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Histologic and molecular evidence of obstructive uropathy in rats with hereditary congenital hydronephrosis

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Abstract Partial obstruction of the upper urinary tract, a frequent challenge for the pediatric urologist, leads to renal damage, if deobstruction is delayed. Several but sometimes unsatisfactory animal models have been developed to study this phenomenon. Obstruction created by surgical manipulation lacks adequate correlation with a developing congenital obstruction. In some animals with congenital hydronephrosis, evidence of renal obstruction is absent. A study of the renal morphology of rats with hereditary unilateral hydronephrosis has exhibited clear evidence of renal obstruction distinguishable from renal dilatation. The renal mRNA expression of renin and transforming-growth factor-β_1 (TGF-β_1) was measured by a semiquantitative RT-PCR technique. In hydronephrotic kidneys, a marked loss of parenchyma, atrophy and dilation of tubuli and collecting ducts and interstitial fibrosis was observed. The mRNA expression of renin was increased significantly in comparison to controls, whereas the contralateral kidneys showed renin activity below control levels. TGF-β_1 expression was markedly increased in hydronephrotic kidneys, whereas contralateral kidneys did not differ significantly from control values. These data suggest the presence of renal obstruction and not only renal dilatation in these rats with congenital hydronephrosis. This colony seems to be a representative animal model to study congenital renal obstruction even in the fetal period without the need of surgical manipulation.

Key words Congenital hydronephrosis · Renal obstruction · Renin-Angiotensin-System · Transforming-growth factor-β_1 · Interstitial fibrosis

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Introduction

Prenatal ultrasonography detects a dilatation of the fetal upper urinary tract in one in 100–200 pregnancies. About one half of these cases persist postpartum. The main problem for the pediatric urologist is to clearly differentiate simple dilatation of the renal pelvis from significant renal obstruction which, left untreated, will cause progressive deterioration of the renal function and will finally lead to end-stage renal failure. The morphologic correlate of renal obstruction is obstructive uropathy. Its incidence is distinctly lower than postpartal hydronephrosis [27].

A complete understanding of the development of obstructive uropathy is essential for a clear differentiation between dilatation and obstruction. Several animal models have been used so far to investigate the pathophysiology of obstruction of the upper urinary tract. Renal obstruction can be introduced by partial or complete surgical ligation of the ureter. However, because of the arbitrary onset, duration and degree of restriction of the urinary outflow from a former healthy kidney, this model represents inadequately the situation of congenital renal obstruction in humans.

Since 1960, hereditary congenital hydronephrosis in various inbred strains of rats has been described by several authors [6, 8, 17, 19, 24, 26]. These animals provide the opportunity for observing the development of congenital hydronephrosis at the onset of the early stages of the disease. Although several of these strains have been characterized morphologically, only two of them were used for studies of the pathophysiology of ureteral obstruction. Animals of both strains showed moderate restriction of urinary outflow of the hydronephrotic kidneys, but no morphologic damage of the renal parenchyma was observed [7, 23]. As obstructive uropathy is characterized by specific structural damage of the affected kidney [10], it is not

clear, whether renal obstruction was present in these animals.

We successfully established a colony of rats with hereditary congenital hydronephrosis that derived from the inbred strain described by Tauchi et al. [26]. Nearly 70% of the offspring of this colony had a congenital hydronephrosis. Ten percent of the littermates had bilateral hydronephrosis. No predisposition to side or sex was found in this strain. Recently, abnormalities of the pelviureteric junction as hypoplasia, splitting or fibrosis of the local smooth muscle fibers were reported, suggesting their participation in the development of the hydronephrosis in these rats [12].

Although this colony of rats has been known for several years, its value as an animal model to study congenital renal obstruction has not been defined yet. Thus, the aim of this study was to determine, whether hydronephrosis in these animals is only an asymptomatic renal dilatation or due to significant renal obstruction. Therefore, unilateral hydronephrotic kidneys, contralateral kidneys and kidneys of healthy animals were investigated for molecular and histopathological evidence of obstructive uropathy.

Materials and methods

Animals

We investigated kidneys obtained from unilateral hydronephrotic or healthy control rats from the colony of rats with congenital hydronephrosis described by Tauchi et al. [26]. The animals were housed in polycarbonate containers and subjected to a 12 h day-night rhythm with free access to water and food. The molecular investigations were performed with kidney tissue from 32 day-old rats, weighing 90–110 g. The histological investigations were performed on specimens from 7.5-week-old animals, weighing 250–270 g. The rats were sacrificed after intraperitoneal injection of ketamine by neck fracture. The kidneys were extracted in toto, fixed in formaldehyde for histopathological investigations or frozen in liquid nitrogen for subsequent molecular investigation.

Histology

Paraffin-embedded formalin fixed sections were cut and stained using routine techniques with periodic acid Schiff (PAS) or Goldners trichrome.

RNA-Preparation

Total cellular RNA (tcRNA) was extracted from frozen tissue by acid guanidinium-isothiocyanate-phenol-chloroform-isoamylalcohol [4] and purified with the Qiagen RNeasy Mini Kit [2]. RNA was quantified by spectrophotometry or fluorometry and checked for integrity as well as quantity on 0.8% agarose gels.

Primers

For two-step reverse transcriptate polymerase chain reaction (RT-PCR) (Gene Amp RNA PCR, Perkin Elmer) 0.25 μg RNA was transcribed and amplified with gene specific primers for renin, TGF-β_1 and β-actin:

		PCR yield (bp)
Renin FW	5′-TCAAAGTCATCTTTGACACG-3′	
Renin RV	5′-AGACAGAAAACACTTCCTCC-3′	385
TGF-1β FW	5′-CAAGTCAACTGTGGAGCAAC-3	
TGF-1β RV	5′-AACCCAGGTCCTTCCTAAAG-3′	448
β-actin FW	5′-CTACAATGAGCTGCGTGTGGC-3′	
β-actin RV	5′-CAGGTCCAGACGCAGGATGGC-3	270 (for β-actin mRNA) 221 (for competitor cRNA)
β-actin deletion primer (FW)	5′-TACAATGAGCTGCGTGTGGCG CCAACCGCGAGAAGATGACCCAG-3′	

PCR Techniques

The mRNA expression of renin and transforming-growth factor-β_1 (TGF-β_1) was measured by a modified semiquantitative RT-PCR-technique. To improve the sensitivity of this relative measurement, the target gene was related to a quantified housekeeping gene β-actin. The quantity of β-actin was determined by competitive quantitative RT-PCR (cqRT-PCR) using a gene specific cRNA competitor fragment which yields a PCR product 49 bp shorter than the usual β-actin amplicon. The deletion in the β-actin sequence was introduced in a PCR based process [28] by a deletion primer consisting of the β-actin FW primer and a nested (49 bp) FW primer appended at the 5′end of the β-actin FW primer. The PCR product carrying the deletion was sliced out of a preparative gel. To generate a cDNA template for T_7 transcription [20] of competitor cRNA, a primer composite of the T_7 promoter region attached to the 5′-end of the β-actin FW-primer was used. In this PCR, the β-actin RV primer 5′-elongated by an oligo $d(T)_{25}$ could produce an oligoA tail in subsequent in vitro cRNA transcriptions (MEGAscript, Ambion). For extraction of DNA from agarose gels and purification of PCR products QIAEX II and QIAquick (Qiagen), respectively, were used.

The competitor cRNA was treated with RNase-free DNase and was extracted once with phenol-chloroform and once with chloroform alone. The cRNA concentration was measured in serial dilutions by spectrophometer (Genequant II Pharmacia) and by fluorometer (Biorad) to yield a 1000 amol/μl stock solution. Competitor cRNA dilutions were added to cqRT-PCR between 10 and 100 amol/μg tcRNA.

Analysis of the PCR products

PCR products were run on 2% agarose gels and analyzed by densitometry (Herolab E.A.S.Y Win32). Ratios of β-actin over competitor intensities (Fig. 1) were plotted against competitor concentrations in a double logarithmic scale for the cqRT-PCR quantification of β-actin. Relative RT-PCR was performed for the target genes renin and TGF-β_1 relating densitometry values of each to densitometry values of β-actin. All PCR were performed in exponential phases. The ratio target/β-actin then was multiplied by the cqRT-PCR results for β-actin of each individual sample. With these results corrected for slight differences in housekeeping, gene expression were defined as ''relative units'' (rU).

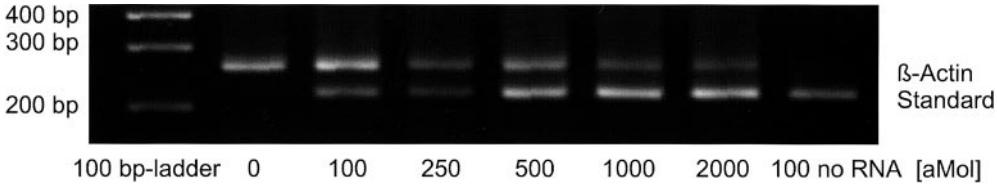

Fig. 1 Representative gel electrophoresis of products from cqRT-PCR, showing bands of β-actin (270 bp) and its competitive standard (221 bp) in different concentrations (100–2000 amol/0.25 μg RNA) for competitive quantification of the β-actin mRNA-concentration of a rat kidney

Statistics

Data are presented as mean ± SE. Comparisons between control, hydronephrotic and contralateral kidneys were performed by one-way analysis of variance, followed by the Scheffe's Test, to determine differences between the groups. Statistical significance was defined as $P < 0.05$.

Results

Morphology

We investigated three hydronephrotic and their three corresponding contralateral kidneys and four control kidneys of animals at the age of 7.5 weeks. Examples of representative pathologic changes are shown in Fig. 2. Compared to contralateral or control kidneys, the hydronephrotic kidneys showed a massive dilation of the renal pelvis and calices and a corresponding thinning of the renal parenchymal width. The papilla was flattened and shortened (Fig. 2). Histologically, we observed dilated and atrophied distal tubuli and collecting ducts. The dilated tubuli were surrounded by increased extracelluar matrix. In addition we observed partly interstitial fibrosis in the widened interstitial space (Fig. 2). The pathologic changes were focused in the medullar area and decreased towards the cortical area. The cortical structures were nearly unaltered. The glomerula showed

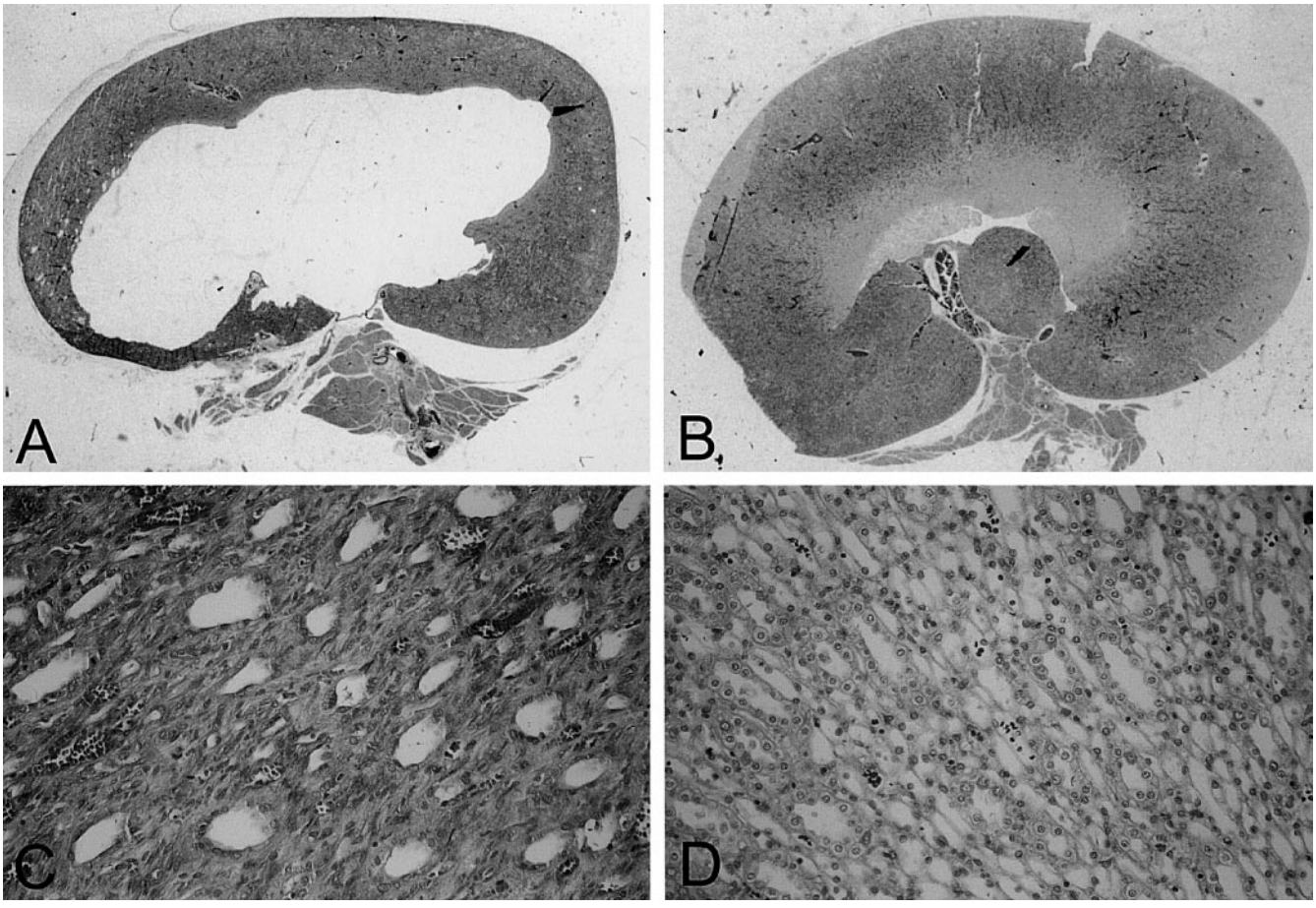

Fig. 2A–D Trichrom-Goldner-stained sections of kidneys from 7.5-week-old rats with congenital unilateral hydronephrosis. **A** Section of a hydronephrotic kidney showing marked loss of renal parenchyma (× 2.5). **B** Section of the contralateral kidney, showing normal renal morphology (× 2.5). **C** Section of the medullar area of a hydronephrotic kidney, showing dilation of the tubules and a widened interstitial space with interstitial fibrosis (× 200). **D** Section of the medullar area of a contralateral kidney with normal renal parenchyma (× 200)

no damage. Histologically there were no differences in morphology between, the contralateral kidney and control kidneys (Fig. 2).

Molecular biology

The mRNA expression of renin of the kidneys from 32 day-old rats with unilateral hydronephrosis was measured by relativeRT-PCR and presented as mean ± standard error of "relative units" as defined in the Material and method section. The renin expression of the hydronephrotic kidney ($n = 5$) was 42.4 ± 8 rU, of the contralateral kidney ($n = 5$) 11.5 ± 6.5 rU and of the control ($n = 5$) 25.6 ± 9.2 rU (Fig. 3). This data indicates a marked increase in the renin gene expression of hydronephrotic kidneys compared with controls ($P < 0.05$ versus control). The renin gene expression of the contralateral kidney was decreased in these animals ($P < 0.05$ versus control).

We analyzed the mRNA expression of TGF-β_1 of kidneys from rats with unilateral hydronephrosis and healthy control animals at the age of 32 days. The mean TGF-β_1 expression of the hydronephrotic kidney ($n = 5$) was 55.3 ± 13.1 rU, of the contralateral 18.7 ± 8 rU and the control ($n = 5$) 14.3 ± 8.8 rU (Fig. 4). We observed a marked increase in TGF-β_1 expression in hydronephrotic kidneys ($P < 0.01$), whereas the TGF-β_1 expression of the contralateral kidneys did not differ from controls.

The mRNA expression of the housekeeping gene, β-actin, of all investigated RNA-samples was measured by cqRT-PCR. The β-actin expression of the hydronephrotic kidneys ($n = 5$) was 280 ± 65 amol/μg RNA, of the contralateral kidneys ($n = 5$) 180 ± 45 amol/μg RNA and of the control kidneys ($n = 5$) 215 ± 55 amol/μg RNA, respectively. The analysis of variance for these values showed no significance.

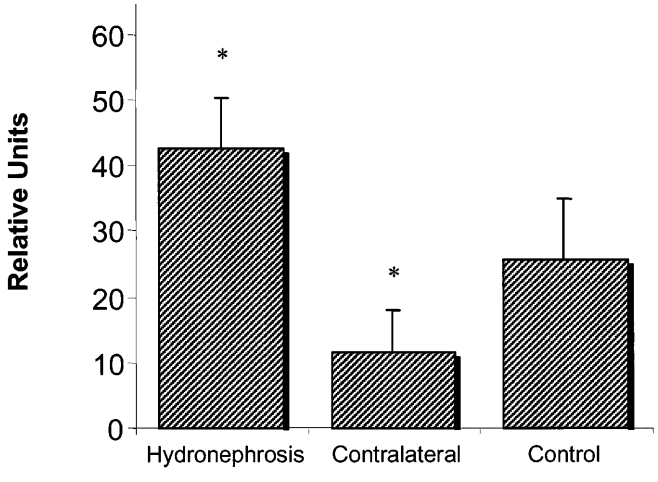

Fig. 3 Messenger RNA expression of renin in control kidneys from healthy rats ($n = 5$), hydronephrotic ($n = 5$) and contralateral kidneys ($n = 5$) from rats with congenital unilateral hydronephrosis. Values are means + SE. Significant differences to control values are $^*P < 0.05$

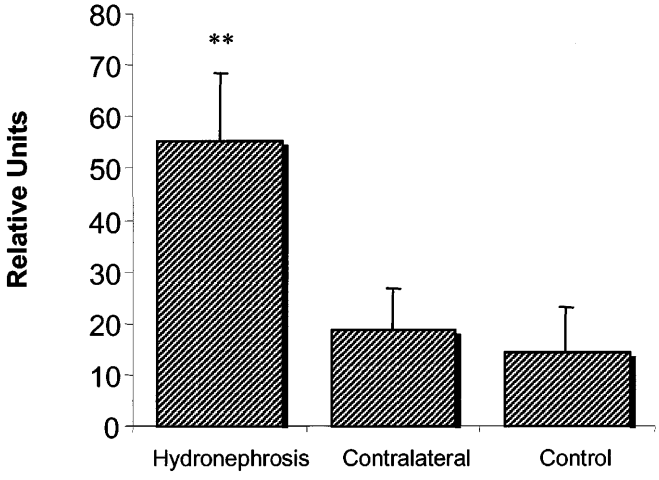

Fig. 4 Messenger RNA expression of TGF-β_1 in control kidneys from healthy rats ($n = 5$), hydronephrotic ($n = 5$) and contralateral kidneys ($n = 5$), from rats with congenital unilateral hydronephrosis. Values are means + SE. Significant differences to control values are $^{**}P < 0.01$

Discussion

In this study, an animal model for congenital renal obstruction is investigated and quantified by the expression of renin and TGF-β_1 as marker genes for this obstructive phenomenon. So far, no representative animal model exists to study the effects of renal obstruction on developing kidney tissue that is analagous to congenital renal obstruction in humans. Frequently used models are newborn rats, undergoing a ureteral obstruction by surgical ligation of one ureter on their first day of life. The development of the rat kidney is incomplete at birth and is complete between the 7^{th} and 10^{th} day of life making it a useful source of material for studying the developing kidney in this period [21].

Chronic complete unilateral obstruction in newborn rats imposed a decrease in glomerular filtration rate (GFR) on the affected side [13], organ weight, DNA content and synthesis of proteins [5]. The kidneys showed an increase in interstitial volume, caused by accumulation of extracellular matrix and collagen deposition, resulting histologically in a tubulointerstitial fibrosis [10]. The interstitial fibrosis is an important marker of obstructive uropathy. Its degree correlates with the impairment of renal function [29]. Furthermore, neonatal renal obstruction leads to a developmental arrest of the kidney, which contributes to an arrested growth of the organ and decrease in renal function [5]. The function and growth of the intact contralateral kidney increased corresponding to the decrease of the obstructed kidney, a phenomenon named "renal counterbalance."

The renin angiotensin system (RAS) is likely to play a mayor role as a mediator of the morphological and functional changes seen in obstruction. The expression of renin mRNA was increased in neonatal obstructed rat

kidneys [5]. The following increase in the activity of angiotensin II leads to a decrease in renal blood flow, causing renal ischaemia that is suggested to be the main reason for the growth arrest of obstructed kidneys [3]. Furthermore, the RAS controls the expression of several growth factors, e.g., TGF [14, 30], which in turn is considered to be a mediator of the development of interstitial fibrosis in obstructive uropathy [5, 16].

However, it remains questionable, whether alterations after surgical ureteral obstruction in neonatal rats can be transferred on congenital renal obstruction, since this phenomenon occurs already in the fetal period. During fetal life, a major part of the subsequent kidney function is accomplished by the placenta. For this reason, even severe renal injury in this period would have no influence on fluid or electrolyte homeostasis of the fetus. Because of the placental dialysis function, renal blood flow is markedly lower in the fetus than after birth. Therefore, the surgical model of neonatal obstruction in the rat is not fully representative for congenital renal obstruction. Animals with hereditary congenital renal obstruction represent a much more promising model, because obstructive kidneys can be investigated in the fetal period as well as postpartum.

Hereditary congenital hydronephrosis in different inbred strains of rats has been reported by several authors [6, 8, 17, 19, 24, 26]. So far, there is insufficient proof that significant obstruction is present in the dilated kidneys of these animals. Therefore, none of these colonies can be regarded undisputedly as animal models for congenital renal obstruction.

In rats from the Tulane colony [8] increased renal pelvic pressure and simultaneously decreased glomerular filtration rate (GFR) of hydronephrotic kidneys were interpreted as signs of renal obstruction [7]. Blockade of angiotensin II markedly improved the restricted GFR, suggesting an important mediator role of the RAS in decreasing the renal function in these animals [1, 9]. Described morphologic alterations of the hydronephrotic kidneys were dilation of the tubuli and flattening of the tubular epithelium without interstitial fibrosis [8]. Because of this lack of marked histologic changes to the affected kidneys as compared to normal kidneys, it is doubtful, whether these animals have significant renal obstruction or only renal dilatation.

Other strains of rats with congenital hydronephrosis showed similar results. Except for dilation of the renal calices, Sellers et al. found unaltered renal morphologic structures in hydronephrotic kidneys from rats of his colony [24]. No histologic data were given for rats with congenital hydronephrosis from the ACI strain [19]. Cohen et al. also described near normal renal architecture in the Brown-Norway strain [6]. Long-term follow-up of rats from this colony with congenital unilateral hydronephrosis showed no deterioration of renal function within 70 weeks [22], suggesting that renal obstruction is absent in the hydronephrotic kidneys, although furosemid renography indicated its presence. Therefore, the authors propagated this colony as an experimental model for asymptomatic unilateral renal dilatation [23].

Only Machado and Lozzio found marked renal damage in hydronephrotic kidneys predominantly in the medullar area from rats of the MRC/H colony. They reported on atrophy of the medulla in the affected kidneys, but there was no description of interstitial fibrosis even in this strain [18]. Rats with unilateral hydronephrosis of the MRC/H colony showed an increased plasma renin concentration. Simultaneously, the renin contents of both the hydronephrotic and the contralateral kidney were decreased. The authors suggested a diminished production or storage capacity for renin in the hydronephrotic kidney [25]. However, this can explain the decreased renin content of the hydronephrotic but not of the contralateral kidney. This phenomenon was not further investigated. Surprisingly, to our knowledge, the most recent investigations in these animals were reported in 1978, although Kentera et al. proposed this inbred strain as a useful animal model for studying the pathogenesis of hydronephrosis from the very beginning of embryonic life [15].

Searching for evidence of renal obstruction in rats of the Imamichi hydronephrosis colony [26], we showed a marked loss of renal parenchyma and its normal organization in the affected kidneys of 7.5-week-old animals with congenital unilateral hydronephrosis. We found areas of tubulointerstitial fibrosis, mainly in the suburothelial areas of the medulla (Fig. 2C). There were no histological differences between the contralateral kidneys and kidneys of healthy control rats (Fig. 2D). Ishitobi et al. described a focal glomerulosclerosis in the affected kidneys in 9 month-old rats from the same colony [11].

Suggesting a major role of the RAS in the development of obstructive uropathy, we focused on changes to the renal renin activity in rats with unilateral congenital hydronephrosis. We observed a significant increase in the mRNA expression of renin in the hydronephrotic kidneys as compared to kidneys of normal rats. Simultaneously, we found a significant decrease in the RAS activity in the contralateral kidney (Fig. 3), which can be interpreted as a sign of ''renal counterbalance''.

The activity of TGF-β_1 in kidneys from rats with hereditary hydronephrosis has not been investigated so far. TGF-β_1 directly stimulates the synthesis of the extracellular matrix and is discussed to be a main mediator of the development of interstitial fibrosis [30], which is the pathomorphologic marker lesion of irreversible renal injury [29]. In animals of our colony with unilateral congenital hydronephrosis we found a markedly increased mRNA expression of TGF-β_1 in the affected kidney, whereas the contralateral mRNA expression was similar to control values (Fig. 4).

Since the morphologic alteration of obstructed renal parenchyma is responsible for the chronification and the progressively deteriorating course of the disease, a representative animal model for congenital renal obstruction has to show excessive morphologic damage of the affected kidney. In hydronephrotic kidneys from this

inbred strain of rats with congenital hydronephrosis we found interstitial fibrosis and an increased renal expression of its mediator TGF-β_1 in addition to structural renal damage. Furthermore, an increased renal expression of renin, a main mediator of the several changes caused by renal obstruction was observed. However, each of the presented changes ito renal morphology, renin or TGF-β_1 mRNA expression alone do not prove the presence of obstructive uropathy in these animals– other renal diseases may show upregulated renin expression, as well as TGF-β_1 expression or interstitial fibrosis. But the combination of a dilated kidney with morphological signs of chronic renal injury, overexpression of renin mRNA as a marker of an activated RAS and overexpression of TGF-β_1 mRNA as a marker of interstitial fibrosis provide solid evidence of renal obstruction underlying congenital hydronephrosis in these rats.

Furthermore, compared to results obtained from rat kidneys after complete surgical ureteral obstruction in the neonatal period [5], our data also suggest marked signs of obstructive uropathy in the hydronephrotic kidneys.

Conclusion

The presented data provide evidence for obstructive uropathy in this inbred strain of rats with congenital hydronephrosis. Although the strain was described for the first time in 1980, until now, it has not been propagated as an animal model to investigate congenital renal obstruction. However, in our opinion this colony of rats will be highly suitable for studying the pathogenesis of renal obstruction without the need for previous surgical manipulation. Therefore, it will be possible to observe congenital obstruction from the very beginning. The morphology of obstructive uropathy is comparable in human and rat kidneys, despite many differences in anatomy and organ development. Thus, rats from the Imamichi hydronephrosis colony with congenital renal obstruction provide an excellent animal model for studying the pathogenesis of congenital renal obstruction in humans.

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