TGF-α Mediates Genetic Susceptibility to Chronic Kidney Disease

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ABSTRACT

The mechanisms of progression of chronic kidney disease (CKD) are poorly understood. Epidemiologic studies suggest a strong genetic component, but the genes that contribute to the onset and progression of CKD are largely unknown. Here, we applied an experimental model of CKD (75% excision of total renal mass) to six different strains of mice and found that only the FVB/N strain developed renal lesions. We performed a genome-scan analysis in mice generated by back-crossing resistant and sensitive strains; we identified a major susceptibility locus (Ckdp1) on chromosome 6, which corresponds to regions on human chromosome 2 and 3 that link with CKD progression. In silico analysis revealed that the locus includes the gene encoding the EGF receptor (EGFR) ligand TGF-α. TGF-α protein levels markedly increased after nephron reduction exclusively in FVB/N mice, and this increase preceded the development of renal lesions. Furthermore, pharmacologic inhibition of EGFR prevented the development of renal lesions in the sensitive FVB/N strain. These data suggest that variable TGF-α expression may explain, in part, the genetic susceptibility to CKD progression. EGFR inhibition may be a therapeutic strategy to counteract the genetic predisposition to CKD.


Human chronic kidney diseases (CKD), regardless of their etiology, are characterized by progressive destruction of the renal parenchyma and loss of functional nephrons, leading to ESRD. Approximately 13% of adults suffer from CKD in industrialized countries and the incidence of ESRD increases by 6% to 8% per year. Therefore, understanding the pathophysiology of CKD is a key challenge for public health.

The mechanisms of CKD progression are poorly understood. Although clinical studies point to the important role of environmental factors in the biologic processes leading to renal deterioration, epidemiologic studies have underscored the importance of genetic components. Indeed, it has been observed that the evolution of CKD varies considerably among individual patients exposed to the same risk factors. Only a proportion of patients with diabetes or hypertension develop renal failure, and this occurs independently of glycemic control or hypertension.1,2 However, the propensity to develop ESRD differs among ethnic groups3–7 and it shows familial clustering.7–10 Similarly, the rate of progression of primary hereditary kidney diseases can vary among members of the same family,11–13 suggesting that genes unrelated to the disease

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(modifiers) might account for the susceptibility to develop ESRD.

Although many studies have sought to discover the modifiers of CKD progression, the gene variants that predispose individuals to ESRD remain largely unknown. The genetic complexity of human populations and the difficulty of standardizing analyses of environmental factors in complex diseases have hampered the identification of these crucial modifiers. Efforts to discover novel modifiers must, therefore, include experimental models.14 Numerous animal models have been developed to elucidate the pathophysiology of CKD. Among these, the remnant kidney model is a mainstay because nephron reduction characterizes the evolution of most human CKD. Consequently, this model recapitulates many features of human CKD, including hypertension, proteinuria, and glomerular and tubulointerstitial lesions. Over the past 50 years, this model has led to the discovery of critical pathways and, more importantly, to the design of therapeutic strategies to slow down CKD progression, that is, the widely clinically used renin-angiotensin system inhibitors.15 More recently, studies in different mouse strains have highlighted the importance of genetic factors. In fact, it has been shown that whereas nephron reduction induces early and severe pathologic lesions in ROP mice, other strains, for example, C57BL/6 or C3H, are resistant to early renal deterioration.16–19 Similarly, we showed that the course and extent of renal lesions after 75% surgical excision of renal mass vary significantly between two mouse strains: whereas the FVB/N mice develop renal lesions, the (C57BL/6xDBA/2)F1 are resistant to early deterioration.20

Here, we combined this experimental model of CKD, experimental crosses, and a whole genome scan to identify a locus that confers increased susceptibility to lesion development in FVB/N mice. Furthermore, we provide evidence that TGF-α, a gene of the locus that codes for a ligand of epidermal growth factor receptor (EGFR), is critically involved in the genetic predisposition to CKD progression.

RESULTS

Genetic Susceptibility To Develop Renal Lesion after Nephron Reduction

To understand how genetic background influences the progression of CKD, we performed 75% reduction of renal mass (Nx) in four different mouse strains and two F1 hybrids and examined remnant kidneys at 2, 4, and 6 months after surgery. Only one strain, FVB/N, displayed severe renal lesions 2 months after Nx. The most striking feature was diffuse tubular lesions consisting mostly of dilations with microcyst formation obliterated by protein casts and focal area of tubular dedifferentiation. They were associated with glomerular lesions characterized by diffuse mesangial sclerosis with segmental/global hyalinization or scarring of the tuft in 50% to 70% of glomeruli. Moreover, mild fibrosis and multifocal mononuclear cell infiltration were observed in the interstitium (Figure 1A). In contrast, C57BL/6, DBA/2, 129S2/Sv, (C57BL/6xDBA/2)F1 (hereafter denoted B6D2F1), and (C57BL/6xSJL)F1 showed only mild glomerular and tubular hypertrophy (Figure 1A). Neither glomerulosclerosis nor tubulointerstitial lesions could be detected up to 6 months after Nx except in a narrow scarring area originating from the surgical excision of the two poles.

Development of renal lesions in FVB/N mice resulted in severe proteinuria (26.8 ± 5.3 mg/d versus 0.9 ± 0.1 mg/d in Nx and control mice, respectively; P < 0.001) and a moderate increase in mean arterial BP (145 ± 8 mmHg versus 116 ± 5 mmHg in Nx and control mice, respectively; P < 0.05). In resistant B6D2F1 mice, proteinuria could not be detected up to 2 months after Nx, whereas moderate hypertension developed (139 ± 5 mmHg). Disease progression was rapid in FVB/N mice with complete destruction of the remnant kidney and ESRD that led to a 50% survival rate at 3 months. The clear bimodal phenotype among the susceptible and resistant strains provides an experimental framework to identify modifiers of CKD progression.

Inheritance Pattern of Renal Deterioration

To determine the inheritance pattern of lesion susceptibility, we performed reciprocal intercrosses between FVB/N and B6D2F1 mice and analyzed the kidneys of their progeny (hereafter denoted G1) 2 months after Nx. Remnant kidneys of G1 progeny were severely damaged, similarly to those of the parental FVB/N strain, but exclusively in G1 males (Figure 1B). In fact, 96% of males developed severe glomerulosclerosis, tubular dilation, and interstitial fibrosis, whereas 96% of females were resistant to lesion development. The severity and the extension of glomerular, tubular, and interstitial lesions were indistinguishable from those of the parental FVB/N strain (Figure 1). This phenotype was observed regardless of the direction of the crosses, thereby excluding a sex-linked inheritance. Complementary reciprocal crosses between either C57BL/6 and FVB/N or DBA/2 and FVB/N mice confirmed these results (data not shown). On the basis of the G1 phenotype, we hypothesized that the FVB/N variant of one or more modifier genes may confer susceptibility to develop renal lesions after Nx in a dominant fashion in males and recessively in females, although a reduced penetrance could not be formally excluded. To test this hypothesis, we backcrossed G1 females to either FVB/N or B6D2F1 males and evaluated renal morphology 2 months after Nx. Among 100 G2 progeny, the percentage of mice developing severe lesions (Figure 1B) was very close to the expected values in the two crosses (Table 1), a result consistent with a major recessive modifying locus for the lesion susceptibility trait. As in the parental strains, the renal phenotype showed a clear bimodal expression with severe glomerular and tubular lesions in the sensitive G2 mice versus mild glomerular and tubular hypertrophy in the resistant G2 animals.

Linkage Mapping of a Modifier Locus for CKD Progression

To map the locus that modifies the renal lesion phenotype, a complete genome scan was performed on females and males from G1xFVB/N and G1xB6D2F1 crosses, respectively, using...
64 informative markers distributed across the mouse genome (average spanning = 20 cM). B6 and D2 alleles were scored as non-FVB for linkage analysis. Data from the two crosses were combined and genotypic distributions were matched with the putative model for modifier inheritance. Significant linkage was found only for a locus on chromosome 6 (Figure 2A). Additional polymorphic markers confirmed significant linkage (lowest P values) to the 17-cM interval between D6Mit209 and D6Mit328 (76 to 113 Mpb) (Figure 2B). Hence, a major modifier locus accounts for CKD progression after Nx in the mouse; we have designated this locus \( Ckdp1 \).

**TGF-\( \alpha \) Is Essential for CKD Progression**

We next try to identify the gene(s) within the locus that might modify CKD progression. Inspection of the \( Ckdp1 \) 95% confidence interval for potential candidates revealed the presence of TGF-\( \alpha \), a ligand of EGFR (Supplemental Table 1). Our recent findings that TGF-\( \alpha \) is a crucial intermediate in angiotensin II–induced renal lesions \(^{21}\) prompted us to examine its expression. TGF-\( \alpha \) mRNA levels significantly increased in remnant kidneys of FVB/N mice relative to sham-operated controls, but were unchanged in B6D2F1 counterparts (Figure 3A). Similarly, TGF-\( \alpha \) protein levels markedly increased 2 months after

**Table 1.** Predicted renal lesion frequencies according to our hypothetical model (dominant allele in FVB/N males) versus the observed renal lesion frequencies of G2 male and female progeny

<table>
<thead>
<tr>
<th>Cross</th>
<th>G1 Female × FVB/N Male</th>
<th>G1 Female × B6D2F1 Male</th>
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<tbody>
<tr>
<td></td>
<td>G2 Male (n = 12)</td>
<td>G2 Female (n = 34)</td>
</tr>
<tr>
<td>Predicted lesions</td>
<td>12 (100%)</td>
<td>17 (50%)</td>
</tr>
<tr>
<td>Observed lesions</td>
<td>11 (92%)</td>
<td>20 (59%)</td>
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G2 mice result from G1 females crossed to either FVB/N or B6D2F1 males. \( n \) = number of animals.
Nx, but exclusively in FVB/N mice (Figure 3B). Immunohis- tochemistry confirmed the increase of TGF-β/H9251 expression (Figure 3C) and showed that TGF-β/H9251 protein is mainly located in ascending loops of Henle and distal tubules. A careful time course analysis revealed that the increase of TGF-β/H9251 preceded the development of renal lesions 6 weeks after Nx (Figure 3D), suggesting that this molecule may be critically involved in the genetic predisposition of FVB/N mice to develop renal lesions.

To test this hypothesis, we treated FVB/N mice with the selective EGFR tyrosine kinase inhibitor, Iressa, for 2 months after Nx. Interestingly, daily administration of Iressa markedly protected remnant kidneys from the deterioration process. In fact, the severity of glomerulosclerosis, tubular dilation, and interstitial fibrosis were markedly reduced in Iressa-treated mice as compared with that in vehicle-treated animals (Figure 4A). Proteinuria was also significantly decreased (Figure 4B), whereas hypertension was unaffected (Figure 4C).

**TGF-α Gene Is Not Mutated in FVB/N Strain**

We next sequenced TGF-α cDNA derived from the three parental C57BL/6, DBA/2, and FVB/N strains. Compared with C57BL/6 sequence (http://www.ensembl.org), alignment of TGF-α coding sequence revealed only a synonymous single-nucleotide polymorphism (SNP) 81A→G in FVB/N and DBA/2 strains and several 3′UTR SNPs (538A→G, 1031A→G, 1316T→C, 2583A→G, 3186G→A) and a polyA 3-bp insertion in the FVB/N and DBA/2 strains. However, all these substitutions are silent mutations. We next screened for regulatory transcriptional mutations in the TGF-α gene using a RNA PCR strategy to detect potential allelic differences in expression of the cognate mRNAs and taking advantage of the neutral polyA 3-bp insertion noted above. To compare relative expression levels of the TGF-α allele in FVB/N and C57BL/6 mice, RNA PCR products spanning polymorphic nucleotides were amplified from total RNA prepared from remnant kidneys of G2 mice. TGF-α PCR products showed equivalent expression from both FVB/N and C57BL/6 allele (FVB/N–C57BL/6 ratio: 0.992 ± 0.017, n = 8). Collectively, these results argue against a cis-acting mutation in TGF-α gene and indicate that another gene within the locus accounts for lesion susceptibility in FVB/N mice by modulating TGF-α expression.

**DISCUSSION**

The genetic factors that control progression to ESRD are largely unknown. By applying genetic and molecular approaches to a CKD experimental model in six strains of mice, we have identified a locus on mouse chromosome 6 that is significantly linked to the evolution of CKD. More importantly, we have discovered that TGF-α, a gene of the locus, is crucially involved in the susceptibility to CKD progression. In fact, pharmacologic inhibition of TGF-α receptor prevented lesion development in the sensitive FVB/N strain, consistent with TGF-α overexpression. However, no mutation could be detected in the TGF-α gene. Collectively, these results identify a new locus linked to CKD progression and strongly suggest that genetic variants that control TGF-α expression are potential modifiers of CKD.

The experimental model of nephron reduction has been widely used to elucidate the mechanisms underlying the progression of CKD. In this study, we used this model to identify candidates that confer susceptibility to CKD progression. Interestingly, we showed that among six different strains of mice, only one, the FVB/N, develops severe renal lesions, whereas the other ones are resistant to early deterioration. This observation is consistent with previous works showing that C57BL/6 mice are particularly resistant to proteinuria and glomerulosclerosis after Nx. That 129/Sv mice are resistant to renal deterior-
surgery. (B) Western blot analysis of TGF-β6D2F1 and FVB/N mice, evaluated by ribonuclease protection assay 2 months after surgery. Because no differences were detected among FVB/N control mice at different time points, only one group is shown. PAS staining, magnification, ×200. Data are means ± SEM; n = 6 for each experimental group. Statistical analysis: ANOVA, followed by Tukey-Kramer test: Nx versus control: *P < 0.05; FVB/N versus B6D2F1 mouse: #P < 0.05.

Figure 3. TGF-α expression increases before the development of renal lesions in FVB/N mice. (A) TGF-α mRNA expression in control (C) and 75% nephrectomized (Nx) B6D2F1 and FVB/N mice, evaluated by ribonuclease protection assay 2 months after surgery. (B) Western blot analysis of TGF-α expression in control and Nx B6D2F1 and FVB/N mice. Extracts of TGF-α−/− kidneys (KO) served as negative controls. (C) Immunostaining for TGF-α in control and Nx B6D2F1 and FVB/N mice. Because no differences were detected between B6D2F1 and FVB/N control mice, only one group is shown. Magnification, ×200. (D) Western blot analysis of TGF-α (upper panel) and renal morphology (lower panel) in control and Nx FVB/N mice 4, 6, and 8 weeks after surgery. Because no differences were detected among FVB/N control mice at different time points, only one group is shown. PAS staining, magnification, ×200. Of note, several studies have suggested evidence of linkage to chromosome 3 also in type 2 diabetic nephropathy.26,29

Our genome-wide scan analysis revealed a major suggestive locus on chromosome 6 that is associated with the development of renal lesions after Nx.18,19 However, in both studies, the percentage of Nx was more severe and the follow-up longer than that in our work, suggesting that the difference in the model used may account for this discrepancy. Supporting this idea, we previously observed that mild lesions appear also in the resistant B6D2F1 strain 8 months after Nx.22 It is worthy to note that the FVB/N genetic background has been reported to be highly permissive to the onset of renal lesions in other experimental models of CKD.23,24

Our genome-wide scan analysis revealed a major suggestive locus on chromosome 6 that is associated with the development of renal lesions after Nx. Interestingly, the interval containing the locus is orthologous to the rat chromosome 4 D4Rat95 marker region. This marker has shown significant linkage to urinary albumin excretion in Munich Wistar Frömter rats,25 a model of progressive CKD. More importantly, our Ckdp1 locus perfectly overlaps with previous reported QTLs in humans. In particular, the locus is orthologous to the human chromosome 2p12-p13 region, where two recent genomewide association studies have identified common variants (rs10206899) associated with renal function and CKD progression in several thousands of patients.26,27 In addition, the Ckdp1 locus is syntenic to the human chromosome 3p14–12 and 3q21–22 regions, which have been linked to proteinuria in patients with type 1 diabetic nephropathy.26,29

On the basis of available mouse genome database (http://www.ensembl.org), we established that 400 annotated genes were encoded within the candidate Ckdp1 interval. Inspection for potential candidates revealed that the locus includes the gene encoding TGF-α, a ligand of EGFR. Interestingly, previous studies highlighted the key role of EGFR activation in CKD.31 Evidence presented here points to TGF-α as the key mediator of the genetic predisposition to CKD progression. In fact, we demonstrated that inhibition of EGFR prevented renal deterioration in the lesion-prone FVB/N strain. Consistently, we observed that TGF-α expression increased in FVB/N mice and, more importantly, the increase preceded the development of renal lesions. The increase of TGF-α was specific because expression of EGF, the principal EGFR ligand synthesized in renal cells, was unchanged by either genetic background or Nx (data not shown). It is worthy to note that transgenic mice overexpressing TGF-α develop severe renal lesions,32,33 whereas transgenic mice broadly expressing EGF do not.34 Hence, it is tempting to speculate that genes encoding molecules involved in regulation of TGF-α are potential modifiers of CKD progression. Supporting this idea, we previously showed that inactivation of JunD, a partner of the AP1 transcription complex, promotes lesion development upon Nx in resistant strains by stimulating the expression of TGF-α.35 Notably, ontology searches for annotated genes within the Ckdp1 locus revealed 23 genes encoding for transcription factors.
However, genetic variants in molecules involved in the post-transcriptional control of TGF-α expression could also act as candidate modifiers. In fact, a complex network of proteins controls the amount of TGF-α in the urine.36,37 Interestingly, we previously showed that angiotensin II causes TGF-α overexpression by favoring the redistribution of its sheddase to the apical membrane.21 Although none of these genes have been shown to modulate the evolution of CKD, further studies are required to determine whether one of them may account for the increased susceptibility to develop renal lesions.

Haplotyping analysis is a powerful tool to identify potential candidates in a chromosomal region. Hence, we applied this strategy to refine the region and identify genes that contain informative SNPs between FVB/N, C57BL/6, and DBA/2. We found 125,294 SNPs within the critical region, among which 9668 are polymorphic between FVB/N and both C57BL/6 and DBA/2. Of these SNPs, 3904 are located within 104 annotated genes. Thirteen genes harbor nonsynonymous coding polymorphisms, among which 10 are expressed in adult mouse kidney (Supplemental Table 1). Notably, one of these potential candidates, Tprkb, has been implicated in human CKD progression by the genomewide association studies mentioned above. This gene encodes for the p53-related protein-kinase-binding protein and is of unknown function. Although we cannot rule out the possibility that additional genes are involved because the genome of FVB/N strain has not been completely sequenced, this bioinformatics analysis provides an initial selection from 400 genes in the initial Ckdp1 interval to a few potential candidates. The functional analysis of the genetic variants will determine if one of them is the modifier of CKD progression in FVB/N mice.

In this study, G1 males and females exhibited a significant sexual dimorphism in susceptibility to lesion development after Nx. That this dimorphism was displayed in reciprocal crosses excludes a direct contribution of the X or Y chromosome to these findings. Several experimental and clinical studies have shown that gender affects the incidence, prevalence and progression of CKD.38 In particular, men are more prone to progressive renal diseases.39 It has been suggested that this disparity may stem from sex hormones,40 but the molecular pathways are not clearly identified. Although elucidating these pathways was beyond the scope of this work, preliminary results suggest that the EGFR pathway might be involved in this sexual dimorphism.

In conclusion, our study identifies a novel molecular pathway critically involved in the genetic predisposition to develop renal lesions after Nx. It also suggests that genes that encode for molecules susceptible to modulate the activation of EGFR pathway could be putative modifiers of CKD progression. More importantly, we have identified a novel therapeutic strategy to counteract the genetic predisposition to CKD progression.

CONCISE METHODS

Animals

Mice used for these studies were FVB/N, C57BL/6, DBA/2, 129S2/Sv, (C57BL/6xDBA/2)F1 (denoted B6D2F1) (Charles River), and (C57BL/6xSJL)F1 (denoted B6SJLF1) (Janvier). The parental FVB/N and B6D2F1 strains were mated to produce the reciprocal (FVB/NxB6D2F1)G1 progeny. The G1 females were crossed to either FVB/N or B6D2F1 males to obtain the G2 progeny. All experiments were performed on 9-week-old mice, fed ad libitum, and housed at constant ambient temperature in a 12-hour light cycle. Animal procedures were conducted in accordance with French government policies (Ministère de l’Agriculture).

Experimental Protocol

Mice were subjected to subtotal nephrectomy (Nx) which consists of the excision of the right kidney and the two poles of the left kidney to achieve 75% reduction of total renal mass. Control mice were subjected to a sham operation with decapsulation of both kidneys. After surgery, mice were fed a defined diet containing 30% casein and 0.5% sodium.

Several groups of mice were investigated in complementary studies. For strain susceptibility studies, 18 and 32 females for each strain were subjected to either sham operation or Nx, respectively. For renal

Figure 4. Pharmacologic inhibition of EGFR prevents lesion development in FVB/N mice. (A) Renal morphology in control (C) and 75% nephrectomized (Nx) FVB/N mice treated with either the pharmacologic inhibitor of EGFR, Iressa, or the vehicle, 2 months after surgery. Because no differences were detected between Iressa- and vehicle-treated control mice, only one group is shown. PAS staining, magnification, ×200. (B and C): Proteinuria (B) and BP (C) in control (white bars) and Nx (black bars) mice treated with either the vehicle or Iressa, 2 months after surgery. Data are means ± SEM; n = 6 for each experimental group. Statistical analysis: Nx versus control: *P < 0.05, **P < 0.01, ***P < 0.001; Iressa versus vehicle: ##P < 0.01 (ANOVA, followed by Tukey-Kramer test).
function and expression studies, additional 6 control and 6 Nx mice for each FVB/N and B6D2F1 strain were studied. For G1 progeny studies, 49 females and 40 males were subjected to Nx. For G2 progeny studies, 12 and 25 males and 34 and 29 females from G1xFVB/N and G1xB6D2F1 crosses, respectively, were investigated after Nx. For pharmacologic EGFR inhibition, 24 FVB/N mice were divided into 4 groups of 6 mice: (i) sham operation + EGFR inhibitor Iressa (100 mg/kg per d by daily gavages; a gift from AstraZeneca); (ii) sham operation + vehicle of Iressa (1% NaCl, 0.5% hydroxypropylmethylcellulose, 1% Tween); (iii) Nx + Iressa; and (iv) Nx + vehicle of Iressa.

Mice were sacrificed 2 months after surgery. In addition, for the strain susceptibility studies, mice were also sacrificed 4 and 6 months after surgery, whereas for the TGF-α time course study mice were also sacrificed at 4 and 6 weeks after surgery. BP was recorded 1 week before sacrifice in awake mice (n = 6 for each experimental group) for 4 consecutive days, using tail-cuff plethysmography and PowerLab/4SP software (AD Instruments). Urine samples were collected from the same animals, using metabolic cages during the 24 hours before sacrifice. At the time of sacrifice, the kidney was removed for morphologic, protein, and mRNA studies. In addition, on G2 progeny, mice were sacrificed at 4 and 6 weeks after surgery. BP was recorded 1 week after surgery, whereas for the TGF-α inhibition, 24 FVB/N mice were divided into 4 groups of 6 mice: (i) sham operation + EGFR inhibitor Iressa (100 mg/kg per d by daily gavages; a gift from AstraZeneca); (ii) sham operation + vehicle of Iressa (1% NaCl, 0.5% hydroxypropylmethylcellulose, 1% Tween); (iii) Nx + Iressa; and (iv) Nx + vehicle of Iressa.

Renal Function
Proteinuria was measured using an Olympus multiparametric autoanalyzer (Instrumentation Laboratory).

Renal Morphology
Kidneys were fixed in 4% paraformaldehyde and paraffin-embedded, and 4-μm sections were stained with periodic acid–Schiff (PAS), Masson trichrome, hematoxylin and eosin, or Picro-Sirius Red. A pathologist examined and evaluated all the sections in a blinded fashion. Twenty randomly selected microscopic fields of the cortex were studied (×200). The severity of renal lesions was evaluated using a Nikon digital camera Dxm/m/1200 and Lucia software (Laboratory Imaging Ltd.), as described previously.35

Genome Scan
Genomic DNA was purified from mouse livers with Qiagen DNeasy kit. Forty-six microsatellite markers were selected to span each autosomal and X chromosome with an average distance between markers of 19.7 cM. The criterion for marker selection was their informativeness to differentiate FVB/N from C57BL/6 and DBA/2 alleles (http://www.cird.jhmi.edu/mouse). When the initial genome scan identified a single locus on chromosome 6, additional microsatellite markers or SNPs (http://www.informatics.jax.org) were performed along the chromosome. Microsatellites were amplified by PCR on a Biometra Uno II thermocycler. The PCR products were analyzed either on 3% agarose gel or by electrophoresis on 4% polyacrylamide, 7 M urea sequencing gel on an ALF Pharmacia Sequencer, or on ABI PRISM 3100 genetic analyzer. SNPs were amplified by PCR on an Eppendorf Mastercycler and analyzed on a Biotage pyrosequencer PSQ96MA.

Linkage Analysis
Because all C57BL/6, DBA/2, and B6D2F1 mice are resistant to lesion development after Nx, we hypothesized that all three strains are homozygous for “resistant” alleles. We pursued the study with B6D2F1 mice and scored B6 and D2 alleles as non-FVB for linkage analysis. Linkage was evaluated in females and males from G1xFVB and G1xB6D2F1 crosses, respectively, with the assumption that, in these mice, a marker closely linked to the putative modifier should be (i) homozygous for the FVB alleles in lesion-prone females and heterozygous in the resistant ones and, conversely, (ii) homozygous for non-FVB alleles in resistant males and heterozygous in the lesion-prone ones. For each marker, linkage was estimated by the Fischer exact test for the fit for the expected renal phenotype (lesions versus no lesions), using Prism Software. Linkage was considered significant when the P values were <0.05. Additional genotyping was performed for the interval that showed linkage P < 0.05. The 95% confidence interval was calculated on the basis of the Bayesian credible interval method (R/QTl).

Gene Sequencing
TGF-α cDNA (primers in 5’ and 3’ UTR sequences) was sequenced in the three parental FVB/N, DBA/2, and C57BL/6 strain. To test the relative TGF-α allele expression, cDNA was reverse-transcribed from kidney RNA of selected G2 mice that brought heterozygous FVB/N alleles (n = 8), then amplified using primers designed around the 3-bp polyA insertion (detected by sequencing in FVB/N and DBA/2 as compared with the C57BL/6 mouse strain), and quantified using an ABI PRISM 3100 genetic analyzer.

Ribonuclease Protection Assay and Real-Time Reverse-Transcription PCR
TGF-α mRNA levels were quantified by a ribonuclease protection assay as described.35 GAPDH was used as the normalization control.

Immunohistochemistry
TGF-α immunostaining on mouse kidney was performed as described.35 The mean extent and intensity of TGF-α staining was quantified with a Nikon digital camera Dxm/m/1200 and Lucia Software in 10 selected microscopic fields of the cortex (×200).

Western Blot Analysis
TGF-α immunoblotting of mouse kidneys was performed as described previously using a rabbit anti–TGF-α antibody (Abcam) diluted 1:500, followed by a donkey horseradish peroxidase-linked secondary antibody (Amersham Pharmacia, Biotech) diluted 1:5000.20 Mouse monoclonal anti–α-tubulin antibodies (Sigma-Aldrich) were used as controls. Protein extracts from kidneys of TGF-α−/− mice were used to confirm antibody specificity.

Data and Statistical Analyses
Data are expressed as means ± SEM. Differences between the experimental groups were evaluated using ANOVA, followed when significant (P < 0.05) by the Tukey-Kramer test. When only two groups were compared, the Mann-Whitney test was used.
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DISCLOSURES

None.

REFERENCES


See related editorial, “Identification of a Major Chronic Renal Failure Susceptibility Locus in Mice: Perhaps EGFR Determines What Happens to eGFR,” on pages 201–203

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