Early glomerular alterations in genetically determined low nephron number

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Benz K, Campean V, Cordasie N, Karpe B, Neuhuber W, Mall G, Hartner A, Hilgers KF, Amann K. Early glomerular alterations in genetically determined low nephron number. Am J Physiol Renal Physiol 300: F521–F530, 2011. First published October 13, 2010; doi:10.1152/ajprenal.00490.2009.—An association between low nephron number and subsequent development of hypertension in later life has been demonstrated. The underlying pathomechanisms are unknown, but glomerular and postglomerular changes have been discussed. We investigated whether such changes are already present in prehypertensive “glial cell line-derived neurotrophic growth factor” heterozygous mice (GDNF+/−) with lower nephron number. Twenty-six-week-old mice [22 GDNF+/−, 29 C57B6 wild-type control (wt)] were used for in vivo experiments with intra-arterial and tail cuff blood pressure measurements. After perfusion fixation, kidneys were investigated with morphological, morphometric, stereological, and immunohistochemical techniques and TaqMan PCR analysis. As expected at this age, blood pressure was comparable between GDNF+/− and wt. Nephron number per kidney was significantly lower in GDNF+/− than in wt (−32.8%, P < 0.005), and mean glomerular volume was significantly higher (+49.5%, P < 0.001). Renal damage scores, glomerular and tubular proliferation, analysis of intrarenal arteries and peritubular capillaries, expression of relevant tubular transporter proteins, as well as gene expression of profibrotic, proinflammatory, or prohypertensive markers were not significantly different between GDNF+/− and wt. Compensatory glomerular hypertrophy in GDNF+/− was accompanied by higher numbers of endothelial and mesangial cells as well as PCNA-positive glomerular cells, whereas podocyte density was significantly reduced. Further electron microscopic analysis showed marked thickening of glomerular basement membrane. In conclusion, lower nephron number is associated with marked early glomerular structural changes, in particular lower capillary supply, reduced podocyte density, and thickened glomerular basement membrane, that may predispose to glomerulosclerosis.

Glial cell line-derived neurotrophic growth factor; hypertension; arteries; capillaries; podocyte; glomerular basement membrane

Nephron number as fixed during nephrogenesis may be one determinant of renal disease and development of hypertension in later life. Various genetic and epigenetic factors are known to influence nephron number, i.e., genes involved in the various steps of nephrogenesis like pax-2, WT-1, and glial cell line-derived neurotrophic growth factor (GDNF), but also maternal or environmental causes like steroid treatment, vitamin A deficiency, malnutrition by either placental insufficiency or protein restriction, etc. (21, 24, 28, 41).

An involvement of the kidney in the development of hypertension has been postulated for a long time and supported by experimental studies in rats (38) and sheep (35). Brenner and colleagues supposed a direct association between nephron number and blood pressure in humans. Their so-called Brenner hypothesis postulated that any reduction in nephron endowment leads to hyperfiltration of the remaining glomeruli, followed by glomerular enlargement with glomerular and then systemic hypertension, resulting in glomerulosclerosis, thereby establishing a vicious circle (7). In line with this theory, an association of low nephron number and development of hypertension has been shown in different animal models (17, 21, 35, 44) as well as in two autopsy studies in humans (23, 27). In the study by Keller et al. (27) in Caucasian patients with essential hypertension the number of glomeruli per kidney was significantly lower than in control subjects matched for age, weight, and sex. In parallel, mean glomerular volume was more than twice as high in hypertensive patients as in normotensive control patients, indicating compensatory glomerular enlargement. The results of this study were confirmed in a Caucasian American (23) population, whereas in African Americans an association between glomerular number and blood pressure was not seen (23). Interestingly, Australian aborigines also have a significantly lower nephron number than the nonaboriginal Australian population, and there is a high propensity of hypertension and renal insufficiency in aborigines (22).

The pathomechanisms, however, that link low nephron number and hypertension are only partly understood. Among potential mechanisms are inappropriate activation of the renin-angiotensin system (RAS), impaired tubular salt handling leading to salt and volume retention, and postglomerular structural changes, i.e., of peritubular capillaries or arteries. GDNF is a crucial factor for nephrogenesis; it directly induces branching of the ureteric bud. Complete deficiency of GDNF in knockout mice leads to bilateral kidney agenesis and death within the first 24 h (9, 37). Heterozygous GDNF (GDNF+/−) mice, however, show moderately reduced nephron number (−30%) (9) and eventually develop mild hypertension in later life (10).

We used the animal model of GDNF+/− mice on a pure C57BL/6 background to study the link between low nephron number and accompanying structural changes in the kidney. This animal model provides the unique opportunity to study the effect of lower nephron number due to one single gene defect independent of all other aspects of fetal programming. We were especially interested in a detailed analysis of possible structural alterations of intrarenal arteries, peritubular capillaries, tubulus system, and glomerular cells, including ultrathin evaluation that had not been performed before. Additionally, potentially relevant prohypertensive systems such as the RAS and endothelin systems and also tubular sodium transporters had not yet been evaluated in the GDNF+/− model, but alterations in models of intrauterine growth restriction (IUGR)
with low nephron number were described previously, making them potential candidate systems.

**MATERIALS AND METHODS**

**Animals**

GDNF+/− mice with pure C57B6 background were kindly provided by Prof. K. Kriegstein and Dr. S. Hermann, Department of Neuroanatomy, University of Göttingen, Göttingen, Germany. The mice were originally described by Pichel et al. (37). Twenty-two GDNF+/− mice (age 26 wk; 11 male, 11 female) were used, with 29 wild-type C57B6 (13 male, 16 female) serving as controls. Mice were housed under maintained conditions (22 ± 2°C, 12:12-h dark-light cycle). All animals were allowed unlimited access to chow (no. 1320, Altromin, Lage, Germany) and tap water. All procedures were done in accordance with the guidelines of the American Physiological Society and were approved by the local government authorities (Regierung von Mittelfranken, AZ no. 54-2531.31-307).

Tail tissue was obtained at weaning for genotyping by PCR analysis. The specific primer sequences were as follows: P1, 5′-CCAGAGATTCACAGGAAAGGGTGTGC-3′; P2, 5′-CAGATA-CATCCACACGGTATAGC-3′; P3, 5′-GACTACGGAGGAG- TAGAAG-3′; and P4, 5′-TATGCCTCCTGGCTTGTCC-3′. The wild-type GDNF allele was detected using the P1 upstream and P2 downstream oligonucleotide. PCR amplification using the P3 and P4 primer pairs detected the mutant GDNF allele. PCR products were analyzed on 1% agarose gels and visualized by UV light after ethidium bromide staining.

Noninvasive blood pressure was measured by regular tail cuff plethysmography (TSE 209000, TSE Systems, Bad Homburg, Germany) as previously described (25). Briefly, all mice were trained with the restrainer over a period of 3 wk (at least 3 times per week). Thereafter, measurements were performed twice per week, and each measurement included a total of 10 consecutive readings of systolic blood pressure and heart rate. In addition, before perfusion fixation all mice were equipped with a carotid artery catheter under isoflurane anesthesia, and intra-arterial blood pressure measurements were then performed in conscious mice 2 h after anesthesia (19).

**Tissue Preparation**

The experiment was terminated by perfusion via the left ventricle under deep ketamine-xylazine anesthesia (3). Both kidneys were taken out, decapsulated, weighed, and dissected in a plane perpendicular to the interpolar axis, yielding slices of 1-mm width. For further morphometric and stereological analyses and electron microscopy five small pieces of the right kidney were selected by area-weighted sampling for embedding in Epon-Araldite. From the resulting resin blocks, semithin sections of 0.5 μm were prepared and stained with methylene blue and basic fuchs. The remaining kidney slices were embedded in paraffin; 2-μm sections were cut (Leitz SM 2000 R microtome, Leica Instruments, Nussloch, Germany), deparaffinized, and rehydrated in decreasing concentrations of ethanol. For immunohistochemistry, endogenous peroxidase activity was blocked [3% H2O2 in Tris-buffered saline (TBS), 20 min, room temperature]; kidney sections were then layered with the primary antibody and incubated at 4°C overnight. The following antibodies were used: PCNA (mouse IgG, clone PC 10, Dako Cytomation, Hamburg, Germany; dilution 1:500), SMA (anti-human smooth muscle actin, monoclonal mouse, Dako; dilution 1:200), 11β-HSD2 (anti-rat 11β-hydroxysteroid dehydrogenase type 2, sheep polyclonal, Chemicon, Temecula, CA; dilution 1:200), calbindin (monoclonal mouse, D-28h, Swant, Bellinzona, Switzerland; dilution 1:500), NCAM (thiazide-sensitive sodium-chloride cotransporter; antibody gift from D. J. Fife, Portland, OR; rabbit, dilution 1:500), NaCa (anti-Na+/Ca2+ exchanger antibody transmembrane segment 5 and 6, monoclonal mouse; Swant; dilution 1:200), desmin (Dako Cytomation; mouse antibody, dilution 1:100), WT-1 (Santa Cruz Biotechnology, Santa Cruz, CA; rabbit antibody, dilution 1:50), nephrin (Acris Antibodies, Herford, Germany; pig antibody, dilution 1:50), collagen IV (Southern Biotechnology Associates, Birmingham, AL; goat antibody, dilution 1:100), CD3 (Neomarkers, Lab Vision, Fremont, CA; rabbit antibody, dilution 1:100), F4/80 (AbD Serotec; Düsseldorf, Germany; rat antibody, dilution 1:100) and MECA-32 (Developmental Studies Hybridoma Bank, University of Iowa, cell supernatant 1:1; see Ref. 11). Thereafter, incubation with the appropriate biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA) and horseradish peroxidase-conjugated avidin-biotin complexes (Vectastain Elite ABC Kit; Vector Laboratories) was performed. Aminoethylcarbazole (AEC) solution substrate (Dako Cytomation) served as chromogen. All sections were counterstained with Mayer’s hemalum solution (Merck, Darmstadt, Germany) and examined by light microscopy.

Detection of the monoclonal mouse antibody was enhanced with the use of a M.O.M. Kit (Vector Laboratories) according to the manufacturer’s instructions to eliminate background staining from endogenous mouse immunoglobulin.

**Serial sections of kidney.** To analyze potential tubular architectural or functional alterations, the distribution of relevant tubular transporters or tubular segment markers (NCC, NaCa, calbindin, 11β-HSD2) was analyzed in five male wild-type and five male GDNF+/− mice by immunohistochemistry on serial sections (1st section: NCC, 2nd section: NaCa, 3rd section: calbindin, 4th section: 11β-HSD2). Thereby, NCC was used as a marker for distal convoluted tubule (DCT), NaCa as a marker for the middle segment of DCT and connecting tubule (CNT), calbindin as a marker for DCT and CNT, and 11β-HSD2 as a marker for CNT.

**Renal innervation.** To rule out changes due to differences in neuronal supply, density and distribution of primary afferent nerve fibers as demonstrated by immunohistochemistry for calcitonin gene-related peptide (CGRP) and of sympathetic axons as demonstrated by immunostaining for tyrosine hydroxylase (TH) were assessed semiquantitatively in the kidney. Twelve-micrometer-thick cryostat sections were mounted on poly-l-lysine-coated slides and incubated with rabbit anti-CGRP (1:1,000; Peninsula, San Carlos, CA) and sheep anti-TH (1:2,000; Novus Biologicals, Littleton, CO) diluted in TBS containing 1% bovine serum albumin and 0.5% Triton X-100 overnight at room temperature. Binding sites were detected with donkey anti-rabbit IgG tagged with Alexa 488 and donkey anti-sheep IgG tagged with Alexa 555 (1:1,000 in TBS for 1 h at room temperature; both from Molecular Probes, Eugene, OR), respectively. Sections were examined by double-channel confocal laser scanning microscopy (Bio-Rad MRC 1000, Bio-Rad, Hemel Hempstead, UK).

**Morphological Investigations of Kidneys**

**Indexes of renal damage.** To quantify mesangial matrix accumulation and sclerosis of the glomerular tuft, a score of 0 to 4 was...
determined on PAS- and H & E-stained paraffin sections as described previously (19): score 0, normal glomerulus; score 1, mesangial expansion or sclerosis involving up to 25% of the glomerular tuft; score 2, glomerular sclerosis of 25–50%; score 3, glomerular sclerosis of 50–75% and/or segmental extracapillary fibrosis or proliferation; score 4, global sclerosis of >75%, global extracapillary fibrosis, or complete collapse of the glomerular tuft. Tubulointerstitial changes, i.e., tubular atrophy, tubular dilatation, interstitial inflammation, and fibrosis, and vascular damage, i.e., wall thickening and necrosis of the vessel wall, were assessed on H & E-stained paraffin sections at a magnification of ×100 with a similar semiquantitative scoring system from 0 to 4 as described previously (5, 45). In brief, for determination of the tubulointerstitial damage 10 fields per kidney were randomly sampled and graded as follows: grade 0, normal tubulointerstitial structure; grade 1, lesions involving <25% of the area; grade 2, lesions affecting 25–50%; grade 3, lesions involving >50% up to 75%; grade 4, tubulointerstitial damage in almost the entire area. Similarly, for the vascular damage score interlobular and smaller arteries were graded according to the following scheme: grade 0, no wall thickening; grades 1, 2, 3, mild, moderate, and severe wall thickening, respectively; grade 4, fibrinoid necrosis of the vascular wall. In addition, in all animals the percentage of interstitial fibrosis was determined by exchanging the reference and lookup sections (42).

Volume density (V_v) of glomeruli and cortex were measured by point counting at a magnification of ×200 on H & E stained sections. Renal cortical volume (V_cortex) was derived from V_v (cortex) × kidney volume.

Glomerular number per kidney and mean glomerular volume were analyzed on semithin sections with unbiased stereological techniques, i.e., the dissector technique (8, 27, 43). From the resin blocks three per animal were randomly chosen and eighty 1-µm semithin serial sections were cut. From these every 20th section was chosen for investigations using the dissector technique (43). All dissector sections were scanned at very high quality with the Mirax scanner and appropriate software (Mirax, Zeiss). A grid was placed over each field of view, and points falling on kidney tissue (P_kid) and glomeruli (P_glom) were counted. The reference and lookup sections were then visualized on two different monitors, thus allowing easy comparison of glomerular number and location. Glomeruli sampled by an unbiased counting frame in the field of view of the reference section that were not present in the lookup section were counted. By comparison of each of these sections with the following and vice versa, four dissector pairs per section were created. Dissector efficacy was increased by exchanging the reference and lookup sections (42).

With this technique the number of glomeruli per area (N_v) as well as Q_v, i.e., the number of glomeruli intersected by one plane within a frame area but not intersected by the other plane, were measured. The number of glomeruli per volume (N_v) was then derived from N_v = Q_v/100 with v(dis) = h × a being the volume of the dissector (h = distance between sectioning planes, a = area of sampling planes). The total glomerular number per kidney (N) was calculated according to N = N_v × r(ref), with r(ref) as the total reference space volume. Mean glomerular volume (V_glom) was calculated according to V_glom = [(V_glom/V_kid) × N/100], with V_glom/V_kid equivalent to P_glom/P_kid.

Analysis of glomerular capillarization and cellularity on semithin sections. Five semithin sections per animal were analyzed in terms of glomerular capillarization and cellularity with the point counting method and a 121-point eyepiece (Zeiss) at a magnification of ×1,000 with oil immersion as previously described (2). In brief, length density (L_v) of glomerular capillaries was determined according to the standard stereological formula L_v = 2 × Q_v, with Q_v being the number of capillary transsects per area of the capillary tuft. Total length of glomerular capillaries per kidney (L_total) was then derived from L_v and the total glomerular volume: V_v = V_glom × V_cortex. Total volume of the capillary tuft (V_kid) was also calculated with point counting.

The number of podocytes and endothelial and mesangial cells per glomerulus was assessed in at least 25 glomeruli from each animal from cell density per volume (N_v) and volume density of the cell type (V_v) according to N_v = kβ × N_v/V_v, with β = 1.5 for podocytes and β = 1.4 for endothelial and mesangial cells, as well as k = 1.

Intrarenal arteries and peritubular capillaries. The length density (L_v) of renal cortical arteries was determined according to the equation L_v = c × Q_v, at a magnification of ×100. Briefly, on paraffin sections Q_v was determined as the number of vessels per cortical area. The area and lumen of cortical vessels were determined with planimetry and a semiautomated image analysis system (Soft Imaging Systems, Münster, Germany). In SMA-stained kidney sections, the minimal and maximal vessel diameters (D_min and D_max, respectively) were measured; c was determined as D_max/D_min. The vessel area A_v and lumen area A_lumen were determined with the equations A_vessel = π × (D_outer/2)^2 and A_lumen = π × (D_inner/2)^2, respectively (D_outer = distance from one outer side of the vessel to the opposite side; D_inner = distance from one inner site of the vessel to the opposite site; D_max = D_outer at the largest vessel diameter; D_min = D_outer at the smallest vessel diameter). The wall area of the vessel was calculated as the difference of the two (4).

In addition, the number of MECA-32-stained peritubular capillaries (Q_A) was counted per each cortical area or medullary area at a magnification of ×20.

Real-Time PCR

To evaluate relative mRNA expression levels, total RNA was obtained from frozen kidney tissue with RNAeasy Mini columns (Qiagen, Hilden, Germany). Primers of the target genes (Table 1) were designed with the primer design software Primer Express 3 (Applied Biosystems, Weiterstadt, Germany) and synthesized (MWG-Biotech, Ebersberg, Germany). Primers were tested for target specificity and amplification efficiency according to standard quality protocols provided by Applied Biosystems. Reverse transcription reactions and real-time PCR were performed with Power SYBR Green (Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. Real-Time PCR data were analyzed with SDS v1.3 software (Applied Biosystems). To compare expression levels among the groups, the relative expression of target gene mRNA levels was calculated with the comparative ΔCt (threshold cycle number) method (31). Normalization was conducted against endogenous 18S mRNA levels, and an additional correction for the determined efficiency of the primer pair was applied to the resulting relative fold changes (49).

Analysis of Data

Data are given as means ± SE. After testing for homogeneous distribution the t-test was used to determine intergroup differences. The results were considered significant if the probability of error (P) was <0.05.

RESULTS

Animal Data

As expected, at age 26 wk wild-type and GDNF+/− mice did not show significant differences in blood pressure, body weight, or relative heart weight (Table 2). The absolute and relative kidney weights of GDNF+/− mice were significantly lower than in wild-type control mice (Table 2). The prede-scribed higher incidence of unilateral renal agenesis in GDNF+/− was seen in 2 of 11 male GDNF+/− and 5 of 11
female GDNF+/−. In these mice a compensatory higher volume of the single kidney was seen, and therefore relative total kidney weight did not differ compared with the group with two kidneys. In addition, as expected GDNF+/− mice showed a significantly lower renal GDNF gene expression compared with control mice (P < 0.05, data not shown).

Renal Innervation

Density and distribution of primary afferent CGRP-positive nerve fibers and sympathetic TH-positive axons in the kidney were not different in GDNF+/− and wild-type control mice.

Table 2. Animal data

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n = 29)</th>
<th>GDNF+/− (n = 22)</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>28.1 ± 0.92</td>
<td>28.3 ± 0.99</td>
<td>ns</td>
</tr>
<tr>
<td>Relative kidney weight, mg/g</td>
<td>12.1 ± 0.40</td>
<td>9.90 ± 0.46</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Relative heart weight, mg/g</td>
<td>5.96 ± 0.31</td>
<td>5.99 ± 0.21</td>
<td>ns</td>
</tr>
<tr>
<td>Mean intra-arterial blood pressure, mmHg</td>
<td>96.7 ± 3.39</td>
<td>102.4 ± 3.17</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are means ± SE. ns, Not significant.

Low Nephron Number Is Accompanied by Early Structural Changes of Glomerular Basement Membrane and Lower Podocyte Density

Nephron number and mean glomerular volume. In GDNF+/− animals (7,813 ± 369) nephron number was significantly (P < 0.0005) lower than in wild-type control animals (11,629 ± 515) (Fig. 1A), confirming previous data in GDNF+/− animals on a mixed background (9, 10). In parallel, mean glomerular cell proliferation assessed by PCNA immunoreactivity was significantly higher in GDNF+/− mice than in wild-type control mice; indicating podocyte rarefaction. In parallel, mean podocyte volume was higher in GDNF+/− mice than in wild-type control mice (12%); whereas the total length of podocytes and GDNF+/− mice showed a significantly lower renal GDNF gene expression compared with control mice (P < 0.05, data not shown).

Fig. 1. A: significantly lower nephron number per kidney in glial cell line-derived neurotrophic factor heterozygous (GDNF+/−) vs. wild-type control mice. B: thickness of the glomerular basement membrane (GBM) in GDNF+/− and wild-type mice. **P < 0.0005.
Table 3. Glomerular cells and capillaries

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n = 10)</th>
<th>GDNF+/− (n = 10)</th>
<th>t-Test</th>
</tr>
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<tbody>
<tr>
<td>N_pod</td>
<td>91 ± 4.6</td>
<td>96 ± 5.2</td>
<td>ns</td>
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<tr>
<td>N_pod</td>
<td>2,591 ± 148</td>
<td>1,946 ± 111</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Mean podocyte volume, μm³</td>
<td>113.3 ± 7.96</td>
<td>135.3 ± 8.60</td>
<td>ns</td>
</tr>
<tr>
<td>N_mes</td>
<td>164 ± 7.6</td>
<td>233 ± 15.0</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>N_Ames</td>
<td>4,053 ± 116</td>
<td>3,908 ± 248</td>
<td>ns</td>
</tr>
<tr>
<td>Mean mesangial cell volume, μm³</td>
<td>76.2 ± 5.24</td>
<td>75.7 ± 5.56</td>
<td>ns</td>
</tr>
<tr>
<td>N_end</td>
<td>146 ± 12.3</td>
<td>190 ± 14.0</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>N_Aend</td>
<td>3,763 ± 270</td>
<td>3,382 ± 204</td>
<td>ns</td>
</tr>
<tr>
<td>Mean endothelial cell volume, μm²</td>
<td>87.9 ± 6.0</td>
<td>85.1 ± 4.60</td>
<td>ns</td>
</tr>
<tr>
<td>L_m, mm/mm³</td>
<td>15,370 ± 291</td>
<td>13,240 ± 473</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>L_end, mm</td>
<td>40.4 ± 3.7</td>
<td>37.8 ± 3.3</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are means ± SE. N_pod, number of podocytes per glomerulus; N_pod, number of podocytes per area; N_mes, number of mesangial cells per glomerulus; N_Ames, number of mesangial cells per area; N_end, number of endothelial cells per glomerulus; N_Aend, number of endothelial cells/area; L_m, length density of glomerular capillaries; L_end, total glomerular capillary length.

GDNF+/− than in wild-type control (0.30 ± 0.08 cells/glomerulus in GDNF+/− vs. 0.15 ± 0.02 in control, P < 0.05).

Podocyte markers. There was no significant difference between the number of WT-1- or nephrin-positive cells per glomerulus in both groups, whereas the number of desmin-positive cells was slightly higher in GDNF+/− than in wild-type control, pointing to mild podocyte damage. However, because of the high standard deviation, this difference did not reach statistical significance (data not shown).

Electron microscopy. By electron microscopy marked thickening of the GBM was observed in GDNF+/− (153.1 ± 2.3 nm) compared with wild-type control (127.9 ± 1.7 nm, P < 0.0001; Fig. 1B) animals. In parallel, enlargement of podocytes with increased cytoplasmic vacuolization and partial fusion of foot processes was seen in GDNF+/− mice compared with wild-type control mice (Fig. 2, E–H).

Lower Nephron Number Is Not Accompanied by Changes of Intrarenal Arteries or Peritubular Capillaries or by Changes in Distribution of Tubular Transporters

Wall thickness and lumen area, number per area, length density, and total length of intrarenal arteries as well as the number of peritubular capillaries per area were not significantly different between the groups, either in the cortex or in the medulla (Table 4).

Immunohistological expression and distribution of relevant tubular transporter systems (NaCa and NCC) or markers of tubular segment differentiation (calbindin and 11β-HSD2) were not different between the two groups (Fig. 3). In addition, no significant differences between GDNF+/− and wild-type littermates were noted in gene expression of tubular sodium transporters [epithelial sodium channel α (ENaC), NCC; data not shown].

Lower Nephron Number in Young GDNF+/− Is Accompanied by Mild Interstitial Activation but Not by Proinflammatory or Prohypertensive Changes in Kidney

No significant differences were found with respect to all renal damage scores, i.e., glomerular [glomerular sclerosis index (GSI)], tubulointerstitial [tubulointerstitial damage index (TISI)], vascular damage (VSI), and mesangiolysis (MSI) (data not shown). In parallel, there was no significant difference in glomerular or tubulointerstitial collagen IV staining between the groups (data not shown). Also, renal tubulointerstitial cell proliferation (assessed as PCNA-positive interstitial cells/mm²) did not differ between GDNF+/− and wild-type littermates (30.1 ± 3.4 in GDNF+/− vs. 32.3 ± 7.3 in control). With Sirius red-stained sections and an automatic image analyzing system, however, the percentage of interstitial fibrous tissue was significantly higher in GDNF+/− animals (1.3 ± 0.27%) than in wild-type control animals (0.17 ± 0.04%, P < 0.0006), indicating early interstitial activation. Interstitial macrophage and T-cell infiltration was not different between GDNF+/− and control (data not shown).

No significant differences between GDNF+/− and wild-type littermates were found in gene expression of markers for fibrosis [fibronectin, TGF-β1], inflammation [monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-6, IL-10, osteopontin], RAS [renin, angiotensinogen, angiotensin (ANG) receptors 1b and 2], endothelin system (ET-1, endothelin receptors A and B), and nitric oxide production (endothelial nitric oxide synthase, inducible nitric oxide synthase) (data not shown).

DISCUSSION

Our detailed analysis of GDNF+/− mice on a C57B6 background compared with wild-type control mice provides some novel findings. First, in GDNF+/− mice with 30% lower nephrin number, compensatory glomerular hypertrophy was accompanied by increased numbers of endothelial and mesangial cells as well as a higher number of PCNA-positive glomerular cells, whereas podocyte density was significantly reduced. Second, electron microscopic analysis showed specific glomerular alterations in GDNF+/− with marked thickening of GBM. Third, despite slightly increased tubular fibrosis, alterations in inflammatory or profibrotic markers as well as in prohypertensive systems and relevant tubular transporter systems could be excluded as potential pathogenetic candidate systems in young GDNF+/− mice.

In addition to the important role of GDNF in kidney development by induction of branching of the ureteric bud from the Wolffian duct (40), GDNF is also relevant for neuronal development. Because GDNF+/− mice had not been analyzed previously with respect to differences in renal innervation that, however, might influence blood pressure or renal damage, we first of all excluded alterations in neuronal supply, density, and distribution of primary afferent nerve fibers in GDNF+/− mice.

We were particularly interested in whether lower nephron number per se is associated with additional early glomerular or postglomerular structural changes, i.e., alterations of the tubular or vascular system that had not been investigated in detail before. To this end, we analyzed kidneys of 26-wk-old GDNF+/− and age-matched wild-type control mice, using morphometric, stereological, and molecular techniques. We used this time point because we did not expect a higher blood pressure at this age, and could thus exclude secondary changes due to already existing hypertension.
In fact, using intra-arterial and repeated tail cuff blood pressure measurements in conscious mice, we did not detect significant differences in blood pressure in 26-wk-old GDNF+/− mice compared with wild-type mice. Also, heart weight as an indirect parameter of higher blood pressure was comparable in the groups.

Using unbiased stereological techniques, we could confirm a significant lower nephron number (33%) in our GDNF+/− model mice compared with their wild-type littermates, which favors them for detailed analysis of renal structure and function, even though various other animal models of low nephron number exist, i.e., due to IUGR, a low-protein diet or diabetes mellitus of the mother, intrauterine glucocorticoid or gentamicin treatment, vitamin A deficiency, etc. (21, 24, 28, 41). In these models low nephron number cannot be dissociated from other programming effects, i.e., programming of several hormonal or metabolic systems. Therefore, the genetically determined reduced glomerular number in GDNF+/− mice provides the unique possibility to analyze the consequences of low nephron number apart from other programming effects. In our model, low nephron number was associated with significantly higher mean glomerular volume, indicating compensatory en--

Fig. 2. Representative paraffin (A and B; magnification ×20), semithin (C and D; magnification ×40), and ultrathin (E–H) sections of wild-type and GDNF+/− mice. A–D: lower glomerular number together with glomerular enlargement can be seen in GDNF+/− (B and D) compared with wild-type (A and C) mice. Semithin sections (C and D) show rarefaction of glomerular capillaries together with mild matrix expansion in GDNF+/− (D). E–H: by electron microscopy marked thickening of the GBM (arrow) and enlargement of podocytes with increased cytoplasmic vacuolization (*) can be seen in GDNF+/− (F and H) compared with wild-type control (E and G) animals. Magnification: ×7,750 (E and F) and ×27,800 (G and H).
largement of glomeruli (+49%). In an earlier study in young GDNF+/− mice at the age of 30 days, a lower nephron number, but not yet an increase in glomerular volume, was found (9). Glomerular enlargement that develops thereafter seems to be independent of ANG II since a comparable increase in glomerular volume was found at 30 and 60 wk in GDNF+/− mice treated with the AT1 blocker candesartan cilexetil (42). Glomerular enlargement is also seen in several other models of lower nephron number and is known to induce premature sclerosis. It is therefore considered a risk factor for kidney disease and renal failure (14, 22).

In our study mild, but significant, expansion of the tubulointerstitial tissue was seen, whereas no differences in interstitial macrophage and T-cell infiltrates or in renal damage scores or gene expression of proinflammatory or profibrotic genes were detected in 26-wk-old GDNF+/− mice. A quantitative evaluation of renal fibrosis and inflammation has not been performed before in this animal model, and the findings are in contrast to models with IUGR, where a relevant increase in renal inflammation and fibrosis was seen, even in young rats with a comparably low nephron number. Thus our data indicate that additional factors of global fetal programming seem to play a more important role in the development of fibrosis and inflammation than nephron number per se. Additionally, the genetic background of the mice should also be taken into consideration, because it was shown that C57BL/6J Os/+ mice with 50% lower nephron number did not develop glomerulosclerosis (20), even after further nephron reduction by unilateral nephrectomy (12). Moreover, 5/6 nephrectomy in C57B6 mice showed significantly lower glomerulosclerosis than in S129 mice (32), and the same was true for DOCA-induced hypertension and kidney alterations (19). Genetic background was one reason why we also had to analyze our model of hypertension and kidney alterations (19). Genetic background and its receptors were found in a mouse model of pronounced hypertension in IUGR rats. With respect to the endothelin system, increased protein expression of ET-1 and the endothelin receptors were found in a mouse model of pronounced reduction of nephron number (−88%) (13). In rats after surgically reduced renal mass, an increased renal endothelin production is well known (6). However, in our study in young GDNF+/− no differences in the renal gene expression of ET-1 and its receptors were found.

Glomerular cellularity, capillarization, and ultrastructure had not been addressed in detail in GDNF+/− mice previously. We therefore analyzed semithin and ultrathin sections and found a significantly lower glomerular capillary density in GDNF+/−, i.e., capillary length per glomerular volume was markedly reduced. This finding is important since it could lead to a higher capillary pressure, which then leads to glomerular and over time to systemic hypertension. In addition, we found early thickening of the GBM in the enlarged glomeruli of GDNF+/− mice compared with wild-type control mice, possibly also indicating a maladaptive process. This is of interest, since a thickened GBM was recently also described in Br/+ mice with deficiency of Sist2 and severely reduced nephron number (−88%), impaired renal function, and hyper-

### Table 4. Intrarenal arteries and peritubular capillaries

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n = 10)</th>
<th>GDNF+/− (n = 7)</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall thickness of intrarenal arteries, μm</td>
<td>17.0 ± 2.4</td>
<td>15.5 ± 1.5</td>
<td>ns</td>
</tr>
<tr>
<td>Wall area of intrarenal arteries, μm²</td>
<td>7,809 ± 1460</td>
<td>6,220 ± 675</td>
<td>ns</td>
</tr>
<tr>
<td>QA, mm²/mm³</td>
<td>3.29 ± 0.6</td>
<td>3.74 ± 0.6</td>
<td>ns</td>
</tr>
<tr>
<td>LV, mm²/mm³</td>
<td>9.73 ± 2.2</td>
<td>12.2 ± 2.7</td>
<td>ns</td>
</tr>
<tr>
<td>Total length of intrarenal arteries per kidney, mm</td>
<td>1,758 ± 538</td>
<td>2,237 ± 478</td>
<td>ns</td>
</tr>
<tr>
<td>No. of peritubular capillaries per cortical area, mm⁻²</td>
<td>1,076 ± 61</td>
<td>1,035 ± 37</td>
<td>ns</td>
</tr>
<tr>
<td>No. of peritubular capillaries per medullary area, mm⁻²</td>
<td>2,378 ± 361</td>
<td>3,224 ± 223</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are means ± SE. QA, number of intrarenal arteries per area; LV, length density of intrarenal arteries.
tension (13). In parallel with the increase in glomerular volume there was a higher number of mesangial and endothelial cells per glomerulus with the cell density being unchanged and also a higher number of PCNA-positive glomerular cells. In contrast, podocyte number per area was significantly reduced in GDNF+/−/− mice compared with control (30%) and podocytes were somewhat larger in size. These alterations were not described previously in this model of genetically determined reduced nephron number but are comparable with results in α5-integrin-deficient mice, in which an ~50% nephron reduction together with a significant increase in the number of mesangial cells, but not of endothelial cells and podocytes, per glomerulus was found (18). Interestingly, in the same study after uninephrectomy of wild-type mice there was a comparable glomerular hypertrophy without any increase in mesangial cells (18), indicating a direct role of low nephron number on proliferation of mesangial cells. A reduced podocyte density as seen in our study in GDNF+/− is of special relevance because...
podocytes are one of the main target cells for renal injury and it has been proposed that in larger glomeruli the area that has to be covered by each podocyte is increased and thereby the podocyte has no longer sufficient preventive qualities (17). In an IUGR (low protein, rat) model with 20% reduced nephron number, the average area covered by one single podocyte was greater in former IUGR diabetic rats than in control diabetic rats, and the authors even found a correlation between podocyte density and albuminuria in former IUGR rats (26). When analyzing podocyte markers in young GDNF+/− mice, we found a comparable number of WT-1- and nephrin-positive cells, whereas, interestingly, a slightly higher desmin positivity was seen in GDNF+/− in wild-type control mice, pointing to mild podocyte damage.

In an interesting experimental study on the correlation of podocyte number and glomerulosclerosis, Wharram et al. (46) analyzed the effect of graduated podocyte loss on the development of proteinuria and renal morphological changes in rats expressing the human diptheria toxin receptor in podocytes, which induces a dose-dependent podocyte loss. Podocyte loss up to 20% resulted in only transient proteinuria and mild mesangial expansion, whereas loss of 20−40% induced focal segmental glomerulosclerosis (FSGS) lesions with mild proteinuria but normal kidney function. Only podocyte loss of >40% induced FSGS and reduced renal function. Since in our study the number of podocytes per area was reduced by ~30%, a similar pathomechanism seems possible, although no proteinuria has been established yet.

In summary, in 26-wk-old prehypertensive GDNF+/− mice with 33% lower nephron number we found no postglomerular structural changes or alterations in the expression of prohypertensive systems, but we detected early cellular and capillary alterations in enlarged glomeruli as well as marked thickening of the GBM in GDNF+/− mice that could provide a predisposition for glomerular injury and hypertension in lower nephron number.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

GLOMERULAR ALTERATIONS IN NEPHRON UNDERDOSING


