J Mol Med (2002) 80:359–366
DOI 10.1007/s00109-002-0326-6

ORIGINAL ARTICLE

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Reduced hypertension-induced end-organ damage in mice lacking cardiac and renal angiotensinogen synthesis

Received: 23 November 2001 / Accepted: 14 January 2002 / Published online: 14 March 2002

Abstract Hypertension-induced damage of kidney and heart is of major clinical relevance, but its pathophysiology is only partially understood. As there is considerable evidence for involvement of angiotensin II, we generated a new mouse model by breeding angiotensinogen (AOGEN) deficient mice with transgenic animals expressing the rat AOGEN gene only in brain and liver. This genetic manipulation overcame the hypotension of AOGEN-deficient mice and even caused hypertension indistinguishable in its extent from the parent transgenic mice with an intact endogenous AOGEN gene. In contrast to normal mice, however, crossbred animals lacked detectable expression of AOGEN in kidney and heart. As a consequence they showed markedly reduced cardiac hypertrophy and fibrosis. Furthermore, hypertension-induced alterations in kidney histology and function were less pronounced in crossbred mice than in equally hypertensive animals expressing AOGEN locally. The dysmorphogenesis observed in kidneys from AOGEN-deficient mice was absent in mice expressing this gene only in liver and brain. Our results support an important role of local AOGEN expression in hypertension-induced end-organ damage but not in the development of the kidney.

Keywords Renin-angiotensin system · Blood pressure regulation · Damage to kidney and heart

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Abbreviations *ACE:* Angiotensin-converting enzyme · *Ang II:* Angiotensin II · *ANP:* Atrial natriuretic peptide · *AOGEN:* Angiotensinogen · *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase · *RAS:* Renin-angiotensin system · *TBS:* Tris-buffered saline · *TGM123:* Angiotensinogen-overexpressing transgenic mice · *TLM:* Angiotensinogen-deficient mice · *TLM123:* Mice lacking murine angiotensinogen and overexpressing rat Angiotensinogen

Introduction

The renin-angiotensin system (RAS) plays a central role in blood pressure regulation and fluid-electrolyte homeostasis. Angiotensinogen (AOGEN) is generated mainly by the liver, secreted into the circulation, and cleaved by renin and angiotensin-converting enzyme (ACE) to yield the octapeptide angiotensin II (Ang II). Ang II exerts multiple biological functions including vasoconstriction, stimulation of aldosterone secretion, sodium retention in the kidney and intestine, and regulation of the sympathetic tone.

Cleavage of AOGEN by renin is the rate-limiting step in the cascade of Ang II production [1]. Therefore AOGEN, the only known substrate for renin, is considered to be an important molecular determinant for blood pressure in mammals including humans [2, 3, 4, 5, 6, 7, 8]. Mice lacking this protein presented drastic hypotension, pathomorphological alterations of the kidney, and reduced survival [3, 4, 5]. In the past decade the role of tissue-based RASs in cardiovascular physiology became to be realized [9]. Fulminantly hypertensive TGR(mREN2)27 rats have normal or even lower plasma AOGEN and Ang II concentrations [10] suggesting that local RASs play an important role in the development of hypertension at least in this model.

End-organ damage is the clinically most important sequel of hypertension causing cardiac and renal failure and stroke. There is accumulating evidence that Ang II contributes to the sequence of events linking high blood pressure to vascular damage, heart hypertrophy, and nephrosclerosis. ACE inhibitors effectively retard the progression of cardiac and renal insufficiency in patients with myocardial infarction, hypertension, or diabetes [11]. Moreover, even low, nondepressoric doses of Ang II receptor AT1 antagonists, or ACE or renin inhibitors blunt the pathophysiological alterations in heart and kidney of hypertensive transgenic animals [12, 13, 14]. AOGEN is synthesized locally in the heart, and its expression is augmented in pressure-overload induced hypertrophy [9]. Furthermore, the local overexpression of AOGEN or AT1A receptors in the heart of mice induces hypertrophy without hypertension, suggesting a local RAS as potent pathophysiological effector system in this organ [15, 16]. Because kidney is a major site of AOGEN expression in mammals, local Ang II synthesis may contribute to the hypertension-induced renal pathology mediated by this peptide [9, 17, 18, 19]. Furthermore, it was recently shown that mice with reduced AOGEN levels are partially protected from renal fibrosis secondary to obstructive nephropathy [20]. Nevertheless, the relative importance of local RASs, the systemic RAS, and high blood pressure in the pathogenesis of end-organ damage is still elusive.

We have previously generated transgenic mice (TGM123) overexpressing the rat AOGEN (rAOGEN) gene in liver and brain and being hypertensive [21]. The aim of our present study was to express rAOGEN tissue-specifically on the background of AOGEN-deficient mice by crossbreeding TGM123 with AOGEN-knockout mice (TLM) [3] thereby elucidating the function of tissue RASs in blood pressure regulation and hypertension-induced end-organ damage.

Materials and methods

Genotyping

Mice were bred according to the schedule shown in Fig. 1A, and littermates were used for the experiments after genotyping. The use of littermates reduced the heterogeneity of the genetic background being a mixture of NMRI [21] and C57BL/6xCBA [3].

For genotyping ear biopsy specimens of mice were digested at 37°C overnight in 80 µl of SSTE buffer (200 mM NaCl, 0.2% sodium dodecyl sulfate, 100 mM Tris-HCl, and 5 mM EDTA, pH8.5) containing 1 mg/ml proteinase K. Samples were diluted tenfold with Tris EDTA buffer (10 mM Tris-HCI, 1 mM EDTA, pH8.0) containing 20 µg/ml RNase to be used for PCR. PCR was performed in 45 µl mixture containing 1× PCR buffer (Life Technologies, Karlsruhe, Germany), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer, and 1 U *Taq* polymerase (Life Technologies) with thermal-cycles including the first denaturation at 95°C for 4 min, 30 cycles of 95°C for 45 s, 60°C for 30 s, and 72°C for 45 s and a final elongation step at 72°C for 5 min. Three pairs of primers specific for rAOGEN (RNANG5: GAGTGAGGCAAGAGGTGTAG; RNANG3: CCCAAGCTCTCAACAAATGGC), mouse AOGEN (mAOGEN) (MMANG5: TGAATGAGGCAGGAAGTGG; MMANG3: CTTGTGTCCATCTAGTCGGG), and the neomycin resistance gene indicative for the AOGEN-knockout genotype (NEOHOM5: CTGCTTGCCGAATATCATGG; MMANG3) were used to genotype the mouse progeny producing amplified bands of 318, 173, and 370 bp, respectively.

Gene expression

RNA isolation was performed using Trizol (Life Technologies) according to the manufacturers instructions. The RNA pellet was resuspended in RNase-free water and kept at –80°C until used.

For RNase-protection assays cRNA probes were generated by in vitro transcription as described previously [22]. The plasmids were pRag0.3G4 for rAOGEN [21], the vector supplied with the RPAII kit (Ambion, Austin, Tex., USA) for β-actin, and pSK-MAO1.10 for mAOGEN, a Bluescript SKII vector (Stratagene) containing a 850 bp *Eco*RI/*Bam*HI fragment covering the boundary of intron 1 and exon 2 of the mAOGEN gene and linearized with *Acc*I. RNase-protection assays were carried out using the RPAII kit (Ambion) as described [22]. The protected fragments were quantified by a phosphoimager (Fuji BAS2000).

For RT-PCR 1–5 µg RNA was pretreated with DNase (2 U/µg RNA) in 10 µl mixture containing 1× PCR buffer and 10 U RNasin at 37°C for 30 min. Reverse transcription was performed in 20 µl mixture containing 1x PCR buffer, 0.5 mM each dNTP, 0.5 µM downstream PCR primer (RNANG3 or MMANG3), 20 U RNasin and 200 U Moloney mouse leukemia virus for 40 min at

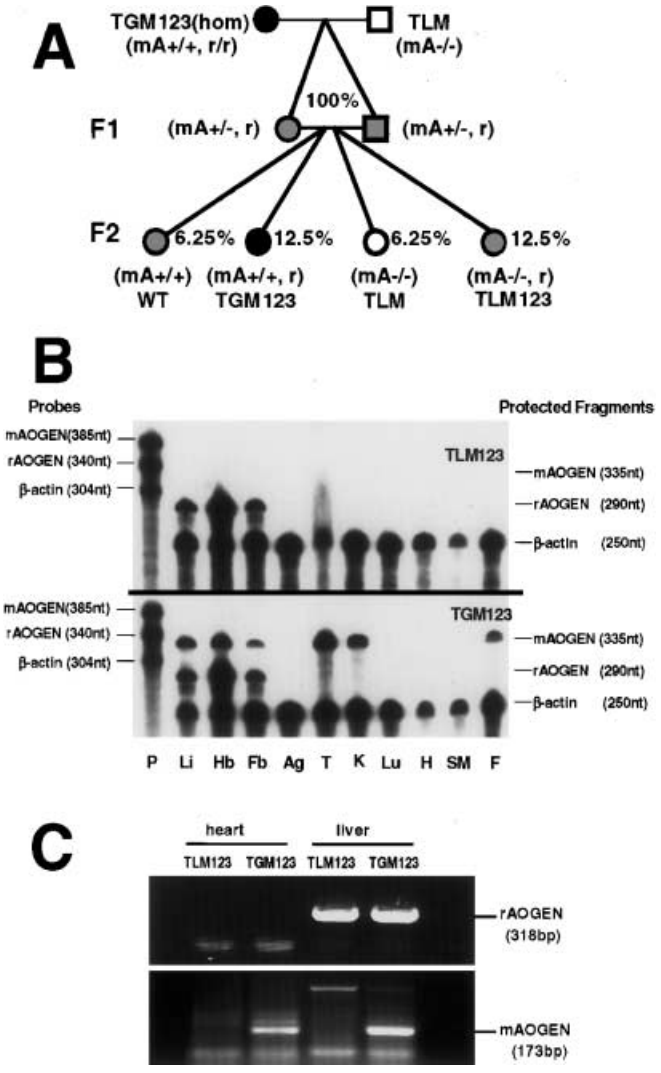

Fig. 1 Breeding schedule (**A**) and AOGEN gene expression (**B**, **C**). **A** *Circle* Female; *square* male; *r* heterozygous for rAOGEN transgene; *r/r* homozygous for rAOGEN transgene; *mA+/+* wild-type for both mAOGEN alleles, *mA+/–* heterozygous, and *mA–/–* homozygous for the disrupted mAOGEN allele. **B** RNase-protection assay showing the tissue-specific expression pattern of mAOGEN, rAOGEN, and β-actin in TLM123 (*above*) and TGM123 (*below*). Total RNA of liver (*Li*, 15 µg), hindbrain (*Hb*, 30 µg), forebrain (*Fb*, 30 µg), adrenal gland (*Ag*, 30 µg), testis (*T*, 30 µg), kidney (*K*, 30 µg), lung (*Lu*, 30 µg), heart (*H*, 30 µg), skeletal muscle (*SM*, 30 µg), and fat tissue (*F*, 30 µg) were hybridized to 385 nt mAOGEN, 340 nt rAOGEN, and 304 nt β-actin cRNA probes. Protected fragments of 335 nt, 290 nt and 250 nt were indicative for the presence of mAOGEN, rAOGEN, and β-actin mRNA, respectively. **C** RT-PCR detecting the presence of mAOGEN mRNA in the heart of TGM123 (*below*) and the absence of both AOGEN mRNAs in the heart of TLM123

42°C. Of the RT products 2–5 µl was used as templates of PCR. The PCR protocol used in RT-PCR was the same as for genotyping described above.

For northern blot 20 µg total RNA and a 780-bp fragment of rat atrial natriuretic peptide (ANP) cDNA, a 1300-bp cDNA fragment of rat α_1 type I collagen, and a 200-bp rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragment labeled by ^{32}P-dCTP (Random primer labeling kit, Stratagene) as probes for mouse ANP, collagen I, and GAPDH mRNA, respectively, were used, as described previously [10].

Plasma renin and AOGEN concentration and urinary albumin

About 0.5 ml blood was collected from 8-week-old mice and transferred into 1.5-ml tubes containing 20 µl 10 mM EDTA and mixed rapidly within 30 s. Plasma preparations were obtained by centrifugation for 5 min at 5,000 rpm and stored at –80°C. As previously described, total plasma AOGEN was measured by an indirect radioimmunoassay measuring the production of AI by excess purified mouse submaxillary gland renin [23]. Plasma renin concentration was determined with the same assay after addition of renin-free mouse plasma. Urine was collected for 3 days in metabolic cages and urinary albumin was measured using a specific enzyme-linked immunosorbent assay by Immundiagnostik (Bensheim, Germany).

Blood pressure and tissue histology and morphometry

Eight-week-old mice were anesthetized by an intraperitoneal injection of chloral hydrate (Aldrich) at a dose of 0.4 mg/g body weight and placed on a heated table to keep their body temperature around 37°C. Cannulation of the femoral artery was performed by PE10 catheters filled with heparinized saline. Blood pressure and heart rate were transmitted to a connected computer equipped with the TSE-BMON system (TSE, Germany) and continuously recorded for 40 min after a period of stabilization. Mean arterial blood pressure and heart rate were calculated by averaging all values recorded.

Hearts of mice were quickly excised, put into saline for 3 min in order to get rid of blood, dried using paper towel, and freed from fat tissue before they were weighed. For morphological analysis hematoxylin/eosin staining and periodic acid–Schiff's staining were performed on both paraffin-embedded and frozen sections of heart and kidney as described [24]. Microscopic images of heart sections of mice were analyzed using software TINA 2.08 (Raytest, Sprockhövel, Germany). More than 100 cardiomyocytes from three individuals of each group were randomly selected, and their diameter was quantified.

Immunohistochemistry

Frozen 6-µm sections of heart and kidneys were air dried overnight, fixed in cold acetone for 10 min, and again air dried for 15 min. After blocking unspecific binding with 10% normal goat serum the sections were incubated overnight at 4°C with a rabbit anti-mammalian collagen type I antibody (Biotrend, Cologne, Germany) diluted to 1 µg/ml in Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl, pH7.6). Then they were washed three times in TBS and incubated in alkaline phosphatase conjugated goat anti-rabbit immunoglobulin diluted 1:50. After washing with TBS for three times, alkaline phosphatase labeling was visualized using naphtol AS-BI phosphate as the substrate and new fuchsin as the chromogen to develop an insoluble red precipitate. Sections were slightly counterstained with hematoxylin, washed, and mounted using glycerol-gelatin for microscopy.

Statistical analysis

All values were expressed as means ±SEM and were evaluated for statistical significance using STATISTICA 5.0 (StatSoft, USA). Data were analyzed by one-way analysis of variance followed by multiple comparison testing using the Student-Newman-Keuls test. Differences at the level of $P<0.05$ were regarded as statistically significant.

Results

AOGEN expression in TLM123

Crossbreeding TGM123 with TLM generated a novel transgenic mouse model (TLM123) lacking the murine AOGEN (mAOGEN) gene and expressing an rAOGEN transgene (Fig. 1A). Species-specific RNase-protection assays (Fig. 1B) and RT-PCR (Fig. 1C) showed that: (a) mAOGEN was expressed in several organs including liver, brain, testis, kidney, fat, and heart of TGM123 but not in TLM123; (b) the tissue-specific expression pattern of the rAOGEN transgene with detectable mRNA only in liver and brain remained unchanged after being introduced into knockout mice; (c) up- or down-regulation of rAOGEN-transgene expression could not be detected in TLM123 compared with TGM123.

We confirmed previous data [3, 21] showing that total plasma AOGEN concentration was more than ten times greater in TGM123 than in controls (P<0.01), and that

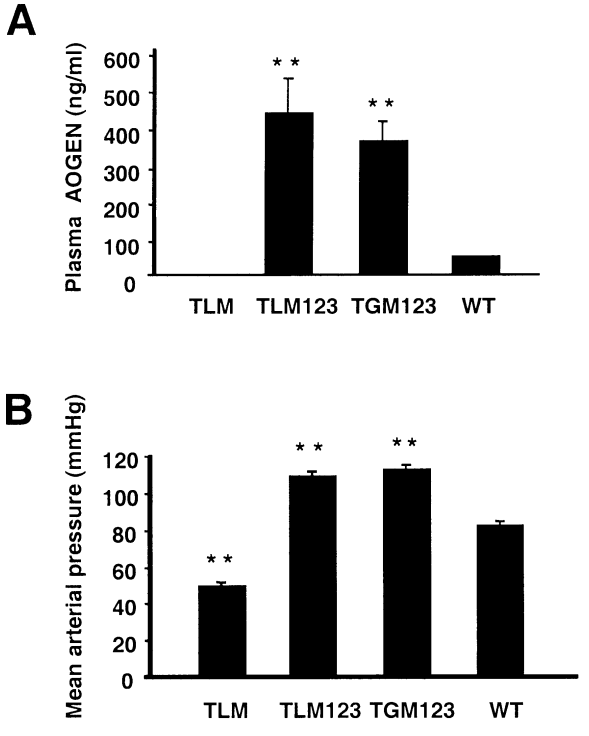

Fig. 2 Plasma AOGEN concentration (**A**) and blood pressure (**B**). There is no difference in the two parameters between TGM123 and TLM123. **P<0.01 vs. wild type; n=6–9

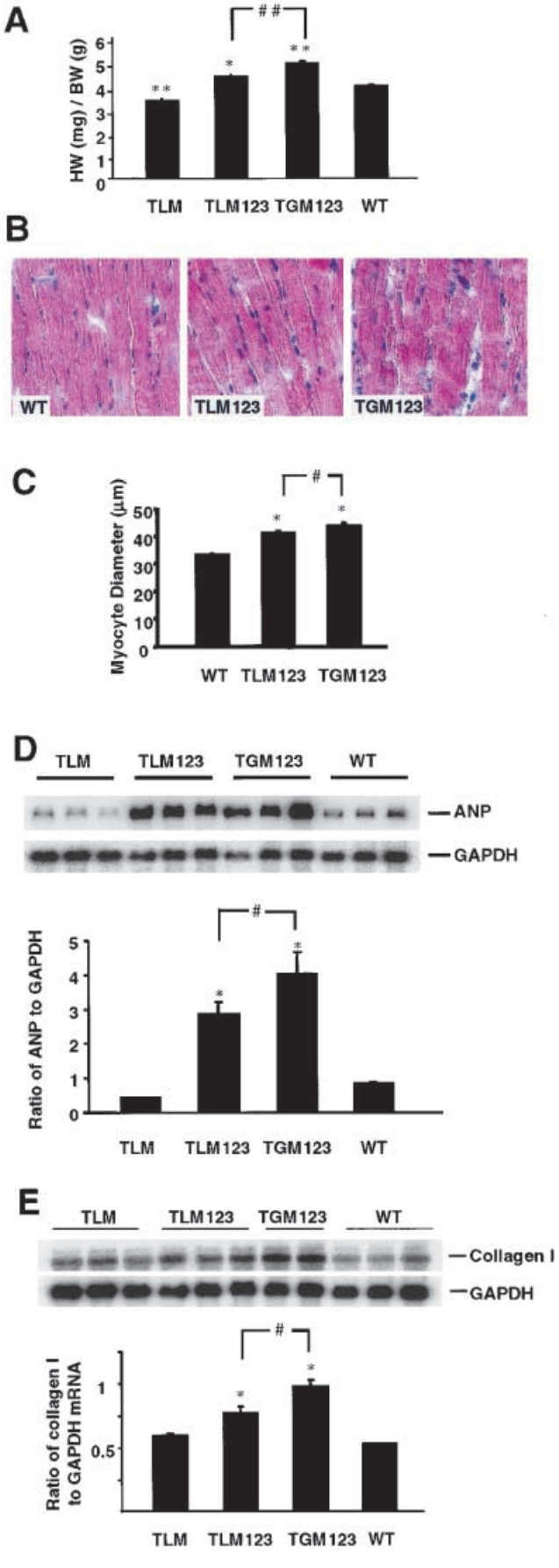

Fig. 3 Cardiac hypertrophy (**A**–**D**) and fibrosis (**E**). Hypertrophy and fibrosis were assessed by determination of relative heart weight [heart weight (HW, mg)/body weight (BW, g) ratios] (**A**), histology (**B**), myocyte diameter (**C**), ANP (**D**), and collagen I (**E**) expression and found to be less pronounced in TLM123 than in TGM123. ANP and collagen I mRNA were detected in the heart by northern blot analysis and normalized to GAPDH expression. *P<0.05 vs. wild type, **P<0.01 vs. wild type, $^{\#}P$<0.05, $^{\#}P$<0.01; n=4–7

TLM were completely devoid of plasma AOGEN (Fig. 2A). Interestingly, plasma AOGEN concentration of crossbred mice TLM123 was elevated to the same level as that of TGM123 (Fig. 2A; $P>0.05$), and plasma renin concentration was equally suppressed by more than 80% in both lines (data not shown).

Hypertension in TLM123

Blood pressure was determined in 8-week-old mice applying an intra-arterial catheter (Fig. 2B). TLM exhibit a mean arterial pressure about 35 mmHg below wild-type controls ($P<0.01$) while it was about 31 mmHg higher in TGM123 males than in matched wild-type ($P<0.01$), again confirming previous results [3, 21]. Interestingly, the rAOGEN transgene in TLM123 not only rescued hypotension of TLM but also elevated blood pressure to the same level as in TGM123 ($P>0.05$), 62 mmHg higher than in TLM ($P<0.01$).

Reduced cardiac hypertrophy in TLM123

While the relative heart weight [heart weight (mg)/body weight (g) ratio] was lower in TLM ($P<0.01$) than in controls, it was markedly higher in TGM123 and TLM123, by 26% ($P<0.01$) and 17% ($P<0.05$), respectively (Fig. 3A). Remarkably, the extent of cardiac hypertrophy was significantly lower in TLM123 than in TGM123 ($P<0.01$). Accordingly, the thickness of the left ventricular free wall (data not shown) and the diameter of single myofibers (Fig. 3B, C) was increased in TGM123 and to a significantly lower extent ($P<0.05$) in TLM123. Expression of the ANP gene in cardiac ventricle is widely used as a marker for cardiac hypertrophy. Figure 3D shows that ventricular ANP expression is enhanced in both TGM123 (540% of control) and TLM123 (376% of control) but, again, was significantly ($P<0.05$) less pronounced in TLM123.

Cardiac fibrosis was analyzed by immunostaining with anti-collagen I antibodies (data not shown). Collagen depositions were found mainly in the perivascular space of coronary vessels in all strains but especially in the TGM123. This finding was confirmed by quantification of collagen I mRNA in the heart by northern blot, again revealing a significantly stronger induction of the gene in TGM123 than in TLM123 (Fig. 3E; $P<0.05$).

Rescued kidney morphology and function

Due to a developmental defect in the urinary outflow tract, AOGEN-deficient mice show obstructive nephropathy (Fig. 4A). Introduction of the rAOGEN transgene rescued this phenotype completely (Fig. 4B). Moreover, the known hyperplastic alterations in renal vessels of TLM [4] (Fig. 4C) are absent in TLM123 (Fig. 4D). Additionally, TLM exhibit collagen depositions inside the

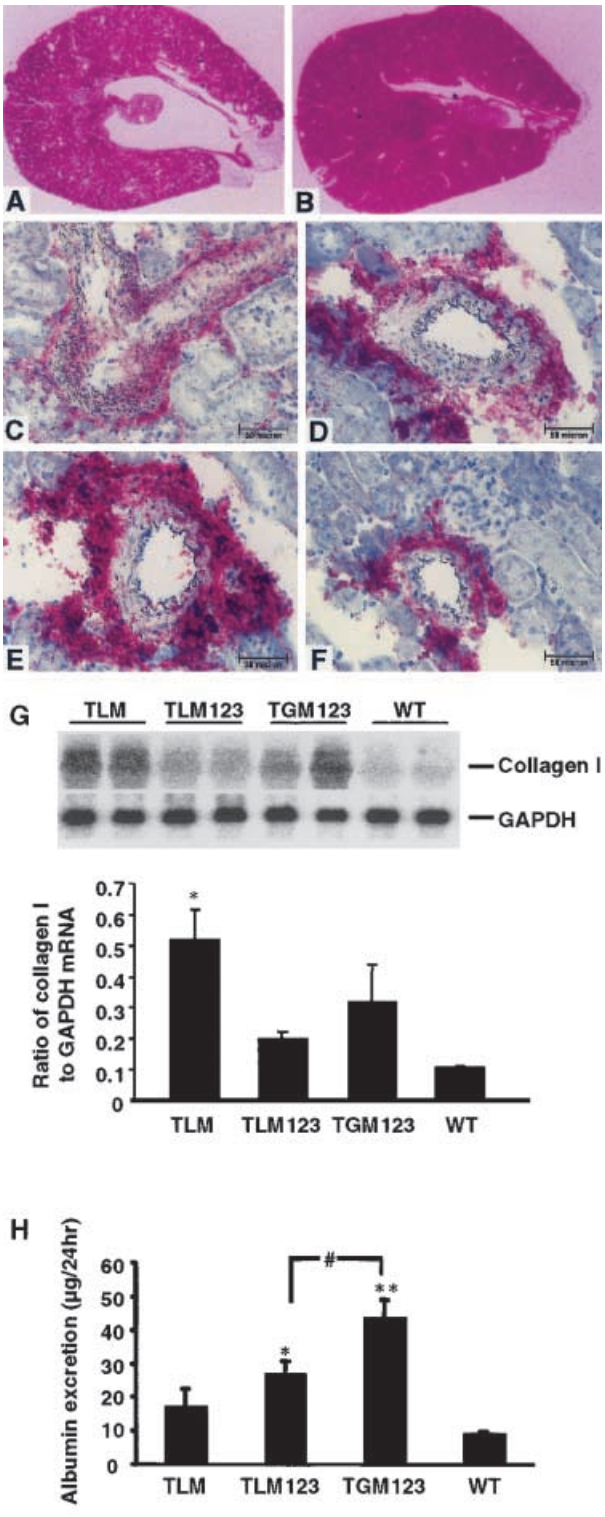

Fig. 4 Renal morphology (**A**–**G**) and function (**H**). Cross-sections through kidneys show an obstructive nephropathy in TLM (**A**), which is not present in TLM123 (**B**). Immunostaining for collagen in TLM (**C**), TLM123 (**D**), TGM123 (**E**), and wild type (**F**) and quantification of collagen I mRNA by northern blot analysis (**G**) revealed less pronounced fibrosis in TLM123 than in TGM123. Albumin concentration in urine (**H**) is increased more moderately in TLM123 than in TGM123. *$P<0.05$ vs. wild type, **$P<0.01$ vs. wild type, $^{\#}P<0.05$; $n=4–7$

vessel wall (Fig. 4C), a phenomenon not observed in TLM123 (Fig. 4D). In contrast, TLM123 develop hypertension-induced perivascular fibrosis (Fig. 4D), but to a much lower extent than TGM123 (Fig. 4E). Accordingly, collagen mRNA levels are drastically increased in TLM and TGM123 but only marginally in TLM123 (Fig. 4G). Periodic acid–Schiff's staining reveals a normal glomerular and tubular morphology in TLM123 in contrast to TLM and TGM123 (data not shown). TLM and TGM123 exhibit a thickening of the glomerular basal lamina, and TGM123 shows hyalin depositions in the tubules and glomeruli. As a functional correlate to these findings, the severe albuminuria observed in TGM123 is markedly attenuated in TLM123 (Fig. 4H).

Discussion

By introducing a rat AOGEN transgene into the genetic background of AOGEN-deficient mice we show that AOGEN expression in liver and brain is sufficient to overcome hypotension and to rescue the developmental defects in the kidney of TLM. These results complement earlier studies in which phenotypic rescue of TLM was achieved by a widespread expression of an AOGEN transgene [25] but not by selective restoration of the renal RAS [26]. Davisson et al. [6] have generated mice carrying in addition to the targeted AOGEN-locus the human genes for renin and AOGEN as transgenes. These mice also became hypertensive indicating, that, in agreement with our results, Ang II deficiency is the cause of the phenotype of animals lacking AOGEN. In contrast to our data showing no difference in blood pressure between TGM123 and TLM123, this parameter depended on the number of alleles of the mAOGEN gene present in each animal. This difference may be explained by the very high concentration of rAOGEN in the plasma of TGM123 rendering mAOGEN negligible for Ang II generation.

Our results further emphasize that AOGEN is an important determinant of blood pressure in mice as it is present in limiting concentrations. Mice with increasing copy numbers of the mAOGEN gene from zero to four exhibit increasing levels of blood pressure which are correlated with the plasma levels of AOGEN [5]. However, this study could not differentiate between the importance of the circulating and the tissue-based RASs for blood pressure determination. In our study AOGEN synthesis was restored only in liver and brain of TLM123, not in kidney, heart, or fat, tissues which normally express mAOGEN [27] (Fig. 1). As blood pressure of TLM123 was indistinguishable from that of TGM123, these tissues do not seem to contribute significantly to the hypertensive phenotype. However, we can not differentiate between the relative importance of the circulating and the central RAS in this respect. Furthermore, our study does not rule out that an activated local RAS can cause hypertension, as it did in the kidney of a recently described transgenic mouse model [28].

In addition to its vasopressor action, Ang II is a potent regulator of development, growth, and remodeling of several organs [9]. It seems to be essential for the full development of cardiac size as we observed a reduced cardiac weight in AOGEN-deficient TLM mice (Fig. 3A). On the basis of our data we cannot decide whether the lack of Ang II directly affects the growth of cardiomyocytes or mediates hemodynamic alterations during development that result in a lower cardiac mass.

The role of Ang II in cardiac hypertrophy is still a matter of debate. We and others have shown that low doses of ACE inhibitors or Ang II receptor AT1 antagonists, which do not influence blood pressure, reduce hypertension-induced cardiac hypertrophy in various animal models [12, 14, 29]. Studies characterizing normotensive transgenic animals overexpressing RAS components exclusively in the heart have produced controversial results. While overexpression of the AT1 receptor [15] and AOGEN [16, 30] induced hypertrophy in transgenic mice, AT1 [31] and ACE overexpression [32] had no such effect in transgenic rats. However, in these rat models pressure-overload induced hypertrophy was augmented, suggesting a modulating role for Ang II in the signal transduction pathway from increased wall stress to cardiomyocyte hypertrophy. Cell culture studies have confirmed such a function showing that AOGEN mRNA levels are elevated and Ang II is secreted by cardiomyocytes subjected to mechanical stress [33, 34, 35, 36]. Moreover, hypertrophic effects of this treatment on cardiomyocytes can be prevented by AT1 antagonists, suggesting an essential role for cardiomyocyte-derived Ang II in this process [36, 37, 38, 39]. Ang II initiates a positive feedback loop on AOGEN synthesis in cardiomyocytes, which may be important as enhancing step in the development of hypertrophy [34, 40]. Nevertheless, it has also been shown that AOGEN-deficient cardiomyocytes in culture [41] as well as mice lacking the AT1A receptor [42] exhibit hypertrophic responses to mechanical stress. In this study we show by several lines of evidence, such as relative heart weight, myocyte diameter and ANP expression, that cardiac hypertrophy is induced by pressure overload in the absence of detectable AOGEN mRNA in cardiomyocytes. However, the extent of the effect is lower than in animals, which exhibit the same blood pressure, but are still capable of synthesizing AOGEN in the heart. Taken these results together, we conclude that Ang II produced from locally synthesized AOGEN is one but not the only mediator linking wall stress in pressure overload to hypertrophic responses of cardiomyocytes.

Furthermore, Ang II has been shown to augment collagen synthesis and induce fibrosis in the myocardium [17]. The markedly lower cardiac fibrosis in TLM123 than in TGM123 reveals that local cardiac AOGEN synthesis is a major source of pathophysiologically relevant Ang II.

The cardiac phenotype of TLM123 supports the existence and functional importance of a local RAS in the heart, in which stretched cardiomyocytes produce

Ang II, inducing hypertrophy in an autocrine or paracrine loop in the same cells and stimulating collagen synthesis and proliferation of cardiac fibroblasts [9]. According to a recent report [43], fibroblast activation by Ang II depends on AT1A receptors on neighboring cardiomyocytes, confirming an autocrine or paracrine action of Ang II on the Ang II secreting or adjacent myocytes which in turn liberate other mediators such as transforming growth factor β.

As in the heart, a paracrine RAS was also postulated to exist in the renal interstitium [9, 44]. Our results provide further evidence for this concept. Collagen synthesis and fibrosis around kidney vessels (Fig. 4) and in the interstitium of cortex and medulla (data not shown) is increased in all hypertensive mice tested but to a significantly lesser extent in animals with blunted local AOGEN expression. The same holds true for glomerular basal lamina thickness. Together, these histological findings explain the functional differences between the kidneys of both equally hypertensive strains. Albuminuria is markedly less pronounced in TLM123 than in TGM123.

The paracrine RAS in the kidney consists of renin liberated from juxtaglomerular cells via the interstitium into the circulation, AOGEN synthesized in proximal tubular cells and ACE present on endothelial, mesangial, and proximal tubular cells. Ang II locally produced by the interaction of these proteins elicits effects in numerous cell types expressing Ang II receptors in the kidney, e.g., afferent arteriolar smooth muscle, macula densa, proximal tubular and mesangial cells inducing a series of events which include the release of inflammatory mediators, endothelin and transforming growth factor β and lead to renal fibrosis and the deterioration of glomerular function [17, 45, 46].

The tissue-specific restoration of AOGEN expression in mice deficient for this protein is a powerful tool for studying the functions of tissue-based RASs. Our study restricted the cause of hypertension in TGM123 to either the central or the circulating RAS and established this transgenic mouse strain as a novel model for the analysis of hypertension-induced end-organ damage. Furthermore, by producing the first animal line (TLM123) with circulating Ang II but blunted cardiac and renal AOGEN synthesis we characterized local AOGEN synthesis in the heart and in the kidney as being involved in the pathogenesis of hypertension-induced end-organ damage in these organs.

Acknowledgements We thank K. Ohta for pGEM3rANP, W. Schneider, and A. Lippoldt for their help with the histological stainings, and G. Born and I. Strauss for their excellent technical assistance. This research was supported by the Deutsche Forschungsgemeinschaft by a grant to M.B. (BA1374/5-1) and a fellowship to N.K. (GRK276).

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