089 ± 0.003</td>
<td>0.110 ± 0.029</td>
<td>0.134 ± 0.030</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>688 ± 17</td>
<td>697 ± 44</td>
<td>684 ± 22</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>46.11 ± 2.25</td>
<td>47.7 ± 3.89</td>
<td>48.67 ± 2.22</td>
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<tr>
<td>Hemoglobin (g/dl)</td>
<td>15.6 ± 0.8</td>
<td>16.0 ± 1.3</td>
<td>16.4 ± 0.7</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>12.4 ± 0.2</td>
<td>12.13 ± 0.17</td>
<td>12.0 ± 1.28</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
<td>63.33 ± 13.20</td>
<td>102.71 ± 0.04</td>
<td>143.25 ± 36.03</td>
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<td>Cholesterol (mg/dl)</td>
<td>70.10 ± 1.03</td>
<td>107.00 ± 12.22</td>
<td>144.25 ± 25.97</td>
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<td>Systolic BP (mmHg)</td>
<td>116 ± 9</td>
<td>119 ± 12</td>
<td>128 ± 2</td>
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</table>

aData are means ± SEM. Plasma creatinine was measured by HPLC. BUN, blood urea nitrogen; ENU, N-ethyl-N-nitrosourea; HbA1c, glycosylated hemoglobin. 
bP < 0.05, cP < 0.005 versus control group.

Discussion

Strong familial factors that predispose to DN were reported previously (26,27). For example, there is a striking difference between the incidence of nephropathy in diabetic siblings of patients with DN versus diabetic siblings of patients without DN. When an index patient with type 1 diabetes had persistent proteinuria, the risk for proteinuria in the patient’s sibling with diabetes was 71 versus only 25% risk when the index patient was not proteinuric (6,27). Likewise, the role of dominant modifiers that predispose to DN was supported by studies of diabetic Pima Native Americans (28). Most recent studies have

focused on associations between certain candidate genes and DN, including the carnosinase gene (29), RANTES receptor gene in immunocompetent cell (30), and engulfment and cell motility 1 gene (31). In more diverse, less homogeneous populations, both recessive and dominant modifiers are likely to play important roles (6). Elucidation of the pathogenesis of DN will be critical to the development of therapeutic interventions that aim to normalize renal function in these patients.

Murine models of DN theoretically offer significant advantages over human studies in the experimental identification of modifier genes. Recent studies indicate that, as in humans, most mice do not develop nephropathy. The genetic background of a mouse (e.g., different inbred mouse strains) determines the predisposition to the development of DN, with some strains being more prone to albuminuria than others (10,11). C57BL/6 mice show relative resistance to nephropathy either from low-dosage streptozotocin (STZ)-induced diabetes or in diabetes that is induced by the Ins2Akita mutation (10). In contrast, in the same studies, STZ-induced diabetic DBA/2 mice exhibited six-fold greater ACR than C57BL/6 mice, suggesting the presence of specific genes that confer relative protection in C57BL/6 mice. Differential susceptibility to DN in inbred mouse strains provides a possible approach for the genetic dissection of this diabetic complication.

To provide insight into the identity of genes that are important for the resistance of C57BL/6 to DN, we undertook a phenotype-driven screen of C57BL/6 diabetic Ins2Akita mice that also inherited a high load of paternally derived ENU mutations. The diabetic Ins2Akita mouse is a model of type 1 diabetes, carrying a heterozygous mutation in insulin-2, resulting in insulin misfolding and autosomal dominant diabetes (19). Homozygous C57BL6 Ins2Akita mice fail to thrive, and they die within 1 to 2 mo, but heterozygous mice are viable and fertile and exhibit hyperglycemia, hypoinsulinemia, polydipsia, and polyuria by 3 to 4 wk of age (19,32), making them an attractive tool for physiologically sensitizing the background so
that new diabetes-dependent renal dysfunction mutations might be identified.

When ENU mutagenized male mice were intercrossed with Ins2Akita female mice, the first-generation offspring (G1) exhibited multiple abnormal phenotypes, including neonatal and juvenile lethality, microphthalmia, and craniofacial and skeletal dysmorphology. Survival of Ins2Akita carriers also was diminished significantly in these G1 progeny. Adverse interaction between the Ins2Akita mutation and ENU-induced mutations could contribute to lethality at embryonic or neonatal stages. However, we did not detect either major anatomic abnormalities or decreased survival in G2 and G3 mice that were generated from G1 founders.

We used albuminuria as the primary screen for nephropathy mutants. In humans, microalbuminuria (AER of 30 to 300 mg/24 h) has been used widely as a marker to identify patients who are at risk for DN (33). In human DN, the onset of microvascular complications or decreased survival in G2 and G3 mice that were generated from G1 founders.

In our study, nonmutagenized C57BL/6 Ins2Akita mice exhibited an average AER of 36 ± 14 µg/24 h, in agreement with a previous report (38). Among G1 progeny that were generated from mating between ENU-mutagenized male and female C57BL/6 Ins2Akita heterozygotes, we identified six Ins2Akita outliers that exhibited elevated ACR. True G1 genetic variants were confirmed by test-crosses of all six outliers with wild-type partners. Of these six diabetic founders, two confirmed genetic variants (ENU20, or Ins2Akita-Nphrp2/H11022) bred true and produced following generations of mice with renal phenotype. Importantly, albuminuria in the two generated lines (ENU20, or Nphrp1, and ENU76, or Nphrp2) was transmitted only to half of diabetic progeny but not to nondiabetic progeny. This is consistent with dominantly heritable mutations that segregate independent of the Ins2Akita mutation and that result in renal disease only in the setting of diabetes. In contrast, increased albuminuria in 50% of all G3 progeny from ENU161 founder was evident in the absence of Ins2Akita mutation. The cause of albuminuria in ENU161 progeny therefore is unlikely to be related to DN, as opposed to the results that were obtained for Nphrp1 and Nphrp2 lines.

The nephropathy that characterizes these two ENU-induced mutant lines was of comparable severity to the reported low-dosage STZ-treated DBA/2, db/db C57BLKS/6, and FVB OVE26 inbred strains that seem to be prone to DN and significantly more severe than in the relatively resistant STZ-treated wild-type C57BL/6 mice (Table 2). Daily AER in Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mice was similar to diabetic DBA/2 and db/db C57BLKS mice at the age of approximately 9 mo (39,40) (Table 2). The AER in FVB/OVE26 mice (41) reportedly exceeded 15,000 µg/24 h, but they also exhibited hydronephrosis, an element that was not observed in our ENU-induced mutants or in STZ-treated FVB mice (10).

Reduction in GFR is a critically important feature of DN that is missing from most models. In these studies, GFR was determined using FITC-inulin clearance (22,23) and was significantly lower in Nphrp1 and Nphrp2 mutant lines than Ins2Akita, a feature that is lacking in db/db C57BLKS (42) and STZ-treated DBA/2 and C57BL/6 mice (10) (Table 2). Renal function decline was confirmed by measurement of BUN and HPLC plasma creatinine in both ENU-induced mutant lines. In this study, the finding of renal functional impairment in diabetic mutant mice is novel and consistent with the identification of two new, robust, and heritable mouse models of DN.

In humans, renal histopathologic alterations that are associated with the development of overt diabetic proteinuria include GBM thickening and mesangial expansion. As albuminuria and renal insufficiency progress, glomerulosclerosis, arteriolar hyalinosis, and tubulointerstitial fibrosis develop (43,44). In mice, pathologic criteria that are predictive of renal insufficiency have not been established, largely because renal failure was not reported previously in mouse models of DN (37). In these studies, in addition to reduced GFR, we observed glomerular lesions consistent with DN in these two ENU-induced DN lines. Light microscopy and ultrastructural evaluation revealed diffuse mesangial matrix expansion, foot process effacement, and increased GBM thickening, consistent with the development of DN. Although we did not observe nodular glomerulosclerosis or arteriolar hyalinosis in ENU-induced mutants, mesangial matrix expansion in Nphrp1/Ins2Akita and Nphrp2/Ins2Akita mice was comparable to that in all analyzed DN mouse models (Table 2). The degree of basement membrane thickening also was greater in Nphrp1 and Nphrp2 than in STZ-treated C57BL/6 (10) and db/db C57BLKS/6 (45) mice and similar to STZ-treated DBA/2 (10) and FVB/OVE26 (46) (Table 2). It is notable that although fasting blood glucose levels and HbA1c values were indistinguishable in the Ins2Akita mutants from Nphrp1 and Nphrp2 lines versus control Ins2Akita mice, GBM thickening was significantly greater in the mutants, suggesting that these novel mutations per se, rather than hyperglycemia alone, contribute to increased GBM deposition.

In addition to albuminuria and renal function decline, hypertension and hyperlipidemia have been implicated in progression of DN. Hypertension may occur early in the course of DN, and BP rises further as GFR falls. The rate of decline in GFR does not seem to be related directly to BP, although antihypertensive treatment seems to retard the rate of decline (47). Nphrp2 offspring showed a trend for increased systolic BP; this did not achieve statistical significance as compared with control Ins2Akita mice. Although mutant mice from both lines were phenotypically similar (with albuminuria, glomerular sclerosis, and renal function decline), it is unlikely that both mutant lines carry identical mutations. These lines derived from different ENU-induced G1 founders. Furthermore, plasma creatinine (P = 0.03), triglycerides (P = 0.048), and cholesterol (P = 0.004) were significantly higher in Nphrp2/Ins2Akita mice than in Nphrp1/Ins2Akita mutants. Dyslipidemia is a common feature of humans with DN (48,49); however, whether this is a consequence of renal injury remains uncertain. Similarly, whether Nphrp1 and Nphrp2 mutations result in primary dyslipidemia or this is a consequence of DN per se remains to be determined.
Table 2. Renal characteristics in mouse models of diabetic nephropathy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Nphrp1/Ins2Akita</th>
<th>Nphrp2/Ins2Akita</th>
<th>Db/db</th>
<th>Db/db</th>
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</thead>
<tbody>
<tr>
<td>GBM (nm)</td>
<td>406 ± 9</td>
<td>358 ± 10</td>
<td>183 ± 5</td>
<td>333 ± 6</td>
<td>333 ± 6</td>
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<tr>
<td>Score</td>
<td>0.71 ± 0.16</td>
<td>1.49 ± 0.58</td>
<td>0.83 ± 0.12</td>
<td>0.35 ± 0.01</td>
<td>0.01</td>
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<tr>
<td>Albuminuria</td>
<td>25</td>
<td>12</td>
<td>25</td>
<td>66</td>
<td></td>
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</tbody>
</table>

References


Conclusion

These studies support the existence of dominant genetic modifiers’ contributing to the development of DN. This pheno-type-driven screen identified two independent heritable dominant mutations that result in renal disease only in diabetic mice. These two new mutant lines (*Nphrp1* and *Nphrp2*) exhibit several critical features of DN, including late onset of increased plasma creatinine, decreased insulin clearance, progressive albuminuria, and histopathologic changes that are characteristic of DN. Insight into phenotypic similarities and disparities and the genetic mechanisms of renal decline in *Nphrp1* and *Nphrp2* mutants should be provided by mapping and identification of the genes that are targeted by these two ENU-induced mutations. It is hoped that this information will accelerate both the understanding of the pathogenesis of DN and progress toward a treatment for this major devastating complication of diabetes.

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Disclosures

None.

References