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Vascular Damage without Hypertension in Transgenic Rats Expressing Prorenin Exclusively in the Liver

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Abstract

We have developed a transgenic animal model to investigate the effects of overexpression of rat prorenin on the cardiovascular system. Two transgenic rat lines were generated in which rat prorenin expression was directed to the liver by a human α1-antitrypsin promoter. Liver-specific expression was confirmed by RNase protection assay. Plasma prorenin concentrations in transgenic rats were increased 400-fold in the males of both lines but were increased only two- to threefold in the females. Thus, transgene expression exhibited sexual dimorphism. Blood pressures were not significantly higher in transgenic rats than in nontransgenic controls. The ratio of heart weight to body weight was greater in male transgenic rats than in the nontransgenic controls. Histological analysis revealed severe renal lesions and hypertrophic cardiomyocytes in transgenic males only. This transgenic model demonstrates a likely role of prorenin in the development of cardiac and renal pathology independent of hypertension. These animals will facilitate studies of the effects of blockade of the renin-angiotensin system and other pharmacological interventions on the development and treatment of cardiac, vascular, and renal lesions induced by changes in this system in the absence of chronic hypertension. (*J. Clin. Invest.* 1996. 98:1966–1970.) Key words: blood pressure • renin-angiotensin system • plasma renin concentration • left ventricular hypertrophy • nephroangiosclerosis

Introduction

Prorenin is the biosynthetic precursor of renin, a key regulatory enzyme of the renin-angiotensin system (RAS).[1] Prorenin was initially detected in amniotic fluid and then in the plasma, where it can be processed into renin after cryoactivation, acid activation, or proteolytic activation (1–3). The cleavage site is located at the amino terminus of prorenin, and renin is released as a 43–amino acid species-specific peptide (4, 5). Juxtaglomerular cells of rat and human kidneys constitute the main site of prorenin synthesis and the exclusive site of its intracellular processing to active renin. Prorenin, which represents up to 90% of the total renin in human plasma, appears to originate from sources other than kidney, since it is still present in plasma after bilateral nephrectomy (6). In addition to serving as a marker for renin biosynthesis, prorenin may increase blood pressure either directly (7), reversibly by activation to renin (8), or by contributing to local RAS activity (9, 10). Alternatively, prorenin may exert a vasodilator effect (11) by limiting the effects of renin. However, the roles of prorenin in physiology and pathology are still unknown.

By targeting expression of the rat prorenin gene to the liver under the control of the human α1-antitrypsin (hAT) promoter (12), we have generated two lines of transgenic rats [TGR(hAT-rpR)]. Cardiovascular lesions were induced in the presence of an excess of circulating prorenin of hepatic origin and in the absence of high blood pressure. These observations strongly suggest RAS and, in particular, prorenin as independent cardiovascular risk factors.

Methods

Transgene construction, DNA preparation, and generation of the transgenic rats. RNA was extracted from Fisher rat kidney with the standard RNAzol protocol (Biotecx Laboratories, Houston, TX). cDNA was obtained by RT-PCR with two rat prorenin-specific primers (Fig. 1). Transgenic rats were generated by pronuclear microinjection of fertilized Fisher F344 rat eggs, which were reimplanted into pseudopregnant foster mothers. Six foster mothers carried 31 pups to term, three of which carried the transgene as demonstrated by PCR and Southern blot analysis of DNA obtained from tail biopsies. Two of these founders transmitted the transgene to their progeny, and the transgenic lines TGR(hAT-rpR) 85-26 and 85-41 were established. In these two lines, segregation of the transgene was consistent with a single insertion site in the genome (confirmed by Southern analysis, data not shown).

Expression of the transgene. RNase protection assays used ^{32}P-labeled RNA prepared by transcription of a 676-nucleotide antisense RNA from the plasmid pBα1-AT-ratRen (after EcoRI digestion) with T7 RNA polymerase. Total mRNA was isolated from adrenal glands, brain, heart, intestine, kidney, lung, muscle, spleen, submaxillary gland, testis, and ovary by homogenization in guanidine isothiocyanate as described (13).

Characterization of phenotype. Phenotype characterization was performed on transgenic TGR(hAT-rpR) 85-26 and 85-41 rats hemi-

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1. *Abbreviations used in this paper:* hAT, human α1-antitrypsin; RAS, renin-angiotensin system; TGR(hAT-rpR), (human α1-antitrypsin-rat prorenin) transgenic rat.

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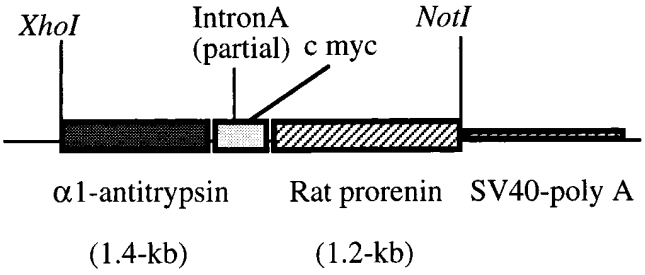

Figure 1. cDNA was obtained by RT-PCR with two rat prorenin-specific primers (synthesized by Oswell DNA Services, Edinburgh, United Kingdom): upstream primer 5′ATATTGTCGACCAGATGGGCGGGAGGAGG3′ and downstream primer 5′ATAATAAGCTTGGCCTAAAACTAGGGTC3′. SalI and HindIII sites were added to 5′ and 3′ ends of rat renin cDNA by the PCR. The resulting amplification product was a 1.2-kb cDNA fragment. Amplification was performed over 30 cycles with 1 min of denaturation at 94°C, 2 min of annealing at 64°C, and 3 min of extension at 72°C. The first untranslated exon and half of the intron were fused to a *c-myc* 3′ splice acceptor (35) and added to pBluescript II SK (−) (Stratagene, La Jolla, CA) after a double digestion with EcoRI/HindIII. The α1-AT gene sequence was obtained from GenBank release 67, Accession No. K02212. The rat prorenin fragment was made cohesive at both ends and ligated to the previous plasmid after digestion with SalI and HindIII hosting the rat renin cDNA. To generate the final plasmid construct pBTGR(hAT-rpR), HindIII and XhoI sites were added to a SV40 polyA signal by PCR amplification of pRagU0.3ARXSV primers upstream 5′CCCGGGGCTCGAGACATGAT3′ and downstream 5′CACATTTGTAAACTTTTACTTGC3′ and cloned into the polylinker of the final construct.

zygous for the rat prorenin and on age- and sex-matched negative control littermates. The rats from each experimental and control group were maintained under identical conditions and had free access to normal food (CRM, SDS, Wilham, Essex, United Kingdom) and tap water.

Renin measurements. Rats were briefly anesthetized in 2% halothane in oxygen (Acoma vaporizer F, International Market Supply, Congleton, United Kingdom). Blood obtained by left ventricular puncture was immediately mixed with 2.5×10^{-3} mol/liter Ω-phenanthroline and 0.1 mol/liter EDTA (10 ml/0.1 ml of whole blood) to inhibit converting enzyme and angiotensinase activity and centrifuged at 4,000 *g* for 10 min. Plasma was stored at −70°C for subsequent assay. Prorenin and active renin were measured as described previously (14, 15). For measurement of renal and liver renin content, tissues were thawed, and renin was mechanically extracted. Protein in tissue extracts was measured according to the method of Bradford (16).

Method for indirect blood pressure measurements. Tail-cuff plethysmography (IITC Life Sciences, Diss, Norfolk, United Kingdom) was performed as described previously (15) on rats anesthetized briefly as described above. This method of indirect blood pressure measurement was validated in a previous study by a simultaneous comparison with direct blood pressure measurements (15).

Histopathology. The kidney was examined histopathologically as described previously (17). In addition, the heart was assessed for the size and number of infarctions, hypertrophied cardiomyocytes, interstitial and perivascular fibrosis, and vascular lesions as described previously (17). The liver was also examined histologically.

In situ hybridization. In situ hybridization was performed as described previously (18). Briefly, a 1.4-kb DNA probe of mouse submaxillary gland renin was used after random primer labeling (19) with d-CTP-^{35}S (Amersham, Les Ulis, France). Control procedures were performed to assess the specificity of the in situ hybridization labeling after digestion with RNase, followed by the previously described technique.

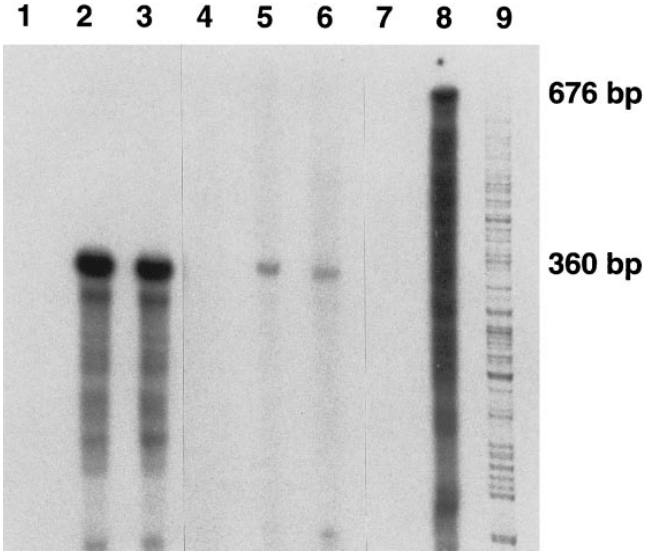

Figure 2. In the RNase protection assay for the males, 10 μg of total RNA was analyzed from nontransgenic rats (lane *1*), transgenic line 85-26 (lane *2*, protected fragment of 360 bp), and transgenic line 85-41 (lane *3*). The gel was exposed for 12 h. For the females, 30 μg of total RNA was analyzed from nontransgenic rats (lane *4*), transgenic line 85-26 (lane *5*), and transgenic line 85-41 (lane *6*). The gel was exposed for 10 d. Yeast tRNA (10 μg), the undigested 676-bp probe, and M13 A track ladder were loaded in lanes *7*, *8*, and *9*, respectively. Samples were dissolved in 30 μl of 80% formamide containing 40 mM Pipes, 400 mM NaCl, 1 mM EDTA, and 350,000 cpm of the gel-purified transcript, denatured at 95°C for 5 min and incubated at 60°C for 20 h. RNase digestion was performed in 350 μl of buffer containing 40 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO), 2 μg/ml RNase T1 (Calbiochem-Novabiochem Corp., La Jolla, CA), 1 M Tris-HCl, 0.5 M EDTA, and 5 M NaCl at room temperature for 30 min. After digestion with proteinase K for 10 min at 30°C, samples were electrophoresed on denaturing 6% polyacrylamide gels.

Statistical analysis. All data are expressed as means±SEM. Comparisons between groups were performed with one-way ANOVA.

Results

Expression of the transgene in the liver. Renin mRNA was detected in livers of transgenic rat lines 85-26 and 85-41 in both sexes but was not found in livers of age-matched negative control littermates, confirming the specificity of the riboprobe. RNase protection assays performed on RNA extracted from liver showed a protected fragment of 360 nucleotides corresponding to rat renin (Fig. 2). Phosphorimaging analysis showed 60-fold higher hepatic transgene expression in males than in females in each line. The specificity of the promoter was checked with RNase protection assays of RNA from adrenal glands, brain, heart, intestine, kidney, lung, muscle, spleen, submaxillary glands, testis, and ovary. As a positive control, liver RNA from the same animal (30 μg of total RNA) was loaded on the same gel as 120 μg of total RNA from the other tissues. After a 3-wk exposure, no expression was detected in tissues other than liver (data not shown).

Plasma prorenin, renin, and angiotensinogen measurements. Plasma prorenin was significantly higher in both lines of transgenic male rats than in the nontransgenic male controls (Table I). Plasma prorenin was also elevated in the females of

Table I. Plasma Prorenin, Active Renin, and Angiotensinogen Measurements

	n	Prorenin	PRA	Angiotensinogen
Males				
Nontransgenic	10	16.6±2.2	1.8±0.5	820.2±67.4
85-26	5	9557.4±1477.4*	2.3±0.6	778.6±17.5
85-41	9	8088.9±963.8*	3.1±0.6	905.1±37.8
Females				
Nontransgenic	8	20.6±5.9	2.5±0.6	714.0±47.7
85-26	3	67.0±8.0*	1.5±1.1	730.0±70.0
85-41	9	52.0±23.1*	2.5±0.7	626.9±24.7

Prorenin, PRA (plasma renin activity), and angiotensinogen measured in ng of angiotensin I/ml/h, $*P < 0.001$ vs. nontransgenic rats.

Table III. Systolic Blood Pressure and Heart Weight/Body Weight Ratio

	n	SBP 5	SBP 10	SBP 15	SBP 20	HW/BW
Males						
Nontransgenic	8	112±4	125±4	103±3	110±4	0.29±0.02
85-26	5	121±9	119±9	117±8	119±9	0.35±0.02*
85-41	7	116±10	133±9	123±7	125±9	0.38±0.02*
Females						
Nontransgenic	6	114±3	115±6	95±3	98±5	0.3±0.02
85-26	2	102±4	104±10	92±8	95±8	0.3±0.02
85-41	5	107±6	123±9	97±5	110±10	0.3±0.02

SBP 5, 10, 15, 20 indicate systolic blood pressure (mmHg) at 5, 10, 15, and 20 wk of age. HW/BW is heart weight to body weight ratio (×100), $*P < 0.01$ vs. nontransgenic rats.

both lines but was 150-fold lower than in the males. Plasma renin activity was not increased in either sex of transgenic rat compared with nontransgenic controls. Angiotensinogen levels were not significantly different in either transgenic line or sex compared with nontransgenic rats. Kidney renin content was significantly lower in transgenic males of both lines than in the nontransgenic males. Conversely, liver renin content was significantly increased in the transgenic rats compared with the nontransgenic controls (Table II).

Blood pressure measurement. Blood pressure was measured in males and females of both lines of transgenic rats (85-26 and 85-41) and in age- and sex-matched negative control littermates from 5 to 20 wk of age, with an interval of 5 wk between each measurement. Systolic blood pressure was significantly greater in males than in females. However, systolic blood pressure of males and females of both transgenic lines was not significantly elevated compared with that of corresponding nontransgenic controls (Table III).

Histology (Fig. 3). Histological lesions were observed exclusively in transgenic males of both lines. The renal lesions were consistent with moderate to severe nephroangiosclerosis (glomerulosclerosis, tubulointerstitial atrophy and inflammation, and arterial wall thickening). Glomerulosclerosis, ranked from mild focal to terminal obsolescent pattern stages, was observed in 26±6% of glomeruli in transgenic males whereas all the glomeruli were normal in the other groups. Cardiac damage was evidenced by hypertrophic cardiomyocytes and subendocardial and pericoronary fibrosis. Aortic wall hypertrophy was observed only in transgenic males and was measured by morphometric image analysis. The thickness of the aorta was greater in transgenic males than in nontransgenic males (0.120±0.004 vs. 0.104±0.005 mm, $P < 0.05$), no difference was observed between transgenic and nontransgenic females (0.095±0.006 and 0.099±0.006 mm, respectively).

In situ hybridization (Fig. 4). The in situ hybridization labeling index of the juxtaglomerular apparatus was lower in transgenic males than in nontransgenic males (1.5%±0.76 vs. 20%±5.0, $P < 0.0001$). Renin expression in the juxtaglomerular apparatus was normal in transgenic females and nontransgenic males and females, but was dramatically reduced in transgenic males of both lines. Transgene expression was localized only in liver cells of transgenic males and more specifically in the hepatocytes of the centrilobular zone.

Table II. Renin Activity in Male Kidney and Liver

	n	Kidney renin	Liver renin
Nontransgenic	7	2.36±0.74	0
85-26	4	0.45±0.26*	0.22±0.04*
85-41	6	0.20±0.09*	0.28±0.17*

Kidney and liver renin measured in ng of angiotensin I/mg protein/h, $*P < 0.001$ vs. nontransgenic rats.

Discussion

Experimental and epidemiological data have demonstrated an important role of the RAS in the pathogenesis of hypertension associated with cardiovascular and renal lesions (20, 21). Angiotensinogen (22), angiotensin I–converting enzyme (23), angiotensin II receptors (24), and active renin (25) are all associated with hypertension and result in vascular damage (9). The role of prorenin remains uncertain (11, 26), although elevated plasma prorenin concentrations have been implicated as a cause of microangiopathy in diabetic patients (27).

To investigate the role of RAS in the pathogenesis of hypertension, TGR (mRen2)27 transgenic rats were generated by random integration of the mouse *Ren 2* renin gene and its flanking sequences into the rat genome (28). These rats were severely hypertensive and had target organ damage. Transgene expression levels varied in different organs involved in blood pressure homeostasis, including adrenals, kidneys, brain, and blood vessel walls, and plasma prorenin was considerably increased (28–31). To further understand the involvement of the RAS in hypertension, we focused our attention on the contribution of elevated prorenin (plasma and tissue) to blood pressure elevation and on the role of extrarenal renin in blood pressure regulation. Therefore, the TGR(hAT-rpR) rat was designed to direct rat prorenin expression to the liver.

Although sexual dimorphism of prorenin levels has been detected in some strains of rats (32), the extent of sexual dimorphism in transgene expression in our study was unexpected because endogenous α1-antitrypsin expression is only severalfold higher in male rats than in females (33). Furthermore, RNase analysis showed that transgene mRNA level in

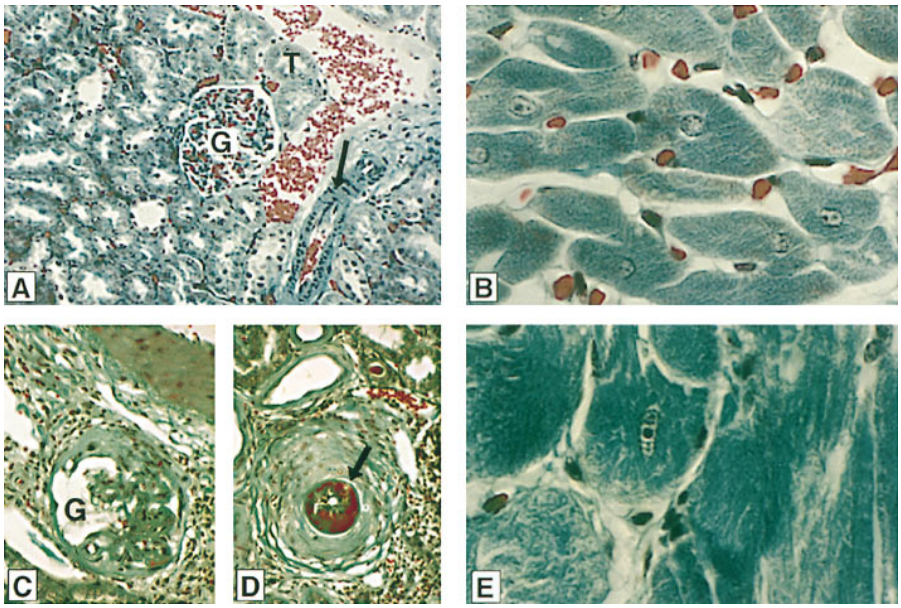

Figure 3. Histopathological analysis of kidney (*A*, *C*, *D*) and heart (*B*, *E*) from transgenic male rat (*top*) and nontransgenic male control (*bottom*). In the nontransgenic control rat, no lesions were observed in the kidney (*arrow*, interlobular artery) (*A*), and cardiomyocytes were of normal size (*B*). In the transgenic male rat, glomerulosclerosis (*C*) and a severe arterial lesion with fibrinoid necrosis (*arrow*) (*D*) were observed in the kidney, and hypertrophic cardiomyocytes were observed in myocardium (*E*). All slides were stained with Mason's trichrome stain. *G*, glomerulus; *T*, tubule. Original magnifications: *A*, *C*, *D*, ×200; *B*, *E*, ×750.

the liver of both transgenic lines was 60-fold higher in males than in females and that the plasma prorenin level was 150-fold higher in the males. This sexual dimorphism provided an internal control for the biochemical and histological characteristics of the transgenic male rats. Prorenin expression in the liver and hepatic and plasma prorenin levels were not increased in age-matched transgenic females and nontransgenic animals, and no histological abnormalities were observed in these same rats. The hAT promoter used in the rat was specific for the liver, and no other sites of expression were detected by RNase protection assays of a large range of tissues.

These transgenic rats manifest a large increase in plasma prorenin concentration as a result of constitutive prorenin secretion by the liver. Plasma renin activity (i.e., active renin concentration) in transgenic male rats is slightly elevated. This result might be explained by factors that influence renin mea-

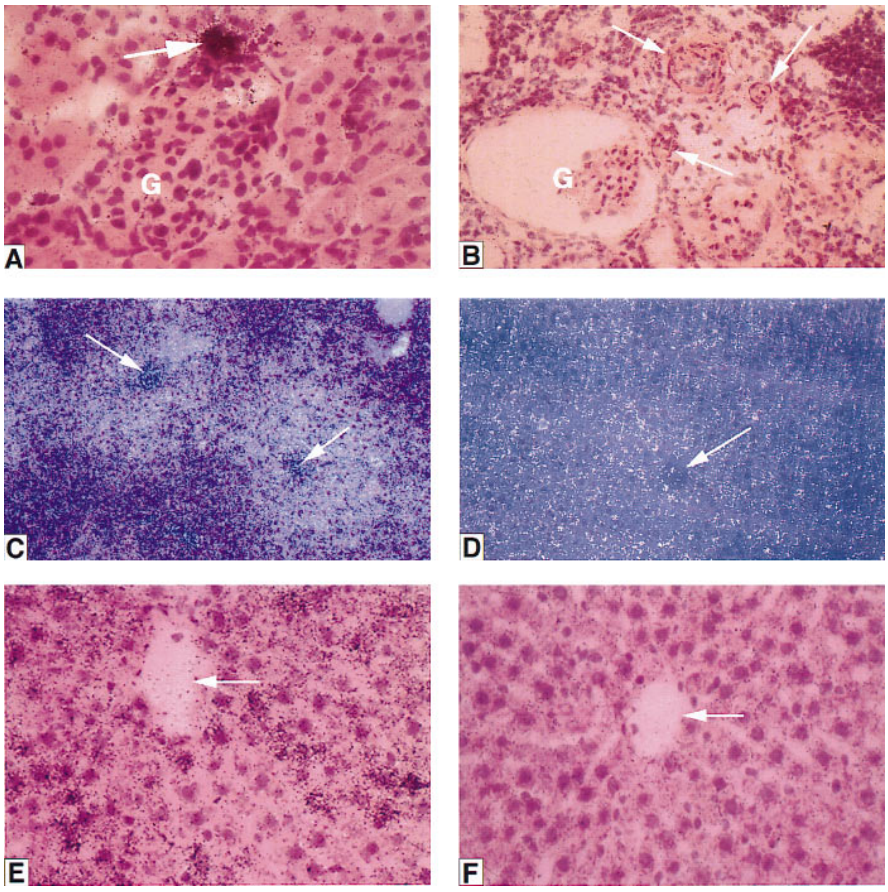

Figure 4. In situ hybridization with a 1.2-kb rat renin cDNA probe labeled with ^{35}S. Frozen sections from nontransgenic male rat kidney (*A*), transgenic male kidney (*B*), transgenic male liver (*C–E*), and nontransgenic female liver (*F*). In the nontransgenic rat (*A*), a strong renin signal was detected in the juxtaglomerular apparatus (*arrow*); original magnification, ×450. In transgenic male rats, renin expression was abolished in the juxtaglomerular apparatus and arterial walls are thickened (*arrows*) (*B*); hepatic expression of the transgene was prominent in centrilobular areas (*C*), but the specific signal was abolished in the presence of RNase (*D*) (*C* and *D*, *arrows*, hepatic vein; dark-field view, ×120); and transgene expression was localized in the hepatocytes (*arrow*, hepatic vein, ×450) (*E*). In transgenic females (*F*), no transgene expression was detected in hepatocytes (*arrow*, hepatic vein, ×450).

surements, including the method of blood sampling, the type of anesthesia used, age- and strain-dependent effects, and the method of renin assay. Therefore, we designed our study to minimize potential interferences dependent on the methodology. Plasma active renin and prorenin were measured after several precautions had been taken (15). However, a degree of in vitro cold activation probably occurred (i.e., the slightly increased level of active renin). This was also confirmed by the fact that the angiotensinogen concentration was not decreased.

Blood pressures were measured in nontransgenic and transgenic rats from either sex or line from 5–20 wk of age. The absence of hypertension in this chronic model is consistent with data from monkeys in which short-term infusion of human prorenin did not increase blood pressure or active renin concentration (6). Even if a minor increase in blood pressure was not detected by tail plethysmography, lesions with the degree of severity seen in our study have only been observed in other animal models with markedly elevated blood pressure levels. Phenotypic changes were observed in the kidney of male rats of both lines, including glomerulosclerosis, tubulointerstitial lesions, and severe vascular lesions. These renal lesions were unexpected. Our observation that renal renin content and prorenin expression were decreased supports the interpretation that local activation of prorenin generates angiotensin II (9, 34).

The TGR(hAT-rpR) rat demonstrates that long-term exposure to elevated prorenin is vasculotoxic. Additional studies will be required to characterize further this new transgenic model and, in particular, to determine the cause of the sexual dimorphism in transgene expression. This model will facilitate the study of the effects of RAS blockade and other pharmacological interventions on the development and treatment of cardiac, vascular, and renal lesions in the absence of chronic hypertension.

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