Diabetic kidney lesions of GIPR<sup>dn</sup> transgenic mice: podocyte hypertrophy and thickening of the GBM precede glomerular hypertrophy and glomerulosclerosis

Nadja Herbach, Irene Schairer, Andreas Blutke, Sabine Kautz, Angela Siebert, Burkhard Göke, Eckhard Wolf, and Ruediger Wanke

Institute of Veterinary Pathology, LMU Munich; Department of Internal Medicine II, Klinikum Grosshadern, LMU Munich; and Chair for Molecular Animal Breeding and Biotechnology/Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, LMU Munich, Germany

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Herbach N, Schairer I, Blutke A, Kautz S, Siebert A, Göke B, Wolf E, Wanke R. Diabetic kidney lesions of GIPR<sup>dn</sup> transgenic mice: podocyte hypertrophy and thickening of the GBM precede glomerular hypertrophy and glomerulosclerosis. Am J Physiol Renal Physiol 296: F819–F829, 2009. First published 11 February 2009; doi:10.1152/ajprenal.90665.2008.—Diabetic nephropathy is the leading cause of end-stage renal disease and the largest contributor to the total cost of diabetes care. Rodent models are excellent tools to gain more insight into the pathogenesis of diabetic nephropathy. In the present study, we characterize the age-related sequence of diabetes-associated kidney lesions in GIPR<sup>dn</sup> transgenic mice, a novel mouse model of early-onset diabetes mellitus. Clinical-chemical analyses as well as qualitative and quantitative morphological analyses of the kidneys of GIPR<sup>dn</sup> transgenic animals and nontransgenic littermate controls were performed at 3, 8, 20, and 28 wk of age. Early renal changes of transgenic mice consisted of podocyte hypertrophy, reduced numerical volume density of podocytes in glomeruli, and homogenous thickening of the glomerular basement membrane, followed by renal and glomerular hypertrophy as well as mesangial expansion and matrix accumulation. At 28 wk of age, glomerular damage was most prominent, including advanced glomerulosclerosis, tubulointerstitial lesions, and proteinuria. Real-time PCR demonstrated increased glomerular expression of Col4a1, Fn1, and Tgfβ1. Immunohistochemistry revealed increased mesangial deposition of collagen type IV, fibronectin, and laminin. The present study shows that GIPR<sup>dn</sup> transgenic mice exhibit renal changes that closely resemble diabetes-associated kidney alterations in humans. Data particularly from male transgenic mice indicate that podocyte hypertrophy is directly linked to hyperglycemia, without the influence of mechanical stress. GIPR<sup>dn</sup> transgenic mice are considered an excellent new tool to study the mechanisms involved in onset and progression of diabetic nephropathy.

Diabetic glomerulosclerosis; animal model

DIABETIC NEPHROPATHY is the leading cause of end-stage renal disease and the largest contributor to the total cost of diabetes care (7). Rodent models of diabetic nephropathy are excellent tools to gain more insight into the pathogenesis of the disease and to test the efficacy of new therapies (1). There are several mouse models of diabetes used for studying diabetes-associated kidney lesions (reviewed in Ref. 3); however, the existing models are only of limited use for the study of diabetic nephropathy (3, 4) and therefore, new diabetic mouse strains are needed (4).

Glucose-dependent insulinotropic polypeptide (GIP) is an incretin hormone, released from endocrine cells of the small intestine after food intake. GIP produces multiple physiological effects, including enhancement of glucose-mediated insulin secretion, insulin gene transcription, and may act as a mitotic and anti-apoptotic factor in pancreatic beta-cells (8, 11, 14, 37, 46).

Recently, we described a novel transgenic diabetic mouse model, exhibiting a dominant-negative GIP receptor (GIPR<sup>dn</sup>) in pancreatic beta-cells (22). The cDNA of the human GIPR was mutated at the third intracellular loop (deletion of amino acids 319-326, Ala→Glu exchange at position 340). The loss of function of the mutated GIPR was demonstrated in vitro. Transgenic mice were then generated, expressing the mutated human GIPR cDNA under the control of the rat preproinsulin 2 gene promoter in pancreatic beta-cells. These GIPR<sup>dn</sup> transgenic mice exhibit an early disturbance in pancreatic islet development (severe reduction of beta-cell mass, disturbed composition of islets, and decreased islet neogenesis), diminished insulin secretion, and early-onset diabetes mellitus, without obesity or insulin resistance (22). In type 2 diabetic patients, the major abnormality of the enteroinsular axis was suggested to be the reduced insulinotropic action of GIP (34), and the volume density of beta-cells in the pancreas (5, 39) and beta-cell mass (39) were found to be reduced in diabetic humans. Therefore, GIPR<sup>dn</sup> transgenic mice resemble important aspects of human type 2 diabetes mellitus.

The aim of the present study was to characterize the age-related sequence of diabetes-associated kidney changes in this novel diabetic mouse model.

RESEARCH DESIGN AND METHODS

Animals. All animal experiments were performed in accordance with institutionally approved and current animal care guidelines. Transgenic mice were generated as previously described (22). Animals investigated in this study were 3-, 8-, 20-, and 28-wk-old hemizygous GIPR<sup>dn</sup> transgenic mice and nontransgenic littermate controls (CD1 background; n = 4 per age, sex, and genetic group if not stated differently). Animals were maintained on a 12:12-h light-dark cycle and received tap water and maintenance diet (1320, Altromin, Lage, Germany) ad libitum. A separate group of mice was fed a calorie-restricted diet (C1009, Altromin) from weaning and kidney lesions were investigated at 43 wk of age.

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Clinical-chemical analyses. Blood was collected from animals that were fasted for 12 h and fed 2 h before sampling. Blood glucose and serum parameters were determined as described previously (25). Serum creatinine levels were determined, using an enzymatic method (Roche Diagnostics GmbH, Mannheim, Germany). Serum insulin levels were analyzed by RIA (CIS Bio International, Gif-Sur-Yvette Cedex, France). Urine glucose was determined semiquantitatively, as described (23).

Spot urine samples were taken between 2 and 3 PM and immediately stored at −80°C until assayed. In addition, daily water intake and 24-h urine excretion were determined at 17 wk of age, using metabolic cages. Urine creatinine concentration was measured, using an automated analyzer technique (Hitachi, Merck, Darmstadt, Germany). Urine albumin concentration was measured, using a mouse albumin ELISA (Bethyl E90–134, Montgomery, TX). For SDS-PAGE, urine samples were diluted to a creatinine content of 1.5 mg/dl, and proteins were denatured (Thermoblock TB1, Biometra, Germany) and separated using a 12% SDS-PAGE gel (Protein III, Bio-Rad, Munich, Germany) together with a broad molecular weight standard (Bio-Rad) and a mouse albumin standard (Biotrend, Cologne, Germany). Silver staining was performed according to a standard protocol.

Blood pressure. Blood pressure was noninvasively measured at 8 and 28 wk of age (n = 4–8 per group) by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT).

Body and kidney weight, kidney processing. The 12-h fasting body weight was determined to the nearest 0.1 g. Animals were killed by ether inhalation and tissue was fixed for histology via orthograde vascular perfusion with 3% glutaraldehyde. After postfixation for 24 h, kidneys were removed carefully, separated from adjacent tissues, weighed to the nearest milligram, cut perpendicular to the longitudinal axis, processed for plastic embedded light microscopy (26), and transmission electron microscopy (TEM) as described previously (27). For light microscopy, kidney samples were embedded in plastic, containing hydroxyethylmethacrylate and methylmethacrylate (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) as described (26). Approximately 1.5-μm-thick plastic sections were cut on a Reichert-Jung 2050 rotary microtome (Leica, Wetzlar, Germany) and stained with hematoxylin and eosin, periodic acid Schiff (PAS), and hematoxylin or periodic acid silver methenamine (PASM), PAS and hematoxylin. For TEM, three 1-mm³ samples of the renal cortex per animal were selected by systematic random sampling (35), postfixed in 1% osmium tetroxide, and routinely embedded in Epon. Semithin sections (0.5 μm) were stained with toluidine blue O and safranin. Ultrathin sections (70–80 nm) were stained with uranyl citrate and examined with a TEM (EM10, Zeiss, Eching, Germany). Silver staining was performed according to a standard protocol.

Morphological analyses. The presence of mesangial expansion and the severity of glomerulosclerosis were scored semiquantiitatively on an arbitrary scale as described previously (10, 13). A total of 100 glomerular profiles from two randomly selected sections of each kidney were investigated and were selected by systematic sampling, according to the unbiased counting rule (18). The mean glomerular volume \( V_{\text{Glom}} \) was determined from the mean glomerular profile area \( A_{\text{Glom}} \) as described (12, 47). An average of 115 glomerular profiles per animal (range: 104–133) was systematically sampled, according to the unbiased counting rule (18).

\[
V_{\text{Glom}} = \frac{\beta}{k} \bar{A}_{\text{Glom}}^{1.5\times f_s}
\]

where the shape coefficient \( \beta = 1.38 \) pertains to spheres, \( k = 1.04 \) is a size distribution coefficient assuming a 15% coefficient of variation (48), and \( f_s \) the individual linear tissue shrinkage correction factor for GMA/MMA-embedded kidney tissue (0.89 ± 0.02).

The glomerular basement membrane (GBM) thickness was determined by the orthogonal intercept method as described (12, 38). A total of six glomeruli were sampled from the center of six semithin sections for electron microscopy.

Capillary loops were photographed in a predetermined manner (by half turns of the stage handle), photographs were developed to a final print magnification of \( \times 46,100 \), covered by a transparent 2.5-cm² grid, and the shortest distance between the endothelial cell membrane and the outer lining of the lamina rara externa underneath the cell membrane of the epithelial foot processes was measured where gridlines transected the GBM. Measurements were undertaken with a logarithmic ruler, which was generated as described (38). The true harmonic mean thickness (\( Th \)) was estimated by

\[
Th = \frac{8}{3 \pi} \times \frac{10^6}{M} \times h
\]

(\( h \), apparent harmonic mean thickness; \( M \), final print magnification).

On average, 462 (390–602) intersections per animal were measured.

The filtration slit frequency (FSF) was determined as described (12) by counting the number of epithelial filtration slits divided by the length of the peripheral capillary wall at the epithelial interface. On average, 520 filtration slits (range: 321–645) were counted per animal.

The physical disector principle was applied for counting podocytes as described, using semithin sections (27, 43). Four dissectors from 12 glomeruli per animal were analyzed. Photographs of glomerular profiles were taken, using a Leica DFC 220 camera (Leica), connected to a microscope (Orthoplan, Leitz, Germany), and printed with a color laser printer (final print magnification \( \times 1,000 \)). The area of the glomerular cross-sections \( \Sigma A_{\text{Glom}} \) was measured, using a Video-plan image analysis system (Zeiss). Podocyte nuclei with transects in the reference section and no corresponding transects in the look-up section were counted \( Q(P) \). The numerical density of podocytes in glomeruli was calculated as

\[
N_{V(P/Glom)} = \frac{\Sigma Q(P)}{h \times \Sigma A_{\text{Glom}}} \times f_s^3
\]

where \( h \) was the disector height (1.5 μm) and \( f_s \) the linear tissue shrinkage correction factor for Epon-embedded murine kidney tissue which was found to correspond to 0.95 ± 0.02.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cob4a1</td>
<td>forward: CGGGGAGAAAAAGGGCCAGAT reverse: TCCTTAACTTGTGCTGTCACA</td>
<td>NM_009931</td>
</tr>
<tr>
<td>F1</td>
<td>forward: TGGGCTCGGGAATGGGAGG reverse: ATGGTGAAGGTTGACAATA</td>
<td>NM_010233</td>
</tr>
<tr>
<td>Lamb2</td>
<td>forward: GGAAACAATTTGCTGCTTTTCT forward: TACCTTGTCCTCTGCTGCTT</td>
<td>NM_008483</td>
</tr>
<tr>
<td>Tgf8</td>
<td>forward: CCCTATTATTTTGGAGCCTGGA reverse: TGTTGTTGATGAGGGGCAAG</td>
<td>NM_011577</td>
</tr>
<tr>
<td>Vegfa</td>
<td>forward: AGGAGGGAGGAGCGTGAGTTA reverse: CAGACTGGCTGCTACGCC</td>
<td>NM_001025250</td>
</tr>
<tr>
<td>Rpl13a</td>
<td>forward: AGGAGGGAGGAGCGTGAGTTA reverse: CAGACTGGCTGCTACGCC</td>
<td>NM_009438</td>
</tr>
<tr>
<td>Mmpr9</td>
<td>forward: CCATCAACAGACAGAGAGAGAGCT reverse: CCTCCCTCCCCGGAGAAGT</td>
<td>NM_023514</td>
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</tbody>
</table>

cDNA-specific primers for amplification of mouse collagen type IV alpha 1 (Cob4a1), fibronectin 1 (F1), laminin beta 2 (Lamb2), transforming growth factor-beta1 (Tgf8), vascular endothelial growth factor-A (Vegfa), and the housekeeping genes murine ribosomal protein 13A (Rpl13a) and mitochon-drial ribosomal protein S9 (Mmpr9).
The number of podocytes per glomerulus \( N_{(P,Glo)} \) was calculated multiplying \( N_{V(P/Glo)} \) and \( V_{(P/Glo)} \). The volume fraction of podocytes per glomerulus \( V_{v(P/Glo)} \) was determined by point counting method. The mean podocyte volume \( v(P) \) was calculated dividing \( V_{v(P/Glo)} \) by \( N_{V(P/Glo)} \).

**Immunohistochemistry.** Animals were killed by ether inhalation and tissue was fixed for immunohistochemistry via orthograde vascular perfusion with 4% paraformaldehyde in PBS (pH 7.4) and kidney samples were embedded in paraffin. The indirect immunoperoxidase technique served to localize fibronectin (rabbit-anti-human fibronectin, Biocarta, San Diego, CA), laminin (rabbit-anti-mouse laminin, DCS, Hamburg, Germany), type IV collagen (rabbit-anti-human placental type IV collagen, Quartett, Berlin, Germany), and TGF-β1 (rabbit-anti-human TGF-β1, Santa Cruz Biotechnology, Heidelberg, Germany). The streptavidin-biotin method was applied to localize VEGF (rabbit-anti-human VEGF, Invitrogen, Karlsruhe, Germany). Incubations took place at 37°C for 2 h in a humidity chamber. Horseradish peroxidase-conjugated pig anti-rabbit IgG and biotinylated goat anti-rabbit IgG were used as secondary antibodies, and DAB was used as chromogen. Specificity controls included substitution of primary antisera with nonimmune serum and omission of the secondary antiserum.

**Glomerulus isolation and real-time PCR.** Isolation of glomeruli from each 7 male 28-wk-old GIPRdn transgenic and control mice was performed essentially as described (45). Total RNA was isolated from the glomeruli isolates, using the RNeasy-Mini kit (Qiagen), including an on-column DNase digestion. Each 500 ng of total glomerular RNA were reverse transcribed (High Capacity RNA-to-cDNA Kit, Applied Biosystems) and analyzed by real-time PCR on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems). cDNA-specific primers for amplification of mouse transforming growth factor beta 1 (Tgfb1), collagen, type IV, alpha 1 (Col4a1), fibronectin 1 (Fn1), laminin, beta 2 (Lamb2), vascular endothelial...
growth factor-A (Vegf-a), the housekeeping genes murine ribosomal protein 13A (Rpl13a), and mitochondrial ribosomal protein S9 (Mrps9) were chosen with the assistance of the computer program Primer Express version 3.0 (Applied Biosystems) and queried by NCBI Blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primer sequences are listed in Table 1. The Col4a1 (36), Tgbf1 (50), Vegfa (19), and the Rpl13a (50) primers, as well as the Fnl forward primer (15), have been published previously. The comparability of amplification efficiencies of the single primers was confirmed by performance of real-time PCR-based standard curve analyses. All real-time PCR measurements were performed in duplicates and included no template controls. Glomerular transcript abundances of Tgbf1, Vegfa, Col4a1, Fnl, and Lamb2 were calculated in relation to the expression of Mrps9 and Rpl13a, using the 2^−\Delta\DeltaCT method (6) and showed comparable results for both housekeeping genes.

Data presentation and statistical analysis. Data were analyzed using the Mann-Whitney U-test. P values <0.05 were considered significant. Data are presented as means and SE or SD as indicated.

RESULTS

Blood, serum, and urine parameters, water intake. The postprandial blood glucose levels of GIPRdn transgenic mice were significantly increased vs. age- and sex-matched littermate controls, irrespective of the age at sampling, and the diabetic phenotype deteriorated with age (Fig. 1A). Accordingly, the daily water intake was largely increased in transgenic mice vs. controls at 17 wk of age (male: 31 ± 5 vs. 4 ± 2 ml/day, P < 0.05; female: 31 ± 8 vs. 5 ± 1 ml/day, P < 0.05). In 8-wk-old and older transgenic mice, serum urea and creatinine levels were increased, whereas total protein and albumin levels tended to be decreased vs. controls (Table 2). The ratios of urine sodium, chloride, calcium, potassium, and total protein, respectively, to urine creatinine were significantly increased vs. age- and sex-matched littermate controls, irrespective of the age at sampling, and the diabetic phenotype deteriorated with age (Fig. 1A). Accordingly, the daily water intake was largely increased in transgenic mice vs. controls at 17 wk of age (male: 31 ± 5 vs. 4 ± 2 ml/day, P < 0.05; female: 31 ± 8 vs. 5 ± 1 ml/day, P < 0.05). In 8-wk-old and older transgenic mice, serum urea and creatinine levels were increased, whereas total protein and albumin levels tended to be decreased vs. controls (Table 2). The ratios of urine sodium, chloride, calcium, potassium, and total protein, respectively, to urine creatinine were significantly increased vs. age- and sex-matched control (Table 3).

Blood pressure. There was no significant difference in systolic, diastolic, or mean blood pressure of 8- or 28-wk-old transgenic and control mice (Fig. 1, B-D).

Urinary albumin excretion. Urine creatinine concentrations were largely reduced in GIPRdn transgenic mice vs. controls (Table 2). At 8 wk of age, 1 male and 1 female GIPRdn transgenic mouse showed albuminuria; at 20 wk, 2 male and 2 female transgenic animals showed albuminuria (data not shown); and at 28 wk, 3 of 4 males and all female transgenic mice showed albuminuria (Fig. 1, E and F). At 17 wk of age, the urine volume was largely increased (Fig. 1G), and the albumin excretion per day was 5.4- and 4.4-fold increased in male and female GIPRdn transgenic mice, respectively, vs. controls (P < 0.05; Fig. 1H).

Body and kidney weight. From 8 wk of age onwards, body weights of male transgenic mice were significantly lower than those of male control mice. The kidney weights of 8-, 20-, and 28-wk-old female transgenic animals were significantly higher than those of female control mice. The kidney weights of male transgenic animals were significantly higher at 28 wk of age vs. controls (Table 4).

Qualitative and quantitative morphological findings of the kidneys. The renal pelvis of GIPRdn transgenic mice was sometimes dilated; however, bilateral hydronephrosis was only observed in one transgenic animal investigated in this study. From 8 wk of age onwards, distended bladders reaching a diameter of up to 2 cm were frequently observed in transgenic mice.

At 8 wk of age, the mean glomerular volume of female GIPRdn transgenic mice was increased by ~24% vs. controls (P < 0.05), and glomerular hypertrophy further progressed to 30% enlargement in 20- and 77% enlargement in 28-wk-old females. The mean glomerular volume was significantly increased in 20-wk-old male transgenic animals (41% increase; P < 0.05) and further progressed to 74% enlargement in 28-wk-old transgenic males vs. controls (Table 4).

At 8 wk of age, some glomeruli of transgenic animals showed mild focal mesangial expansion and matrix accumulation. At 20 wk of age, glomeruli of transgenic animals showed more pronounced mesangial expansion and matrix accumulation. Glomerular alterations of 28-wk-old transgenic animals were apparent in a diffuse segmental to panglomerular pattern.

Table 2. Serum parameters and urine creatinine from mice fed standard diet

<table>
<thead>
<tr>
<th>Na, mmol/l</th>
<th>Cl, mmol/l</th>
<th>Urea, mg/dl</th>
<th>Crea, mg/dl</th>
<th>TP, g/dl</th>
<th>Alb, g/dl</th>
<th>Urine Crea, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m, wt (4)</td>
<td>151 (12)</td>
<td>111 (9)</td>
<td>51 (4)</td>
<td>0.34 (0.06)</td>
<td>4.3 (0.3)</td>
<td>2.9 (0.1)</td>
</tr>
<tr>
<td>m, tg (4)</td>
<td>159 (14)</td>
<td>119 (11)</td>
<td>52 (8)</td>
<td>0.40 (0.06)</td>
<td>4.1 (0.2)</td>
<td>2.9 (0.1)</td>
</tr>
<tr>
<td>f, wt (4)</td>
<td>147 (41)</td>
<td>109 (28)</td>
<td>51 (4)</td>
<td>0.32 (0.02)</td>
<td>4.2 (0.1)</td>
<td>3.0 (0.1)</td>
</tr>
<tr>
<td>f, tg (4)</td>
<td>144 (28)</td>
<td>107 (18)</td>
<td>54 (8)</td>
<td>0.27 (0.03)</td>
<td>4.0 (0.3)</td>
<td>2.9 (0.3)</td>
</tr>
<tr>
<td>8 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m, wt (4)</td>
<td>179 (17)</td>
<td>128 (12)</td>
<td>94 (12)</td>
<td>0.32 (0.01)</td>
<td>5.7 (0.1)</td>
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<tr>
<td>m, tg (4)</td>
<td>184 (6)</td>
<td>137 (7)</td>
<td>149* (23)</td>
<td>0.46* (0.03)</td>
<td>4.2* (0.4)</td>
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<tr>
<td>f, wt (4)</td>
<td>178 (15)</td>
<td>129 (12)</td>
<td>103 (21)</td>
<td>0.36 (0.10)</td>
<td>5.5 (0.6)</td>
<td>3.5 (0.4)</td>
</tr>
<tr>
<td>f, tg (4)</td>
<td>163 (30)</td>
<td>118 (20)</td>
<td>133* (7)</td>
<td>0.47* (0.10)</td>
<td>4.7 (0.5)</td>
<td>3.0 (0.3)</td>
</tr>
<tr>
<td>20 wk</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>m, wt (4)</td>
<td>158 (2)</td>
<td>117 (5)</td>
<td>89 (14)</td>
<td>0.31 (0.01)</td>
<td>5.5 (1.0)</td>
<td>3.1 (0.5)</td>
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<tr>
<td>m, tg (4)</td>
<td>150* (4)</td>
<td>110* (2)</td>
<td>135* (27)</td>
<td>0.47* (0.03)</td>
<td>4.8 (0.5)</td>
<td>2.7 (0.1)</td>
</tr>
<tr>
<td>f, wt (4)</td>
<td>159 (3)</td>
<td>115 (3)</td>
<td>57 (5)</td>
<td>0.34 (0.02)</td>
<td>5.6 (0.4)</td>
<td>3.5 (0.3)</td>
</tr>
<tr>
<td>f, tg (4)</td>
<td>147* (6)</td>
<td>107* (5)</td>
<td>121* (29)</td>
<td>0.47* (0.7)</td>
<td>4.8* (0.3)</td>
<td>2.9* (0.2)</td>
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<tr>
<td>m, wt (4)</td>
<td>164 (1)</td>
<td>118 (1)</td>
<td>49 (3)</td>
<td>0.32 (0.02)</td>
<td>6.2 (0.2)</td>
<td>3.9 (0.2)</td>
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<tr>
<td>m, tg (4)</td>
<td>156* (5)</td>
<td>115 (4)</td>
<td>91* (16)</td>
<td>0.50* (0.02)</td>
<td>5.5* (0.2)</td>
<td>3.1* (0.1)</td>
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<tr>
<td>f, wt (4)</td>
<td>157 (4)</td>
<td>118 (2)</td>
<td>50 (6)</td>
<td>0.33 (0.02)</td>
<td>6.0 (0.3)</td>
<td>3.9 (0.1)</td>
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<tr>
<td>f, tg (4)</td>
<td>162 (4)</td>
<td>120 (3)</td>
<td>69* (7)</td>
<td>0.48* (0.02)</td>
<td>5.7 (0.5)</td>
<td>3.4* (0.3)</td>
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</table>

Data represent means and (SD). m, male; f, female; tg, transgenic; wt, wild-type; (t), number of animals investigated; co, control; Na, sodium; Cl, chloride; Crea, creatinine; TP, total protein; Alb, albumin. Bold numbers: *P < 0.05 vs. age- and sex-matched control.
including mesangial expansion, hyalinosis and sclerosis, adhesions between the capillary tuft and the capsule of Bowman, as well as distortion of capillaries and occasional cystic dilatation of Bowman’s capsule (Fig. 2). Accumulation of acellular hyaline PAS-positive material was observed in a nodular fashion in the periphery of the glomerular tuft; however, peripheral displacement of nuclei seen in human nodular diabetic glomerulosclerosis was not observed. Focal global sclerosis was observed in some individuals.

From 20 wk of age onwards, the glomerulosclerosis index was significantly increased in GIPR<sup>dn</sup> transgenic mice vs. controls, and at 28 wk of age, the glomerulosclerosis index was fourfold increased in male and female transgenic mice vs. controls (Table 4).

Immunohistochemically, an increased deposition of the extracellular matrix proteins collagen type IV, laminin, and fibronectin was evident in glomeruli of transgenic animals, and staining intensity for TGF-β1 in podocytes was significantly increased vs. controls. Staining pattern and intensity for VEGF were equal in glomeruli of transgenic and control mice (Fig. 3).

At 8 wk of age, the numerical volume density of podocytes was 38% lower in female transgenic mice (P < 0.05) and 16% lower in male transgenic mice (P < 0.05), and the mean podocyte volume was significantly increased in both female (64%) and male (29%) GIPR<sup>dn</sup> transgenic mice vs. their respective controls (Table 5).

The GBM thickness of 8-wk-old transgenic mice was significantly increased, whereas the FSF was unchanged vs. controls (Table 5).

At 28 wk of age, podocytes of transgenic mice were enlarged, showed pseudocysts and vacuolization, microvillous transformation, and focal foot process effacement. The GBM was thickened, both homogenously (male: 203 ± 8 vs. 239 ± 16 nm; female: 187 ± 8 vs. 244 ± 8 nm), and in a nodular manner (Fig. 4), and the FSF was slightly reduced in male, and significantly reduced in female transgenic mice (male: 2,079 ± 208 vs. 1,913 ± 117 per mm; female: 2,017 ± 124 vs. 1,743 ± 158 per mm).

Tubulointerstitial lesions were most prominent in 28-wk-old GIPR<sup>dn</sup> transgenic animals, including protein reabsorption droplets in proximal tubular epithelia and hyaline casts in distal tubules (Fig. 2C) as well as focal tubular atrophy, focal interstitial fibrosis, and mononuclear cell infiltration (Fig. 2D). Furthermore, glycogen-rich granules were observed in proximal tubular epithelia (data not shown).

Quantitative real-time PCR. Relative glomerular expression levels of transcripts coding for collagen type IV alpha 1, fibronectin 1, and TGF-β1 were significantly increased in male GIPR<sup>dn</sup> transgenic mice vs. controls (Fig. 3).

Dietary intervention study. Dietary intervention was performed to show that the renal phenotype of GIPR<sup>dn</sup> transgenic mice is caused by the diabetic milieu (23). Long-term dietary intervention normalized blood glucose levels of most transgenic mice vs. controls, and there were no differences in serum insulin levels at 43 wk of age (Table 6). Four of nine male and one of nine female transgenic mice exhibited fasting glucosuria at 43 wk of age. There were no differences in the serum parameters investigated from transgenic mice fed the calorie-restricted diet at 43 wk of age vs. controls (Table 6). Total protein and serum albumin levels were also unchanged (data not shown). Daily water intake of transgenic mice at 17 wk of age was largely reduced by the calorie-restricted diet compared with transgenic mice fed the normal diet but was still slightly higher vs. controls (male 9 ± 2 vs. 6 ± 1 ml/day, P < 0.05; female: 8 ± 3 vs. 5 ± 1 ml/day, P < 0.05; modified from Ref. 23). At 17 and 43 wk of age, urine creatinine was also lower in transgenic mice fed the calorie-restricted diet, but creatinine levels were fourfold higher than those of 28-wk-old mice fed the normal diet [Tables 2 and 7; 17-wk male: 13 ± 1 vs. 20 ± 6 mg/dl, not significant (n.s.); female: 15 ± 6 vs. 22 ± 8 mg/dl, n.s.].

Long-term dietary intervention resulted in normalization of kidney weights, and the mean glomerular volumes were nearly identical, comparing 43-wk-old transgenic and control mice (Table 7).

---

**Table 3. Clinical-chemical parameters of 24-h urine**

<table>
<thead>
<tr>
<th></th>
<th>Na/Crea</th>
<th>Cl/Crea</th>
<th>Ca/Crea</th>
<th>K/Crea</th>
<th>TP/Crea</th>
<th>Crea, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>m, wt (8)</td>
<td>30 (13)</td>
<td>53 (21)</td>
<td>0.51 (0.35)</td>
<td>87 (16)</td>
<td>5.0 (1.2)</td>
<td>37.0 (7.6)</td>
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<tr>
<td>m, tg (7)</td>
<td>99* (31)</td>
<td>153* (39)</td>
<td>1.66* (1.36)</td>
<td>287* (59)</td>
<td>27.6* (7.6)</td>
<td>27.6* (1.0)</td>
</tr>
<tr>
<td>f, wt (7)</td>
<td>40 (25)</td>
<td>35 (24)</td>
<td>0.30 (0.11)</td>
<td>97 (47)</td>
<td>1.20 (0.8)</td>
<td>42.2 (15.6)</td>
</tr>
<tr>
<td>f, tg (7)</td>
<td>116* (32)</td>
<td>224* (58)</td>
<td>4.03* (2.08)</td>
<td>336* (107)</td>
<td>26.7* (11.1)</td>
<td>1.7* (0.6)</td>
</tr>
</tbody>
</table>

Data represent means and (SD). Ca, calcium; K, potassium. Bold numbers: *P < 0.05 vs. age- and sex-matched control.

---

**Table 4. Body and kidney weights, mean glomerular volume, and glomerulosclerosis index**

<table>
<thead>
<tr>
<th></th>
<th>Body wt, g</th>
<th>Kidney wt, mg</th>
<th>V&lt;sub&gt;Glu&lt;/sub&gt;</th>
<th>Glomerulosclerosis Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 wk</td>
<td>8 wk</td>
<td>20 wk</td>
<td>28 wk</td>
</tr>
<tr>
<td>m, wt (4)</td>
<td>14.7 (2.8)</td>
<td>29.3 (4.7)</td>
<td>36.0 (6.5)</td>
<td>48.7 (6.3)</td>
</tr>
<tr>
<td>m, tg (4)</td>
<td>12.2 (0.6)</td>
<td>22.4* (4.0)</td>
<td>26.6* (1.8)</td>
<td>32.3* (5.3)</td>
</tr>
<tr>
<td>f, wt (4)</td>
<td>13.8 (2.2)</td>
<td>21.1 (1.2)</td>
<td>27.9 (2.1)</td>
<td>29.9 (1.3)</td>
</tr>
<tr>
<td>f, tg (4)</td>
<td>11.2 (0.7)</td>
<td>20.9 (1.2)</td>
<td>26.1 (1.3)</td>
<td>27.3 (3.0)</td>
</tr>
</tbody>
</table>

Data represent means and (SD). V<sub>Glu</sub>, mean glomerular volume. Bold numbers: *P < 0.05 vs. age- and sex-matched control.
Furthermore, transgenic mice fed the carbohydrate-restricted diet only showed an up to 1.7-fold increase of the glomerulosclerosis index vs. controls at 43 wk of age (Table 7).

**DISCUSSION**

Diabetic nephropathy is a major health concern worldwide and is characterized by albuminuria, progressive renal failure, and characteristic pathologic changes of the kidney starting with moderate changes such as glomerular hypertrophy and subsequent thickening of the GBM. Later in the course of the disease, progressive structural and functional damage occurs, leading to proteinuria, and end-stage renal disease (33). Diabetic animal models are essential to get insight into the pathogenesis of the disease and to establish effective treatment strategies. Existing mouse models of diabetic kidney disease do not fulfill the requirements of a model for human diabetic nephropathy (3).

In the present study, we characterized the age-related sequence of clinical changes and structural alterations of the kidneys of GIPRdn transgenic mice. We determined increased serum levels of urea and creatinine and reduced levels of total protein and albumin in transgenic mice vs. controls from 8 wk of age onwards. Azotemia could be caused by decreased glomerular filtration due to advanced kidney changes; however, morphological changes suggesting renal failure were not readily observed, therefore, azotemia is more likely due to severe polyuria, resulting in sodium-chloride loss and exsiccosis (21) or catabolism of muscular tissue due to insulin defi-

**Fig. 2.** Light microscopy of the kidneys. Representative glomerular profiles of male co (A, E) and male tg mice (B, C, D, F) at 28 wk of age. PASM-PAS stain ×10 objective (A–D); ×25 objective (E, F). The tg animals exhibit glomerular hypertrophy, mesangial expansion, and matrix deposition (B, C), tubular atrophy, mononuclear infiltration, proteinaceous casts (arrows; C, D), and segmental glomerular hyalinosis (arrowheads; C, F).

**Fig. 3.** Immunohistochemistry of the kidneys and real-time PCR. Representative glomerular profiles of 28-wk-old co (A, D, G, J, M) and tg mice (B, E, H, K, N) at 28 wk of age. Collagen type IV ×40 objective (A, B); laminin ×40 objective (D, E); fibronectin ×40 objective (G, H); TGF-β1 ×40 objective (J, K); insets ×100 objective; VEGF ×40 objective (M, N). Tg animals exhibit increased mesangial deposition of the extracellular matrix proteins collagen type IV (B), laminin (E), and fibronectin (H) and increased staining of podocytes for TGF-β1 (arrows point to podocyte shown in inset; arrowheads show examples of podocytes; K). Glomerular expression of transcripts coding for collagen type IV alpha 1 (C), laminin beta 2 (F), fibronectin (I), TGF-β1 (L), and VEGF (O) in isolated glomeruli of m tg (n = 7) and co mice (n = 7) aged 28 wk. The expression of the housekeeping gene ribosomal protein 13A (Rpl13a) served as internal reference. Differential glomerular expression of Tgfb1, Col4a1, and Fln1 was significantly increased in tg animals. Data are means ± SE. *P < 0.05.
GIPRdn transgenic mice. Glomerular hypertrophy is thought to complete normalization of renal and glomerular hypertrophy in models of insulin-deficient diabetes (29, 32). Interestingly, glomerular size does not increase with age in other mouse models. In GIPRdn transgenic mice, renal hypertrophy progressed until 28 wk of age. In contrast, mean podocyte volume; GBM, harmonic mean thickness of the glomerular basement membrane; FSF, filtration slit frequency; n.d., not done. Bold numbers: *P < 0.05 vs. age- and sex-matched control.

Renal hypertrophy is a characteristic early diabetes-associated finding of humans and diabetic animal models (2, 41). Whole kidney enlargement may occur within 4 days of diabetes onset, probably in response to increased glucose and fluid filtration and their active reabsorption (2). In nonobese diabetic (NOD) mice, renal hypertrophy occurred 10 days after diabetes onset and reached a plateau at day 20 (32). In GIPRdn transgenic mice, renal hypertrophy was detected at 8 wk of age (5–6 wk after diabetes onset), and reached a plateau at 20 wk of age. Similar development of renal hypertrophy was shown in OVE26 mice (51) and endothelial nitric oxide synthase-deficient mice (30). Dilation of the renal pelvis and bladder was evident in diabetic GIPRdn transgenic mice but unlike other diabetic mouse models, hydrenephrosis was very rarely observed (20, 51).

Like renal enlargement, glomerular hypertrophy is a feature of early type 1 diabetes mellitus and occurs within days of experimental diabetes (2). The mean glomerular volume of GIPRdn transgenic mice was significantly increased and glomerular hypertrophy progressed until 28 wk of age. In contrast, glomerular size does not increase with age in other mouse models of insulin-deficient diabetes (29, 32). Interestingly, acceleration of the diabetic phenotype resulted in almost complete normalization of renal and glomerular hypertrophy in GIPRdn transgenic mice. Glomerular hypertrophy is thought to precede thickening of the GBM (2, 33); however, in male GIPRdn transgenic animals, GBM thickness was slightly but significantly increased at 8 wk of age, when glomerular size did not differ from controls. In early stages of diabetes of the NOD mouse, glomerular size also did not differ from controls but electron microscopy revealed an irregular thickening of the GBM; however, GBM thickness was not quantified (32). In many other diabetic animal models, thickening of the GBM was assessed at late time points where glomerular hypertrophy was already evident (17, 29, 40).

The increase in the mean glomerular volume of aging GIPRdn transgenic animals was associated with progressive glomerular alterations. This finding is in line with observations in other diabetic mouse models (29, 30, 51). However, in streptozotocin diabetic rats, the glomerulosclerosis index 6 mo after diabetes induction was only one-third that of 20-wk-old GIPRdn transgenic mice, whereas in the diabetic SHR/N-cp rat model, a similar degree of glomerular damage was determined (17).

We could demonstrate an increased glomerular expression and mesangial deposition of the extracellular matrix proteins collagen type IV and fibronectin. Furthermore, an increased expression of Tgfb1 was detected by real-time PCR in glomeruli of GIPRdn transgenic animals. TGF-β1 is thought to be a major player in the development of diabetic nephropathy (52). TGF-β1 is known to increase the synthesis of extracellular matrix proteins and to decrease matrix degradation (52, 53) and could therefore account for increased deposition of extracellular matrix proteins of GIPRdn transgenic mice.

The mean podocyte volume was significantly increased in male and female transgenic mice vs. controls. Since in male GIPRdn transgenic mice, the glomerular volume was not yet increased at 8 wk of age, and since blood pressure was unaltered, adaptive growth of the podocytes due to glomerular hypertrophy (49) or mechanical stress cannot be the cause for podocyte hypertrophy. We rather suggest a direct effect of hyperglycemia on glomerular epithelial cell growth in this age group (28). Hypertrophy of renal cells is thought to contribute to diabetes-associated kidney lesions, such as glomerulosclerosis, tubular atrophy, and interstitial fibrosis. Podocyte hypertrophy, caused by high-glucose concentrations, may eventually lead to podocyte loss and glomerulosclerosis (16, 31).

The numerical volume density of podocytes in glomeruli was significantly reduced in both male and female GIPRdn transgenic mice. Dalla Vestra et al. (9) suggested that the numerical volume density of podocytes is functionally more relevant than absolute numbers in type 2 diabetic patients, since the numerical volume density of podocytes but not absolute numbers is inversely related to albumin excretion rate. Podocytes in enlarged glomeruli have to cover a larger GBM area and are subjected to increased mechanical stress and injury. Widening of foot processes and increased albumin excretion, loss of podocytes, and glomerulosclerosis are the consequences (9).

In addition to glomerular lesions, GIPRdn transgenic mice developed tubulointerstitial changes, such as tubular atrophy, focal interstitial fibrosis, and mononuclear cell infiltration. Tubulointerstitial lesions occur in late stages of diabetic nephropathy in humans (2) and few experimental animals, such as diabetic SHR/N-cp rats and the OVE26 diabetic mouse (17, 31).
but in many diabetic animal models, e.g., Akita, db/db, and ob/ob mice, only mild kidney changes are observed (3, 42).

Due to the early-onset and severity of the diabetic phenotype, and the relatively long life span of GIPR<sup>dn</sup> transgenic mice, the development of diabetic kidney disease may be more robust than in other diabetic models. In addition, the CD1 genetic background may be more susceptible to developing diabetic nephropathy than other genetic backgrounds (44).

In summary, GIPR<sup>dn</sup> transgenic mice develop progressive diabetes-associated kidney lesions with many parallels to the

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**Fig. 4.** Electron microscopy of glomeruli. Representative electron micrographs of podocytes of an 8-wk-old co (A) and a tg mouse (B): podocytes of the tg animal show hypertrophy, pseudocysts (●), and microvillous transformation (arrow). 28-wk-old co (C) and tg (D) mouse: podocytes of the tg animal show hypertrophy, pseudocysts (●), vacuolization (arrow), microvillous transformation, and focal foot process effacement. The glomerular basement membrane (GBM) is thickened both homogenously and in a nodular manner. Electron micrographs of the GBM of an 8-wk-old co (E) and a tg animal (F), showing mild thickening of the GBM of the tg mouse with unchanged filtration slits, and of a 28-wk-old co (G) and tg mouse (H) showing homogenous and nodular thickening of the GBM as well as focal foot process effacement. A, B: print magnification ×14,100. C, D: print magnification ×6,300. E, F, G, H: print magnification ×68,000.
human disease, i.e., renal, glomerular, and podocyte hypertrophy, thickening of the GBM, reduction of the numerical density of podocytes, progressive glomerulosclerosis associated with albuminuria, and tubulointerstitial changes. Our findings particularly in male GIPR<sup>dn</sup> transgenic mice suggest that the increase in podocyte size is directly related to hyperglycemia and not associated with mechanical stress. Furthermore, podocyte hypertrophy is an early event, preceding other structural changes of the podocyte in this novel mouse model of diabetic nephropathy.

GIPR<sup>dn</sup> transgenic mice may therefore serve as an excellent new model for studying the pathogenesis of diabetic nephropathy, the sequence of structural, functional, and molecular changes of glomerular cells, and for testing the efficacy of new therapies.

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GRANTS

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REFERENCES


Table 6. Serum parameters from 43-wk-old mice fed a calorie-restricted diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucose, mg/dl</th>
<th>Insulin, μU/ml</th>
<th>Na, mmol/l</th>
<th>Cl, mmol/l</th>
<th>Urea, mg/dl</th>
<th>Crea, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>m, wt (8)</td>
<td>70 (39)</td>
<td>15.4 (3.0)</td>
<td>142 (2)</td>
<td>111 (9)</td>
<td>64 (20)</td>
<td>0.30 (0.18)</td>
</tr>
<tr>
<td>m, tg (8)</td>
<td>116 (61)</td>
<td>10.6 (5.5)</td>
<td>143 (3)</td>
<td>119 (11)</td>
<td>67 (11)</td>
<td>0.40 (0.17)</td>
</tr>
<tr>
<td>f, wt (8)</td>
<td>97 (60)</td>
<td>13.0 (5.7)</td>
<td>144 (3)</td>
<td>109 (28)</td>
<td>44 (12)</td>
<td>0.27 (0.08)</td>
</tr>
<tr>
<td>f, tg (11)</td>
<td>121 (106)</td>
<td>16.4 (10.0)</td>
<td>143 (3)</td>
<td>107 (18)</td>
<td>64 (28)</td>
<td>0.27 (0.12)</td>
</tr>
</tbody>
</table>

Data represent means and (SD).

Table 7. Parameters from 43-wk-old mice fed a calorie-restricted diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Body wt, g</th>
<th>Kidney wt, mg</th>
<th>V(1Cl) × 10&lt;sup&gt;3&lt;/sup&gt; µm&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Glomerulosclerosis Index</th>
<th>Urine Creatinine, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>m, wt (5)</td>
<td>38.1 (4.2)</td>
<td>631 (103)</td>
<td>486 (30)</td>
<td>0.6 (0.1)</td>
<td>40.2 (628.8)</td>
</tr>
<tr>
<td>m, tg (6)</td>
<td>28.6* (4.7)</td>
<td>710 (113)</td>
<td>452 (72)</td>
<td>1.0* (0.1)</td>
<td>10.8 (8.3)</td>
</tr>
<tr>
<td>f, wt (5)</td>
<td>30.0 (2.7)</td>
<td>498 (98)</td>
<td>424 (27)</td>
<td>0.7 (0.1)</td>
<td>25.2 (9.2)</td>
</tr>
<tr>
<td>f, tg (7)</td>
<td>28.6 (3.1)</td>
<td>611 (79)</td>
<td>512* (67)</td>
<td>1.1* (0.2)</td>
<td>12.5* (9.9)</td>
</tr>
</tbody>
</table>

Data represent means and (SD). Bold numbers: *P < 0.05 vs. age- and sex-matched control.
Diabetic nephropathy in transgenic mice expressing a dominant negative glucose-dependent insulinotropic polypeptide receptor (GIPRdn).


Herbach N, Röthel B, Kemter E, Pichl L, Klaften M, de Angelis Nauck MA, Baller B, Meier JJ.


