Ablation of renin-expressing juxtaglomerular cells results in a distinct kidney phenotype

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Ablation of renin-expressing juxtaglomerular cells results in a distinct kidney phenotype. Am J Physiol Regul Integr Comp Physiol 286: R474–R483, 2004. First published October 16, 2003; 10.1152/ajpregu.00426.2003.—Renin-expressing cells are peculiar in that they act as differentiated cells, producing the hormone renin, while they also seem to act as progenitors for other renal cell types. As such, they may have functions independent of their ability to generate renin/angiotensin. To test this hypothesis, we ablated renin-expressing cells during development by placing diphtheria toxin A chain (DTA) under control of the Ren1d mouse renin promoter by homologous recombination in a two-renin gene strain (Ren2 and Ren1d). Renin-expressing cells are essentially absent from kidneys in homozygotes (DTA/DTA) which, unlike wild-type mice, are unable to recruit renin-expressing cells when homeostasis is threatened. In contrast, renin staining in the submandibular gland (SMG), which expresses mainly Ren2, is normal. Homozygous mice survive normally, but the kidneys are small and have morphological abnormalities: 25% of the glomeruli are hyperplastic or atrophic, tubules are dilated and atrophic, and areas of undifferentiated cells exist near the atrophic glomeruli and tubules. However, in contrast to the very abnormal renal vessels found when renin-angiotensin system genes are deleted, the kidney vessels in homozygotes have normal wall thickness and no decrease in lumen size. Homozygotes have severely reduced kidney and plasma renin concentrations and females have reduced blood pressure. Homozygotes have elevated blood urea nitrogen and potassium levels, which are suggestive of altered renal function. We conclude that renin cells per se are necessary for the morphological integrity of the kidney and may have a role in maintenance of normal kidney function.

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Diphtheria toxin A chain; vasculature; angiotensin; homologous recombination; recruitment

The juxtaglomerular (JG) cells in the adult kidney are located in the wall of the afferent arteriole at the entrance to the glomerulus, which is the filtering unit of the kidney (1, 34). These unique cells have both endocrine and smooth muscle cell characteristics. Their endocrine function involves the secretion of renin, a key hormone enzyme of the renin-angiotensin system (RAS), which regulates blood pressure, fluid homeostasis, and electrolyte balance. JG cells contain numerous granules in which renin is stored and also myofilaments, peroxisomes, and few mitochondria (34). They are connected to arteriolar smooth muscle cells, endothelial cells, and other JG cells by gap and myoendothelial junctions (34).

Although they are few in number, JG cells exert a major effect on homeostasis. Despite their important role in renin production and secretion, very little is known about the JG cells themselves. In fact, the only known specific markers for these cells are renin and the small transcript of the gene Zis (18). Deletion studies suggested a role for renin (perhaps through angiotensin generation) in the maintenance of normal kidney structure (6, 29, 37). However, JG cells may have effects in the kidney that are independent of renin production but dependent on their physical presence. Gene deletion studies do not permit assessment of the role of JG cells per se in the morphological and functional integrity of the kidney. To address this problem, it would be ideal to generate a model devoid of JG cells.

One way to remove only a particular cell type is to cause diphtheria toxin A chain (DTA) protein to be expressed in the cell. DTA catalyzes the ADP-ribosylation of elongation factor 2 (EF2), which eventually results in inhibition of protein synthesis and cell death (9, 17, 36). By placing DTA expression under the control of cell specific promoters, ablation of a number of specific cell types has been accomplished (1, 14, 15, 19, 24, 26, 30, 31).

The present study was designed to address the role of JG cells in the structural and functional development of the kidney. To ablate JG cells specifically, we replaced the Ren1d gene with DTA by homologous recombination. We report here that ablation of JG cells results in renal morphological abnormalities. In this model, the vascular hypertrophy evident when other genes of the RAS were deleted was not seen, which suggests a direct (non-RAS mediated) effect of JG cells on vascular growth.

MATERIALS AND METHODS

Construction of Ren1d-DTA targeting vector. The targeting vector (Fig. 1C) was constructed from three fragments. The 1,242-bp 5’ targeting fragment was generated by PCR of 129J mouse genomic DNA as previously described (29). The DTA coding sequence was excised from the pbIB30-DT-A plasmid (kindly supplied by Dr. Ian Maxwell) with HindIII and XbaI and was inserted into the HindIII and XbaI cut pEGFPN1 vector (Clontech, Palo Alto, CA) to acquire a polyA addition signal. (This construct, which has a cytomegalovirus promoter driving DTA expression, was introduced into As4.1 kidney cells. There was a high level of cell death in the DTA-transfected cells, which confirmed that the DTA functioned as expected.) The DTA-SV40 polyA fragment was excised with Xhol and SspI and cloned into pBlueScript (Stratagene, La Jolla, CA) at the Xhol and EcoRV sites. The blunt-ended 5’ DNA PCR product was then cloned into a blunted Ncol site that contained the ATG initiating codon of DTA. The DNA sequence of the PCR product and the sequence across the junction with DTA were confirmed by sequencing. The 3’ targeting DNA was the 8.9-kb HindIII genomic fragment previously described (29). The 5’ targeting fragment was excised with SpeI and

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cloned into the targeting vector osdupdel (a gift from O. Smithies) at an NheI site 5' to a Neo<sup>+</sup> gene selectable marker, and the 3' fragment was cloned into a HindIII site 3' to the marker.

**Gene targeting in ES cells.** Gene targeting was carried out using conventional procedures (20) in TC1 ES cells (a gift from Dr. Philip Leder). Targeted colonies were provisionally identified by PCR analysis (22) using a 5' primer (5'-GCC AGG GTC AGG TCA CTT C) located upstream of any sequences in the targeting vector and a 3' primer (5'-AGC GGG TTT TCA TCT ACA G) located at position 376 in the 5' flanking DNA and a 3' primer (5'-GTA GTA GAA GGG GGA GTT GTG) located at position 1,579 in the first intron of Ren<sup>1d</sup> (4). This PCR reaction generates a 1.2-kb fragment if the Ren<sup>1d</sup> gene is deleted (as in a homozygous Ren<sup>1d</sup>-DTA animal).

**RNA extraction and RT-PCR analysis.** Total RNA was extracted from kidneys and submandibular glands (SMGs) using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s directions. Contaminating DNA was removed using the DNA-free kit (Ambion, Austin, TX). The cDNA was prepared from 2 µg of RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY) and an oligo(dT)15 primer. PCR for renin mRNA used a 5' primer (5'-GTC AAA GTA and 5'-AGA TTC CAT TCA AAA TGG GCA CTC TT) located in exon 1 and a 3' primer (5'-ATT AGG TTA ATA TGC AGG TCT CG) located at position 376 in the 5' flanking DNA and a 3' primer (5'-GTA GTA GAA GGG GGA GTT GTG) located at position 1,579 in the first intron of Ren<sup>1d</sup> (4). This PCR reaction generates a 1.2-kb fragment if the Ren<sup>1d</sup> gene is present and no product if the Ren<sup>1d</sup> gene is deleted (as in a homozygous Ren<sup>1d</sup>-DTA animal).

**Histological analysis.** Animals (adults, 2–4 mo old) were anesthetized with tribromethanol. The kidneys were removed, weighed, and

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**Fig. 1.** Targeting diphtheria toxin A chain (DTA) to the renin gene. A: strain 129 mouse renin locus shows the two renin genes. Relevant restriction sites are shown: PstI (P), HindIII (H), and SacI (S). Genomic DNA fragments detected by the renin 5' probe in Ren<sup>1d</sup> and Ren<sup>2</sup> are shown above the map. Numbers indicate lengths (in kb). B: expanded map of the Ren<sup>1d</sup> gene shows intron/exon structure and relevant restriction sites. Numbered black boxes are exons. C: targeting construct shows 5' and 3' homology regions flanking DTA and the locations of the neo-resistance cassette and Tk gene. Regions in which recombination can take place are shown (dashed lines). TK, thymidine kinase. D: targeted gene. DTA replaces exons 1–7 and part of intron 7 of the Ren<sup>1d</sup> gene. Probes used: renin 5', 0.6-kb renin 5' DNA; DTA, 0.7-kb DTA DNA. E: Southern blot of DNA from targeted ES cells (PstI and SacI digests) and mouse genomic DNA (SacI digests). For PstI digests (left), renin 5' probe was used. The 2.5-kb Ren<sup>1d</sup> and 4.4-kb Ren<sup>2</sup> bands are present in +/+ and +/+DTA as expected. In +/DTA, the additional band is at 2.7 kb, which is the size predicted for targeting DTA to Ren<sup>1d</sup>. For PstI digests (right), DTA probe was used. Only the 2.7-kb band is present in +/DTA. For SacI digests, the renin 5' probe on ES cells and mouse DNA was used. Only the normal genomic bands (6.7-kb Ren<sup>1d</sup> and 6.9-kb Ren<sup>2</sup>) are present in +/+ , whereas an additional 5.3-kb targeted band is present in +/DTA ES cells and +/DTA and DTA/DTA mice.
either frozen immediately in liquid N\textsubscript{2} for RNA extraction or fixed in Bouin’s fixative or 10% formalin. The fixed tissue was dehydrated and embedded in paraffin, and 5-\mu m sections were cut. Tissues were processed for immunohistochemistry and stained with hematoxylin and eosin to assess morphology, with periodic acid Schiff reagent to assess basement membrane integrity, and with Masson’s trichrome stain to assess collagen deposition.

**Immunohistochemistry.** Bouin’s fixed kidney tissue sections (5 \mu m thick) were deparaffinized in xylene and graded alcohols, and immunostaining was performed for renin (using a 1:10,000 dilution of goat anti-rat-renin polyclonal antibody; a gift of Dr. T. Inagami, Nashville, TN), \alpha-smooth muscle actin (\alpha-SMA; a 1:10,000 dilution of a monoclonal anti-\alpha-SMA-specific antibody isotype IgG\alpha; Sigma, St. Louis, MO), CD3 [a 1:200 dilution of CD3-\epsilon (M-20), sc-1127; Santa Cruz Biotechnology, Santa Cruz, CA], and proliferating cell nuclear antigen (PCNA; a 1:10,000 dilution of NCL-PCNA; Novocastra Laboratories, Newcastle upon Tyne, UK) using the appropriate Vectastain ABC kit (Vector Laboratories, Burlingame, CA) as previously described (12). Apoptosis was detected by the terminal deoxynucleotidyl transferase uridine nick end label (TUNEL) technique on formalin fixed tissue using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (catalog no. S7100, Serologicals, Norcross, GA).

**Morphometric measurements.** The number of renin-positive cells per kidney section was calculated as [(number of renin-positive glomeruli/total number of glomeruli) \times number of renin-positive cells per JGA] and was then expressed as a percent of the wild-type number. To estimate the percent of damaged glomeruli in the mutants, images from hematoxylin and eosin-stained sections were captured, and measurements of the diameter of the glomeruli were made using the Image-Pro Plus program. The glomeruli measured were those that showed the vascular or urinary pole (meaning a cut at the middle of the glomerulus; Refs. 2, 23), and the maximal profile was identified as the largest diameter with at least two smaller sections preceding and succeeding the glomeruli (23). The total number of glomeruli per section was also determined, and glomeruli were grouped by size (normal or large and hypercellular or shrunken). For measurements of vessel diameter, the outer and inner diameters of vessels of different calibers were measured in \alpha-SMA stained sections.

**Stimulation of RAS.** To stimulate expression of renin, adult animals were treated for 11 days with the angiotensin-converting enzyme (ACE) inhibitor captopril (0.5 g/ml) in drinking water. At the end of the treatment period, kidneys and other organs were collected for measurement of renin activity.

**Intra-arterial blood pressure measurements.** Animals were anaesthetized with isofluorane and placed on a thermostatically controlled heating table at 38°C, and a polyethylene catheter was threaded down the right carotid artery so that the catheter tip was at the aortic root. Mean arterial pressure was continuously recorded from this catheter by means of a Gould transducer coupled to a Biopac recorder. Measurements were taken over a 10-min period.

**Renin measurements.** After measurement of intra-arterial blood pressure, blood was collected via the catheter into EDTA plasma separator tubes (Microtainer, Becton Dickinson, Franklin Lakes, NJ) and stored on ice until separation by centrifugation for 5 min at 14,000 g at 4°C. Plasma samples were snap-frozen in liquid N\textsubscript{2} and stored at –80°C. Kidneys for tissue renin measurements were snap-frozen in liquid N\textsubscript{2} and stored at –80°C. Plasma and kidney renin concentrations were measured by angiotensinogen substrate as previously described (29).

**Blood chemistry measurements.** After the blood sample was collected for renin measurements, blood was collected through the catheter into heparinized plasma separator tubes (Microtainer). The basic metabolic panel for blood urea nitrogen (BUN), potassium, sodium, calcium, glucose, chloride, and CO\textsubscript{2} was performed by the University of Virginia Hospital clinical lab.

Animals. Mice used in these experiments were in an inbred 129SvEv background. Chimeric animals produced from the 129 strain ES cell injections were crossed to 129SvEv (Taconic) females to generate essentially inbred 129 offspring. Mice were fed regular mouse chow (Prolabo 2000, PMI Feeds, St. Louis, MO) and tap water ad libitum and were housed in a temperature-controlled (22 ± 2°C) environment on a 12:12-h light-dark cycle. All procedures were performed in accordance with the guidelines of the American Physiological Society and were approved by the University of Virginia Animal Care and Use Committee.

**Statistical tests.** All values are expressed as means ± SD. Statistical significance was assessed by t-test, Mann-Whitney rank sum test, or ANOVA as appropriate using the SigmaStat program (SPSS, Chicago, IL).

**RESULTS**

**Generation of the Ren1\textsuperscript{d}-DTA targeted mouse.** The 5’ DNA sequences of both Ren1\textsuperscript{d} and Ren2 are similar, so the targeting construct was designed to have the larger region of Ren1\textsuperscript{d} homology at its 3’ end to favor recombination with the Ren1\textsuperscript{d} allele (see Fig. 1). After electroporation of the targeting construct into ES cells, PCR analysis identified 4 of 123 (3.2%) dual drug-resistant colonies. Figure 1 (A–D) illustrates the pattern of bands expected in genomic Southern blots of wild-type and targeted DNA. In a PsI digest of wild-type DNA, the renin 5’ probe detected a 2.5-kb Ren1\textsuperscript{d} band and a 4.4-kb Ren2 band (Fig. 1, A and B). If DTA is correctly targeted to Ren1\textsuperscript{d}, the size of the PsI fragment is 2.7 kb (Fig. 1, C and D), whereas if it is targeted to Ren2, the band is 4.6 kb. In SacI digests, a 6.8-kb Ren1\textsuperscript{d} band and a 6.9-kb Ren2 band (Fig. 1, A and B) were detected in wild-type DNA, and the DTA band was 5.3 kb (Fig. 1, C and D). A Southern blot of PstI digests of targeted ES cells (Fig. 1E) showed the expected genomic Ren1\textsuperscript{d} and Ren2 bands as well as a 2.7-kb band that confirmed the targeting of DTA to Ren1\textsuperscript{d}. The DTA probe hybridized only with the 2.7-kb DTA-targeted band in the ES cells (Fig. 1E). In SacI digests, the wild-type genomic band and the 5.3-kb DTA-targeted band were present. These results show that the DTA in the ES cell clones was correctly targeted to the Ren1\textsuperscript{d} gene and that DTA and surrounding DNA from the targeting construct remained intact.

One of the targeted ES cell lines was injected into mouse blastocysts to generate a targeted transgenic animal. The chimera were bred to 129SvEv mice (Taconic) that had the same genetic background as the ES cells to produce the isogenic heterozygotes that were intercrossed to produce isogenic wild-type, heterozygous, and homozygous targeted animals. In the following, the genotypes are designated as Ren1\textsuperscript{d}/Ren1\textsuperscript{d} or +/- for wild type (Ren2 Ren1\textsuperscript{d}/Ren2 Ren1\textsuperscript{d}), Ren1\textsuperscript{d}/DTA or +/DTA for heterozygotes (Ren2 Ren1\textsuperscript{d}/Ren2 Ren1\textsuperscript{d}-DTA), and DTA/DTA for homoyzogotes (Ren2 Ren1\textsuperscript{d}-DTA/Ren2 Ren1\textsuperscript{d}-DTA). The Southern blot of SacI digests of the targeted mice (Fig. 1E) has the pattern of bands as in the ES cells, which confirms that DTA and the surrounding DNA from the targeting construct were preserved intact in the mouse.

**Expression of DTA ablates JG cells.** The number of cells that contained renin, as assessed by immunostaining, was markedly reduced in heterozygous and homozygous animals. Not only was there a lower percent of JGAs positive for renin, but the number of renin-positive cells per JGA was also significantly reduced. In contrast to the plump renin-positive cells in Ren1\textsuperscript{d}/Ren1\textsuperscript{d} kidneys, the occasional renin-positive
cells found in DTA-expressing animals were smaller and thinner (Fig. 2). In DTA/DTA kidneys, renin staining was observed in only 2.2% of the JGAs (Table 1), and two of five animals had no renin-positive cells. These very few renin-positive JGAs in DTA/DTA kidneys contained an average of one renin-positive cell. Ren1\textsuperscript{d}/DTA kidneys had more renin-positive cells than DTA/DTA kidneys. However, both the percent of renin-positive JGAs (Table 1) and the number of renin-positive cells per JGA were significantly less than in the wild-type [for Ren1\textsuperscript{d}/Ren1\textsuperscript{d}, 3.78 ± 0.41 (n = 65 in 4 animals); for Ren1\textsuperscript{d}/DTA, 1.86 ± 0.25 renin-positive cells/JGA (n = 48 in 3 animals; P < 0.001 compared to Ren1\textsuperscript{d}/Ren1\textsuperscript{d})].

Wild-type animals normally have a pool of renin precursor cells that can be converted to renin production when the body requires more renin (11, 13, 21). Treatments with agents that inhibit the response to angiotensin II (such as the ACE inhibitor captopril) result in an increase in the number of JG cells that express renin and an accompanying increase in the number (recruitment) of renin-expressing cells along the afferent arteriole, in pericytes, and sometimes in the glomerulus. To test whether renin precursors were killed by DTA, we examined the effects of ablation on recruitment of renin-expressing cells. Our results show that wild-type animals had the expected increased proportion of JGAs that express renin as well as an increased number of afferent arterioles with renin expression extending along their length (Table 1). In adult Ren1\textsuperscript{d}/DTA animals, the proportion of renin-positive JGAs also increased, and there was some recruitment along the afferent arterioles (Table 1). In contrast, the DTA/DTA animals showed no evidence of recruitment; i.e., there was no increase in the proportion of JGAs that were positive for renin staining nor was there expression of renin along the afferent arterioles (Table 1).

Gene expression analysis. Renin mRNA expression in Ren1\textsuperscript{d}/DTA and DTA/DTA kidneys was assessed by RT-PCR of total RNA and digestion of the resulting products with BstXI to distinguish the Ren1\textsuperscript{d} (392 bp) and Ren2 (234 bp) products. The results revealed that mRNAs for Ren1\textsuperscript{d} and Ren2 are present in wild-type (as expected) and also in Ren1\textsuperscript{d}/DTA kidneys (which have some cells that contain renin protein), and that only Ren2 is present in DTA/DTA kidneys (Fig. 3A). The absence of the Ren1\textsuperscript{d} transcript in DTA/DTA animals confirms that in the homologous recombination, inserted DTA resulted in deletion of the Ren1\textsuperscript{d} gene and killed Ren1\textsuperscript{d}-expressing cells. RT-PCR for DTA mRNA demonstrated that DTA mRNA was present in the kidneys of the Ren1\textsuperscript{d}/DTA and DTA/DTA targeted animals and absent from the wild-type animals (Fig. 3B).

Specificity of ablation of Ren1\textsuperscript{d} cells. Renin is present at high level in SMGs and is predominantly Ren2 protein based on the almost exclusive expression of Ren2 mRNA in SMGs (10). To further confirm that the DTA targeting affected only Ren1\textsuperscript{d}-expressing cells, we performed RT-PCR for renin mRNA in the SMGs of male and female animals. All three genotypes showed the presence of Ren2 mRNA (Fig. 4A). Immunostaining for renin in the SMGs confirmed the abundant presence of renin protein in homozygotes of both sexes in the same pattern as in the wild-type animals (Fig. 4B).

Morphological and histological analysis of kidneys. Homozygous animals exhibited a range in the severity of the phenotypic abnormalities. The kidneys in all homozygotes were much smaller than those of wild-type animals and weighed significantly less (Fig. 5 and Table 2). In the most extreme cases, the kidneys were shrunken, their surfaces lacked the normal shiny appearance of fresh tissue, and they exhibited a granular aspect with patches of white, fatty material. The cortical region was reduced in thickness and the demarcation of the inner and outer medullary bands was lost (Fig. 5). In those DTA/DTA kidneys that showed a less-extreme phenotype, with the exception of the reduced size, the kidneys

Table 1. Renin expression in kidneys of control and captopril-treated mice

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<th>Genotype</th>
<th>Control</th>
<th>Captopril Treated</th>
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<tr>
<td></td>
<td>Ren\textsuperscript{+} JGA</td>
<td>Ren\textsuperscript{+} Afferent Arterioles</td>
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<tr>
<td>Ren1\textsuperscript{d}/Ren1\textsuperscript{d}</td>
<td>0.461 ± 0.041* (4)</td>
<td>0.00 ± 0.00 (4)</td>
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<tr>
<td>Ren1\textsuperscript{d}/DTA</td>
<td>0.187 ± 0.037* (6)</td>
<td>0.17 ± 0.41 (6)</td>
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<tr>
<td>DTA/DTA</td>
<td>0.022 ± 0.023† (5)</td>
<td>0.00 ± 0.00 (5)</td>
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Values are means ± SD; n (in parentheses), no. of animals. Ren\textsuperscript{+} JGA, no. of renin-positive juxtaglomerular apparatuses/total no. of glomeruli; Ren\textsuperscript{+} Afferent Arterioles, no. of afferent arterioles with renin immunostaining extending >30 μm from the glomerulus. *P < 0.05 compared to control of same genotype; †P < 0.05 compared to Ren1\textsuperscript{d}/Ren1\textsuperscript{d}.

Fig. 2. Renin immunostaining. Arrows indicate glomeruli. Renin staining is in dark red/brown. Note the thinner renin-positive cells in +/DTA and absence of renin staining in DTA/DTA.
Renin mRNA was amplified from total kidney RNA by RT-PCR. A: renin products were digested with BstXI to distinguish Ren1d and Ren2 transcripts. Diagnostic bands for Ren1d (392 bp) and Ren2 (346 bp) are indicated. Diagnostic band for Ren1d is absent from the DTA/DTA sample. U, undigested PCR product that contains both Ren1d (552 bp) and Ren2 (549 bp) products. B: DTA mRNA (459 bp product) is present in Ren1d/DTA and DTA/DTA samples but not Ren1d/Ren1d samples.

Fig. 4. Renin mRNA and protein in the submandibular gland (SMG). A: renin mRNA was amplified by RT-PCR and the product was digested with BstXI to distinguish Ren1d and Ren2 transcripts. Only Ren2 mRNA was detected in the SMG of all three genotypes. B: immunostaining for renin. There was no difference in the level of renin staining between +/- and DTA/DTA. In addition, the characteristic range of renin-staining intensity in the SMG was the same in DTA/DTA as in +/-.

Fig. 5. Comparison of Ren1d/Ren1d and DTA/DTA kidneys. DTA/DTA kidney is markedly smaller than Ren1d/Ren1d kidney, the cortical region is reduced, and the demarcation of the inner and outer medullary bands (arrows) is less distinct.

At the microscopic level, the outer surface of the DTA/DTA kidneys was irregular in appearance, and the macroscopically

visible white areas contained what appeared to be fat cells (Fig. 6). Nearly 25% of the glomeruli in the DTA/DTA kidneys showed evidence of damage (Fig. 6): 8.3% were large and hypercellular, and 16.3% were shrunken [glomerular radius for Ren1d/Ren1d, 71.6 ± 11.4 μm (n = 47 from 5 animals); for DTA/DTA, enlarged glomeruli, 80.3 ± 6.4 μm² (n = 25 from 3 animals); shrunken glomeruli, 49.3 ± 6.5 μm² (n = 31 from 3 animals); *P < 0.001 compared to Ren1d/Ren1d]. Staining with Masson’s trichrome showed that there was an increase in collagen deposition in the glomeruli that appeared shrunken and also in some interstitial areas. In the cortex, there were areas of tubular dilatation and atrophy. Staining with periodic acid Schiff reagent revealed reduced brush borders in the proximal tubules, thus confirming the observation of tubular atrophy. The shrunken glomeruli in the DTA/DTA kidneys were mainly located in areas that contained abnormal tubules.

Deletion of other RAS genes results in vascular abnormalities, especially thickening of the smooth muscle layer of the vessel walls. To assess whether ablation of JG cells resulted in renal vascular abnormalities, we immunostained renal tissue for α-SMA. In DTA/DTA animals, there was the expected staining of all vessels, but also many α-SMA-positive cells were observed within the glomeruli, which is typical of immature kidneys (5). The vessels in the DTA/DTA kidneys (afferent arterioles and interlobular arteries) were not at all thickened compared with those in Ren1d/Ren1d kidneys (Fig. 7 and Table 3). This was in contrast to the very significant thickening of the vessel wall and decrease in vessel lumen in the kidneys of animals that lack the genes for angiotensinogen (20, 33), ACE (16), or angiotensin II type 1A and 1B receptors (AT1A/AT1B; Refs. 28, 35).

All of the DTA/DTA kidneys had areas of “undifferentiated” cells adjacent to the tubules in the outer cortex (see Fig. 6). These cells were not dividing (by PCNA staining), and only a few of them were infiltrating lymphocytes (by CD3 staining). Staining with Masson’s trichrome showed that there was an increase in collagen deposition within these areas of undifferentiated cells. Assays for apoptosis in Ren1d/Ren1d kidneys identified only a few apoptotic cells in the tubules. In DTA/DTA kidneys, apoptotic nuclei were seen in the dilated tubules and the areas of undifferentiated cells, whereas there were no...
apoptotic cells in the JGAs or around the glomeruli [no. of apoptotic cells per kidney section: for Ren1d/Ren1d, 2.25 ± 1.55, n = 4 animals; for DTA/DTA, 21.1 ± 12, n = 4 animals; *P < 0.05]. In summary, there was a subset of cells that were neither dying nor infiltrating cells, which for the lack of a better name (and based on their appearance) we designated as undifferentiated cells.

**Physiological parameters.** The Ren1d/DTA and DTA/DTA animals appeared normal externally and had no apparent excess mortality in the first 6 mo of life compared with the wild-type animals. The three genotypes were obtained in the expected 1:2:1 Mendelian ratio from crosses of heterozygotes: 18 Ren1d/Ren1d, 35 Ren1d/DTA, and 16 DTA/DTA.

The body weights of heterozygotes and homozygotes (both males and females) were not different from wild-type animals (see Table 2). However, the kidneys in homozygotes of both sexes were smaller than in the wild-type animals (see Table 2). The heart weights in heterozygotes and homozygotes of both sexes were not different from wild-type animals, which indicates that the reduction in kidney size was organ specific.

The blood pressure values in the female homozygous mice were significantly reduced compared with the wild-type animals (Table 4). In the female heterozygotes, the pressure measurements tended to be lower than the wild type, but the difference did not reach statistical significance (*P = 0.12). In the male animals, the blood pressure values of the homozygotes and heterozygotes were not different from the wild-type animals (see Table 4), although there was a tendency toward lower pressures in the homozygous males.

**Renin concentration.** The renin concentration in the kidneys was significantly reduced in the homozygous males and females to 10–20% of the wild-type measurements and in heterozygous females to 60% of wild-type levels [for males: Ren1d/Ren1d, 1.03 ± 0.14 (n = 5 mice); Ren1d/DTA, 0.91 ± 0.59 (n = 7 mice); and DTA/DTA, 0.11 ± 0.15* μg Al·mg⁻¹·h⁻¹ (n = 5 mice); for females: Ren1d/Ren1d, 2.02 ±

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<th>Genotype</th>
<th>Body wt</th>
<th>Kidney wt</th>
<th>Heart wt</th>
<th>Body wt</th>
<th>Kidney wt</th>
<th>Heart wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>26.0±4.1 (5)</td>
<td>0.184±0.024 (10)</td>
<td>0.130±0.012 (5)</td>
<td>20.6±4.8 (7)</td>
<td>0.136±0.028 (14)</td>
<td>0.106±0.022 (7)</td>
</tr>
<tr>
<td>Ren1d/DTA</td>
<td>25.2±2.0 (7)</td>
<td>0.164±0.019 (14)</td>
<td>0.127±0.030 (7)</td>
<td>18.6±2.0 (8)</td>
<td>0.116±0.014 (16)</td>
<td>0.090±0.007 (7)</td>
</tr>
<tr>
<td>DTA/DTA</td>
<td>22.8±3.6 (5)</td>
<td>0.135±0.03* (10)</td>
<td>0.127±0.022 (5)</td>
<td>19.4±2.6 (7)</td>
<td>0.115±0.026* (14)</td>
<td>0.089±0.015 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SD; *P < 0.05 compared to Ren1d/Ren1d.

Fig. 6. Abnormalities in DTA/DTA kidneys. Sections were stained with hematoxylin and eosin. **Left, top:** section from +/+ kidney shows edge of cortex and glomeruli (arrows). **Left, bottom:** section from DTA/DTA kidney with fat (F) cells on the outer surface of the cortex, shrunken glomeruli (arrows), and an area of undifferentiated cells between and surrounding the shrunken glomeruli. **Right, top:** enlarged glomerulus (arrow). **Right, middle:** shrunken glomerulus (arrow). **Right, bottom:** shrunken glomeruli (arrows) in disorganized, congested area. All pictures are at the same magnification.
0.47 (n = 7 mice); Ren1d/DTA, 1.29 ± 0.31* (n = 8 mice); DTA/DTA, 0.46 ± 0.48* µg Al·mg⁻¹·h⁻¹ (n = 7 mice); *P < 0.01 compared with Ren1d/Ren1d mice of same sex]. The plasma renin concentrations (PRC) in both heterozygous and homozygous females were significantly lower than those of wild-type animals. In heterozygotes, the level was reduced to 10% of wild type, and in homozygotes, to 1% of wild type [for Ren1d/DTA, 27.8 ± 19.9 (n = 6 mice); for Ren1d/DTA, 3.2 ± 2.2* (n = 8 mice); for DTA/DTA, 0.37 ± 0.38* µg AI·ml⁻¹·h⁻¹ (n = 7 mice); *P < 0.01 compared with Ren1d/ Ren1d mice]. In males, the PRC values were extremely variable; nevertheless, the mean values for the homozygotes were reduced to 30% of wild type [for Ren1d/Ren1d, 67.1 ± 53.8 (n = 5 mice); for Ren1d/DTA, 65.9 ± 40.2 (n = 7 mice); for DTA/DTA, 20.0 ± 19.4 µg AI·ml⁻¹·h⁻¹ (n = 5 mice)].

Blood chemistry. Homozygous male mice had significantly elevated BUN levels compared with the wild-type animals, and the levels in homozygous female mice were modestly but not significantly elevated [in males: for Ren1d/Ren1d, 30.0 ± 6.1 (n = 5 mice); for DTA/DTA, 40.4 ± 6.5* mg/dl (n = 5 mice); *P < 0.05; in females: for Ren1d/Ren1d, 30.2 ± 6.8 (n = 4 mice); for DTA/DTA, 37.6 ± 14.4 mg/dl (n = 7 mice); P = 0.140]. Potassium levels were significantly elevated in homozygous males and females [in females: for Ren1d/Ren1d, 4.75 ± 0.31 (n = 4 mice); for DTA/DTA, 6.54 ± 1.35* mmol/l (n = 5 mice); in males: for Ren1d/Ren1d, 4.38 ± 0.78 (n = 5 mice); for DTA/DTA, 5.34 ± 1.21* mmol/l (n = 5 mice); *P < 0.05 compared with Ren1d/Ren1d mice of same sex]. There were no differences in the values for sodium, calcium, glucose, chloride, and CO₂ between wild-type and DTA/DTA animals of either sex.

**Table 3. Diameter and vessel wall thickness of afferent arterioles and interlobular arteries**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Afferent Arterioles, µm</th>
<th>Interlobular Arteries, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diameter</td>
<td>Wall Thickness</td>
</tr>
<tr>
<td>Ren1d/Ren1d</td>
<td>15.1±3.7 (31)</td>
<td>4.8±1.2 (31)</td>
</tr>
<tr>
<td>DTA/DTA</td>
<td>14.7±2.9 (54)</td>
<td>4.9±1.1 (54)</td>
</tr>
</tbody>
</table>

Values are means ± SD; n (in parentheses), no. of arterioles or arteries.

**Table 4. Mean arterial pressure measurements**

<table>
<thead>
<tr>
<th></th>
<th>Mean Arterial Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ren1d/Ren1d</td>
</tr>
<tr>
<td>Males</td>
<td>77.6±11.2 (5)</td>
</tr>
<tr>
<td>Females</td>
<td>71.7±8.2 (6)</td>
</tr>
</tbody>
</table>

Values are means ± SD; n (in parentheses), no. of animals. *P < 0.05 compared to Ren1d/Ren1d.

**DISCUSSION**

Absence of renin-expressing cells causes renal morphological abnormalities. The ablation of renin cells from the kidneys results in almost total elimination of the local expression of renin and significant morphological alterations. This is in contrast to the effects of simple deletion of one renin gene (Ren1d or Ren2) in a two-renin-gene animal that results in minimal [Ren1d (6, 29)] or no [Ren2 (32)] changes in kidney morphology. [Note that mice have two alternative genotypes at the renin locus: some strains have only one renin gene, Ren1c, whereas others have two renin genes, Ren1d and Ren2 (3, 8).] In the DTA/DTA animals, the elimination of nearly all renin-expressing cells results in very reduced kidney renin levels, which ultimately will result in low levels of tissue angiotensin II. The phenotype of the DTA/DTA kidneys has some distinct differences from that of kidneys of the RAS deletion mice while also sharing some common features. The differences suggest that the absence of the renin cells and not just the lack of angiotensin II is responsible for the phenotype. Some of the morphological alterations in the kidney (tubular dilatation, disorganization, atrophy, and focal areas of interstitial fibrosis) can be attributed to the lack of angiotensin II, because they are similar to the changes seen in animals that are missing other components of the RAS that also affect the active angiotensin pathway. Mice with the single Ren1c gene (37), ACE (16), and AT1A/AT1B (28, 35). Not all of the effects, however, can be attributed to low levels of angiotensin II. Unlike the other RAS component mutants [including the single Ren1c gene (37)], there is no evidence of atrophy of the renal papilla in our animals.
A very striking difference from the other RAS gene deletions is that the vasculature in the DTA/DTA kidneys shows no thickening of the vessel walls or reduction in the size of the vessel lumen. In contrast, all of the RAS gene deletion animals (Atg, ACE, and AT1A/AT1B) share the same renal vessel abnormalities: the vessels are thickened, the lumens are obstructed, and the vascular tree is stunted (16, 20, 28, 33, 35). In these RAS gene deletion animals, there is also an increased number of renin-expressing cells along the renal arterioles, suggesting that either renin or the cells that contain it are involved in the vessel hypertrophy. Our DTA/DTA animals, which do not have arteriolar hypertrophy, also lack renin-expressing cells and are incapable of recruiting more renin cells in response to stress such as stimulation by captopril. These observations suggest that the renin cells per se may have a role in vessel hyperplasia. This participation could be a direct effect if the physical presence of the cells is important or indirect if the cells secrete a factor(s) that stimulates smooth muscle growth. The latter is supported by the observation that renin cells surround the hyperplastic vessels in ACE deletion mice but are not usually present in the hyperplastic portions of the vessels (16).

The DTA/DTA kidneys also have additional alterations different from those reported for the other RAS mutants. The kidneys are small and have a shrunken and granular surface. There is glomerular damage, with 25% of the glomeruli being either large and hyperplastic or atrophic. The areas of undifferentiated cells present in the DTA/DTA kidneys are composed of a mixture of quiescent cells, apoptotic cells, and a few lymphocytes. This is in contrast to the other RAS mutants, which contain lymphocytic infiltrates rather than foci of undifferentiated cells [Atg (7) and AT1A/AT1B animals (28)]. These findings highlight the importance of renin/renin cells on the development and maintenance of the structural integrity of the kidney.

Ren1d and Ren2 are synthesized in the same cells in the kidney. In the two renin gene strains of mice, Ren1d and Ren2 mRNA are both present in the kidney (6, 10, 29, 32) and are expressed at sexually dimorphic levels (21) with Ren1d expression being higher than Ren2 (accounting for 70% of the renin transcripts in males and 80% in females). Studies of the Ren1d deletion animals (29) showed that both proteins are generally made in the same cells, because the pattern of renin immunostaining in the kidney when only the Ren2 gene is present is similar to that seen when both genes are present. The DTA/DTA animals have essentially no renin protein containing cells (only 1% of the wild-type number). These remaining renin-positive cells most likely are those few cells that expressed only the Ren2 gene.

Ren1d/DTA animals, contrary to expectations, still retain some renin-positive cells, although the numbers are greatly reduced from wild-type levels (80% reduction compared with wild type). It appears that the effects of DTA expression on renin cells are dosage sensitive. It may be that the renin-expressing cells require a higher level of DTA expression to be killed, and in heterozygous cells, this level is not consistently achieved. Alternatively, a subset of cells may be protected by modifications of the chromosomal region around the Ren1d DTA allele such that its effective expression is reduced, and two copies of Ren1d-DTA are necessary for full lethality.

Analysis of the mRNA present in the kidneys revealed the presence of both Ren1d and Ren2 mRNA in Ren1d/DTA samples but only Ren2 in DTA/DTA samples (which have no Ren1d coding region). The Ren2 mRNA is most likely from the small population of Ren2-expressing cells that never express Ren1d, and in Ren1d/DTA kidneys, additionally from Ren1d-expressing cells that have not yet died. The detection of DTA mRNA in both Ren1d/DTA and DTA/DTA kidneys indicates the presence of some cells attempting to synthesize Ren1d protein (i.e., they have transcription driven by the Ren1d promoter) that do not survive long enough to make the protein. This result suggests that there may be a pool of cells in these kidneys that can be mobilized to replace the ablated renin cells. These cells begin to make renin mRNA but die before accumulation of renin protein due to the action of DTA.

Renin expression in SMGs is rather different. Ren2 is highly expressed, whereas Ren1d mRNA can only be detected by ribonuclease protection assays (27). In the SMGs of the Ren1d/DTA and DTA/DTA animals, there is a high level of Ren2 mRNA expression. As expected, renin protein is present in the same epithelial location and in equal abundance to the wild-type SMGs. In addition, there are no morphological changes evident in the DTA/DTA SMGs. These results show that cells expressing Ren2 are not damaged and demonstrate the specificity of the ablation of Ren1d-expressing cells.

Mice that lack JG cells have a sexually dimorphic drop in blood pressure. DTA/DTA females have a significant reduction in blood pressure of 25 mmHg and the males have slightly reduced pressure measurements. These findings are in agreement with Ren1d gene deletion studies in a two-renin-gene animal (6, 29). In the Ren1d-deletion animals thus far reported, the blood pressure values of the males are unaffected (6) or modestly reduced (29), whereas the females have a significant reduction of 13 mmHg, which is much less than the 30 mmHg seen in the other RAS gene deletions. In Ren1d-deletion mice (which do not have Ren2), the blood pressure measurements in both homozygous males and females are similarly reduced by ~30 mmHg (37). This suggests that Ren2 plays a role in the maintenance of blood pressure at least in the male animals.

The pressure changes in the DTA/DTA females are closer to those of the other RAS deletion animals. These differences were reflected in the PRC measurements, which were decreased to a much greater extent in females than in males. Again, the androgen responsive Ren2 gene may have a stronger compensatory effect in males than in females when renin levels are extremely low but the rest of the RAS is functional.

Ablation of JG cells and renal function. The combination of hypoplasia, glomerular damage, and tubular damage is manifested functionally by elevated BUN and potassium levels suggestive of decreased renal function. It is likely that this decrease reflects both the structural damage mentioned above and the known functional effects (or lack thereof) of angiotensin on renal hemodynamics. The contribution of each of these factors remains to be determined. Because renin is expressed at low levels in the adrenal gland, it is possible that the elevated potassium levels are indicative of adrenal and/or mineralocorticoid insufficiency. Future studies of adrenal function including measurement of aldosterone levels must be performed to address this issue. In addition to the kidney damage sustained by the DTA/DTA animals, these mice lack the ability to respond to challenges
to homeostasis as evidenced by their inability to recruit renin-expressing cells in response to captopril treatment. We previously suggested that recruitment of renin-expressing cells is a fundamental mechanism to maintain blood pressure homeostasis (11, 21). It is likely that future experiments on these animals will demonstrate that they are unable to maintain blood pressure in response to stressful situations such as dehydration or mild hemorrhage.

In summary, we have used gene targeting to ablate renin cells with DTA and produced a mouse model devoid of JG cells. We expect that this model will be a useful tool for researchers interested in renal physiology and pathology.

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REFERENCES


