Tubular gelatinase A (MMP-2) and its tissue inhibitors in polycystic kidney disease in the Han:SPRD rat

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Tubular gelatinase A (MMP-2) and its tissue inhibitors in polycystic kidney disease in the Han:SPRD rat. Thickening of the tubular basement membrane is one of the hallmarks of polycystic kidney disease (PKD). The present study was conducted to investigate the potential role of the matrix metalloproteinase-2 (MMP-2) and its specific tissue inhibitors (TIMP-1 and TIMP-2) in the accumulation of matrix components in PKD. As a model of PKD, two-month-old heterozygous Han:SPRD rats, which are at an early stage of cystogenesis, were used. MMP-2, but not MMP-9 (gelatinase B) nor MMP-3 (stromelysin) could be detected in proximal tubules of the normal rat kidney. The presence of the inhibitors TIMP-1 and TIMP-2 was confirmed on the mRNA level. In tubules from PKD rats MMP-2 activity was lower (31 ± 8 vs. 58 ± 7 U/prep., $N = 9$, $P < 0.05$), mRNA of MMP-2 was reduced 4.2 ± 0.6-fold ($N = 4$, $P < 0.05$) and enzyme protein was depressed 3.8 ± 0.8-fold ($N = 4$, $P < 0.05$). By contrast, TIMP-1 mRNA was 9.0 ± 1.1-fold and TIMP-2 mRNA 3.8 ± 0.7-fold ($N = 4$, $P < 0.05$) elevated over controls. Cyst fluid from homozygous rats contained MMP-2 protein and activity. These findings indicate that tubular MMP-2 activity is reduced in PKD, due to down-regulation of MMP-2, up-regulation of TIMP-1 and TIMP-2, and luminal secretion of the enzyme. It is conceivable that these alterations relate to the enhanced matrix accumulation observed in the evolution of PKD.

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common hereditary disorders in humans, which is estimated to afflict 1 in 1000 live births. This disease accounts for up to 10% of all cases of end-stage renal disease [1]. The phenotypic expression of ADPKD is characterized by innumerable cysts scattered throughout the cortex and medulla of both kidneys. The cysts progressively enlarge and lead to the onset of chronic renal failure by the sixth decade in approximately one-half of the affected individuals [2]. Three major pathophysiological events have been identified to explain cyst formation: enhanced proliferation of tubular epithelia, transepithelial secretion of fluid into the cyst and abnormal turnover of tubulointerstitial matrix [3–6].

The turnover of extracellular matrix is dependent on a balance between its synthesis and degradation, and consequently enhanced matrix deposition may be either due to increased synthesis and/or reduced activity of proteinases responsible for the remodeling of the matrix components. Several lines of evidence suggest the involvement of metalloproteinases (MMPs) in matrix turnover, including the glomerular extracellular matrix [7–11]. The gene family of MMPs includes the collagenases, gelatinases and stromelysins, which are Zn-dependent endopeptidases with the combined ability to degrade all the components of ECM at physiological pH [12–14]. These enzymes are specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), of which TIMP-1, TIMP-2 and TIMP-3 have been described [15–18].

Several studies have highlighted the putative role of MMPs and their specific inhibitors (TIMPs) in the degradation of extracellular matrix including the matrix of the glomerulus [8, 9]. By contrast, little is known concerning tubular metalloproteinases. Walker, Kaushal and Shah [19] described a laminin-degrading activity in rat renal tubular homogenates, which could be inhibited by EDTA but not by TIMP. In a later paper the authors identified this metalloproteinase as meprin, which had been shown previously to degrade azocasein and the insulin B chain, but was not known to cleave extracellular matrix components [20]. MMP-2 and MMP-9 were identified by zymography in conditioned media from cultured rat and mouse tubular epithelial cells [21, 22] as well as from cystic *cpk/cpk* mouse cells [23].

The objective of the present study was twofold: (1) to define MMPs and TIMPs from normal proximal tubules; and (2) to investigate whether the levels of these proteinases and their inhibitors were altered in a model of heterozygous PKD rats (Han:SPRD) in an early stage of cystogenesis.

Methods

Animals

Homozygously (cy/cy) and heterozygously (cy/+) affected male Han:SPRD rats as well as healthy littermates (+/+) were used in this study. This model of ADPKD is derived from a spontaneous mutation in the Sprague-Dawley strain and the disease is transmitted in an autosomal dominant pattern. Homozygous animals develop excessive cysts with gross renal enlargement and die early (after 4 weeks) from renal failure. By contrast, heterozygously afflicted rats suffer from slow, progressive renal cystic disease and death usually occurs after 12 to 21 months [24–26].

Heterozygously affected Han:SPRD rats (cy/+) were investigated at the age of two months. Age-matched, unaffected littermates (+/+) were used as control animals. The presence of enlarged kidneys was checked by abdominal palpation and further controlled by histopathology to identify affected animals. Cyst

fluid was obtained from homozygous three-week old Han:SPRD rats. All animal experiments were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Isolation and characterization of proximal tubules

Animals were anesthetized with hexobarbital (150 mg/kg), the kidneys removed, and tubules isolated according to the method of Spiro [27]. In brief, renal cortex from one kidney of each animal was minced and resuspended in ice-cold saline. A sample of the total homogenate was reserved and the rest of the homogenate was passed through a stainless steel sieve (250 μm pore size, Linker, Kassel, Germany). The sieved material was suspended in ice-cold saline and poured over a 50 μm nylon sieve (Schweizerische Seidengazefabrik, Zurich, Switzerland), thereby allowing cell debris and small fragments to pass through, while retaining glomeruli and tubules. Separation of tubules from glomeruli was achieved by passage on a 150 μm nylon sieve. The tubuli which were retained by this sieve were resuspended on 50% Percoll and centrifuged at 12,200 g for 30 minutes at 4°C according to Vinay, Gougoux and Lemieux [28]. This preparation contained proximal tubules obtained from a single kidney after sieving and Percoll gradient separation.

The fraction containing proximal tubules was resuspended in 0.9% saline, washed three times and then examined by light microscopy. A sample from each preparation was also processed for histological evaluation after formalin fixation and periodic acid-Schiff (PAS) staining. This allows for clear differentiation between proximal (granular yellow cytoplasm and pink brush border membrane) and distal tubular epithelia (clear cytoplasma, no brush border membrane) [28]. The purity of the proximal tubular preparation was 95%. Isolated proximal tubules were either used for RNA isolation or were disrupted by sonication (5 bursts, 2 seconds each) at 4°C for the measurements of enzyme activities.

Cyst fluid samples

Cyst fluids from homozygous polycystic kidneys were obtained by puncture and were free of leukocytes. Proteolytic enzyme activities were measured in unconcentrated supernatants after centrifugation and filtration through a 0.2 μm Durapore membrane which consists of polyvinylidenfluoride and displays very low protein binding (Millipore, Eschborn, Germany). Prior to zymography cyst fluids were concentrated 10 times using microconcentrators (Microcon 10, Amicon, Beverly, MA, USA).

Gelatinolytic activity

Gelatinolytic enzyme activities were measured fluorometrically as described by us previously [29]. Denatured (gelatin) rat tail collagen type-1 was used as substrate. Latent enzyme was activated by preincubation with 0.5 mM of oxidized glutathione. The activity of gelatinase was determined in the presence of 4 mM PMSF for inhibition of serine proteinases. After 18 hours of incubation at 25°C, the digested fragments were labeled with 2% fluorescamine. Fluorescence was measured by excitation at 390 nm and emission at 475 nm. The assay was quantified by determening a calibration curve relating the fluorescence emission reading to an equivalent concentration of H-Leu-Leu-OH (Bachem, Heidelberg, Germany). One unit was defined as 1.0 nM amino groups cleaved from the gelatin molecule per minute.

Zymograms

SDS substrate gels were prepared by including gelatin or casein at a final concentration of 1 mg/ml in standard 10% acrylamide gels according to Laemmli [30]. Electrophoresis was performed under non-reducing conditions at 4°C and 20 mA/gel. Thereafter, gels were washed for one hour at room temperature in 2.5% vol/vol Triton X-100 with two changes, and then incubated over night at 37°C in 50 mM Tris-HCl/0.1 M NaCl/10 mM CaCl$_2$/4 mM PMSF/0.05% Brij/pH 7.6 in the presence or absence of 20 mM EDTA. Staining was achieved with Coomassie Blue R-250. A high-molecular mixture of protein (205 to 29 kD; Sigma, Munich, Germany) was used as molecular standards.

Western blot analysis

Prior to electrophoresis homogenates of proximal tubules containing equal amounts of protein or samples of cyst fluid were activated with 2 mM APMA (4-aminophenolmercuric acetate) (Sigma) for 18 hours at 25°C and applied to a gelatin-Sepharose column (1 ml) equilibrated in 10 mM Tris/HCl pH 7.6/5 mM CaCl$_2$/0.04% Brij 35 (vol/vol) and washed with the same buffer. Bound material was eluted with equal volume of the same buffer plus 10% DMSO. The gelatin-binding fraction was further concentrated 50-fold using Microcon 10 concentrators. Samples (20 μl) and standard purified human MMPs (100 ng) were subjected to electrophoresis on 10% (wt/vol) gels under reducing conditions. Proteins were transferred electrophoretically at 100 V for one hour to nitrocellulose membranes using the Bio-Rad Mini Trans-Blot Apparatus as recommended by the manufacturer. The membranes were blocked by incubation with 1% (wt/vol) BSA in PBS containing 0.1% (vol/vol) Tween 20 (PBST) for two hours. After washing in PBST for five minutes, the nitrocellulose was incubated over night at 4°C with the primary antibody in a dilution of 1:500 (rabbit polyclonal antibody to human MMP-2; Anawa, Zurich, Switzerland) in PBS containing 0.05% Tween 20 and 1% BSA (PBSTB). Thereafter, the nitrocellulose strips were washed for one hour in PBST and incubated in PBSTB containing the second antibody in a dilution of 1:1000 (anti-rabbit IgG, peroxidase-linked, species specific whole antibody) from Amersham (Amersham, UK) for one hour. The strips were then extensively washed in PBST and incubated in ECL Western blotting detection reagent (Amersham) and exposed to Kodak X-OMAT AR film.

Northern blot analysis

Northern blots were performed on total RNA extracted from proximal tubules isolated from single animals with the acid guanidinium thiocyanate phenol chloroform extraction method [31]. Total RNA was quantitated by UV spectrophotometry at 260 nm and 280 nm. RNA samples (20 to 40 μg per lane) were denatured and electrophoresed through 1.2% agarose formaldehyde gels and then transferred by pressure blotter (Posi Blot, Stratagene, La Jolla, CA, USA) onto nylon filters (GeneScreen, DuPont-NEN, Boston, MA, USA). The RNA was fixed to the filters by ultraviolet irradiation for two minutes at 254 nm (UV Stratalinker, Stratagene).

All probes were radiolabeled with [^{32}P]dCTP (3,000 Ci/mmol) using random decamer oligonucleotide primers and Klenow enzyme [32] with the DECAprime DNA labeling kit (Ambion, Austin, TX, USA). Membranes were prehybridized at least for two hours at 42°C in 5 × SSPE/50% deionized formamide/5×

Table 1. Body weights, single kidney weights and renal function in heterozygous PKD and control rats

Han:SPRD animals	Heterozygous PKD	Controls
Body weight *g*	331 ± 6	334 ± 9
Kidney weight *g*	2.1 ± 0.12[a]	1.1 ± 0.05
Serum creatinine *mg/dl*	0.61 ± 0.03	0.54 ± 0.02
Creatinine clearance *μl/min/100 g*	472 ± 25	491 ± 24

Data are given as means ± sem from 9 animals in each group.
[a] *P* < 0.05 for heterozygous PKD vs. healthy offspring (2 months)

Table 2. Gelatinolytic activities in proximal tubules from heterozygous PKD and control rats

	− Glutathione − EDTA	+ Glutathione − EDTA	+ Glutathione + EDTA
		U/tubular preparation	
PKD	3.0 ± 0.7	31.2 ± 8.3[a]	1.2 ± 0.3
Controls	5.6 ± 0.9	57.9 ± 6.9	2.4 ± 0.4

Data are given as means ± sem from 9 animals in each group. All experiments were performed in the presence of 4 mM PMSF.
[a] *P* < 0.05 for PKD animals vs. healthy offspring (2 months)

Denhard's/0.5% SDS/50 μg/ml herring sperm DNA. Hybridization was carried out overnight at 42°C. The filters were washed twice for 15 minutes in 2 × SSPE at room temperature and then agitated in 2 × SSPE/2% SDS at 65°C for 30 minutes and exposed to Kodak X-OMAT AR film at −80°C for one to four days. After autoradiography, the filters were stripped and hybridized according to the same procedure with a human GAPDH cDNA probe (1.2 kb *Pst*I restriction fragment of pBR 322). A 0.24 to 9.5 kb RNA ladder (Gibco BRL, Gaithersburg, MD, USA) was used as reference for RNA size. The autoradiographs were digitized using an EPSON GT 6000 scanner (Epson, Tokyo, Japan). For all RNA samples the density of each individual RNA band was normalized for that of a GAPDH mRNA band to correct for the difference in RNA loading and/or transfer. Data were expressed in relative optical density units.

Plasmids containing cDNA for mouse MMP-2 (pBR 322), MMP-9 (pBluescript II SK) were kindly provided by Dr. K. Tryggvason (University of Oulu, Finland) and cDNAs for mouse TIMP-1 and TIMP-2 (pBS) were a gift from Dr. K. Leco (University of Calgary, Canada). The plasmid containing cDNA for rat MMP-3 (pUN 121) was obtained from ATCC (Rockville, MD, USA).

Restriction fragments isolated from these plasmids were as follows: *Eco*RI 2.8 kb fragment for MMP-2; *Eco*RI 1.7 kb fragment for MMP-3; *Eco*RI-HindIII 1.8 kb fragment for MMP-9; *Bam*HI/*Hin*dIII 825 bp fragment for TIMP-1; and *Hin*dIII/*Xho*I 700 bp fragment for TIMP-2.

Analytical methods

Each preparartion of isolated tubules and samples of total cortical homogenates were assayed for fructose-1,6-diphosphatase activity according to the method of Pontremoli et al [33]. Results were expressed as U (nM of substrate converted per min at 37°C) per whole kidney cortex. Analysis of serum and urine samples for creatinine was performed using a Technicon autoanalyzer (Bayer Diagnostics, Munich, Germany).

Statistics

Results were expressed as means ± sem. Statistical analysis was performed using the unpaired Student's *t*-test. Significance was accepted at the 5% level.

Results

Kidney weight and function in heterozygous PKD rats

The body wt was comparable in two-month-old polycystic (331 ± 6 g) and control animals (334 ± 9 g). Kidney weight was considerably higher in PKD rats (2.1 ± 0.1 g) than in control animals (1.1 ± 0.05 g) and the enlarged kidneys could be readily detected by abdominal palpation. Polycystic animals displayed normal glomerular filtration rates, as measured by creatinine clearance. Clinically these rats appeared healthy and showed no signs and symptoms of uremia (Table 1).

Characterization of isolated proximal tubules

Light microscopy of PAS-stained preparations revealed that proximal tubules were isolated to a purity of 95% and no glomeruli were detectable. To demonstrate that the isolation procedure resulted in comparable preparations from both PKD and control animals, the activity of fructose-1,6-diphosphatase, a specific marker for proximal tubular epithelial cells [25], was measured both in the kidney cortex homogenate and in the final proximal tubule preparation. Enzyme activity in the cortical homogenate was comparable in PKD and control rats (PKD 294.4 ± 23.8 vs. controls 301.6 ± 21.7 U/kidney cortex; N = 9). In parallel, isolated tubules from the initial homogenate also displayed more or less identical fructose-1,6-diphosphate activities (PKD 226.1 ± 22.4 vs. controls 234.9 ± 18.9 U/tubular prep.; N = 9). This represents a 77.7% and 76.8% recovery, respectively, and indicates that the isolation resulted in comparable tubular preparations both from normal and diseased kidneys. Furthermore, the amount of protein in both preparations was comparable (PKD 5.7 ± 0.8 vs. 6.2 ± 0.6 mg per tubular preparation; N = 9).

Tubular gelatinolytic activities

Gelatinolytic activities found in proximal tubules from control and PKD animals were mainly due to latent forms of enzymes, since measurements without activation by oxidized glutathione resulted in activities of less than 10% of total gelatinolytic activity in proximal tubules of both control and PKD animals (Table 2). The results also show that tubules prepared from PKD rats contained significantly lower gelatinolytic activities (PKD 31.2 ± 8.3 vs. Controls 57.9 ± 6.9 U/tubular preparation; N = 9, P < 0.05). These assays were performed in the presence of the serine proteinase inhibitor PMSF (4 mM). When EDTA was added, it resulted in a more than 95% enzyme inhibition, indicating that these activities were indeed due to metalloproteinases (Table 2).

To further define this gelatinolytic activity samples were subjected to gelatin and casein zymography. With gelatin as a substrate, bands of enzyme activity were readily identified at 72 kD, 68 kD and a 62 kD from both healthy and PKD rats (Fig. 1, lanes A, B). This suggests that the activity was due to the proenzyme and two active forms of gelatinase A. These bands were not obtained if EDTA was added. Furthermore and in agreement with the fluorometric analysis, proximal tubules from PKD animals displayed lower intensity bands than those from
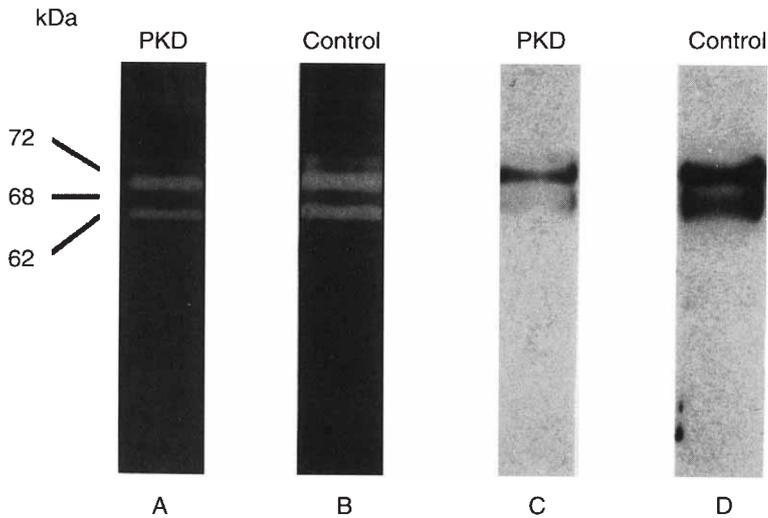
Fig. 1. *Zymogram (lanes A and B) and Western blot analysis (lanes C and D) of gelatinase A (MMP-2) in proximal tubules isolated from PKD and control rats.* Tubular MMP-2, yielding a triplet of gelatinolytic activity (72, 68 and 62 kD), which was reduced in PKD (lane A) as compared to healthy rats (lane B). MMP-2 protein from PKD (lane C) and healthy animals (lane D) were recognized by Western blotting (68 and 62 kD bands) using a polyclonal antibody to human MMP-2. Enzyme protein, measured by densitometry, was 3.8-fold lower ($N = 4$; $P < 0.05$) in tubular preparations of PKD animals.

controls (Fig. 1, lane A vs. B). This semiquantitative estimation became possible because equal volumes of the final tubular preparation from PKD and control animals were subjected to electrophoresis. No other bands at 92 kD were detected. For the detection of stromelysin casein zymography was performed, but did not reveal any activity bands in tubular preparations from both healthy or PKD rats.

Western blotting of tubular MMP-2

For further characterization of the gelatinolytic activity, Western blot analysis was conducted using a polyclonal antibody against human MMP-2, which cross reacts with the rat protein [9]. The antiserum recognized protein bands of 68 and 62 kD, which exactly corresponded with the activity bands detected by zymography (Fig. 1 lanes C, D). Under the conditions of the experiment the protein bands of MMP-2 in tubular preparations from PKD animals were markedly less in comparison to control animals (Fig. 1, lanes C vs. D). Densitometric evaluation showed that tubular MMP-2 protein from PKD was 3.8 ± 0.8-fold ($N = 4$; $P < 0.05$) lower as compared to tubular control samples.

Tubular mRNA expression of MMP-2 and TIMPs

Northern blot analysis of MMP-2 mRNA expression in proximal tubules from PKD animals was also performed. As can be seen in Figure 2 there was down-regulation of the MMP-2 gene in tubules from PKD rats as compared to controls. In contrast, a different expression pattern for both tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) was observed. In comparison to healthy controls, TIMP-1 (Fig. 3) and TIMP-2 (Fig. 4) mRNA expression in proximal tubules was up-regulated in PKD rats. Densitometric analysis of MMP-2 mRNA showed that these signals were 4.2 ± 0.6-fold lower ($N = 4$; $P < 0.05$) in proximal tubules from PKD rats. On the other hand, the contents of mRNA for TIMP-1 and TIMP-2 were considerably increased (9.0 ± 1.1-fold [$N = 4$; $P < 0.05$] and 3.8 ± 0.7-fold [$N = 4$; $P < 0.05$], respectively) in proximal tubules from PKD as compared to control rats. Regarding MMP-3, no mRNA was detectable by Northern blot analysis.
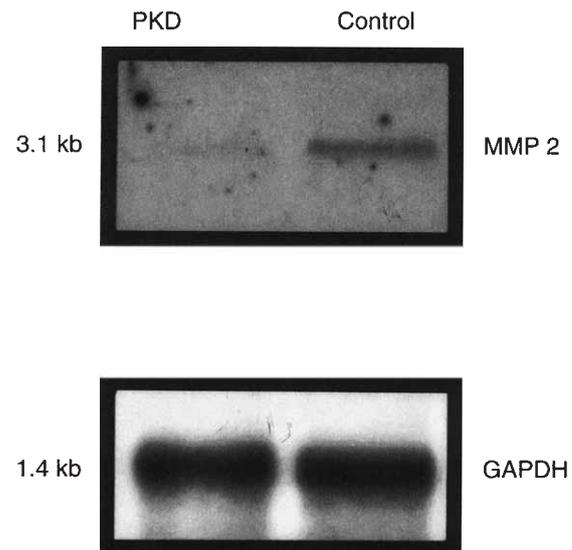

Fig. 2. *Northern blot analysis of tubular MMP-2 mRNA of PKD and control rats.* As measured by densitometry, the levels of mRNA coding for MMP-2 were 4.2 ± 0.6-fold lower in PKD ($N = 4$; $P < 0.05$) as compared to healthy controls. Expression of GAPDH mRNA in the same membrane is shown on the lower panel.

MMP-2 in cyst fluid

Cyst fluid from homozygous PKD rats showed gelatinolytic activity of 735 ± 127 mU/ml after activation by glutathione, while in the absence of glutathione the activity was only 51.5 ± 9.8 mU/ml, indicating that 93% of the enzyme was present in the latent form. These activities was determined after preincubation with 4 mM PMSF and 1 mM E-64 in order to inhibit serine and cysteine proteinases, respectively. In the presence of EDTA the total enzyme activity declined to 4.1 ± 0.5 mU/ml. Gelatin zymography of cyst fluid samples showed bands at 72, 68 and 62 kD (Fig. 5, lane A). Incubation with 20 mM EDTA completely
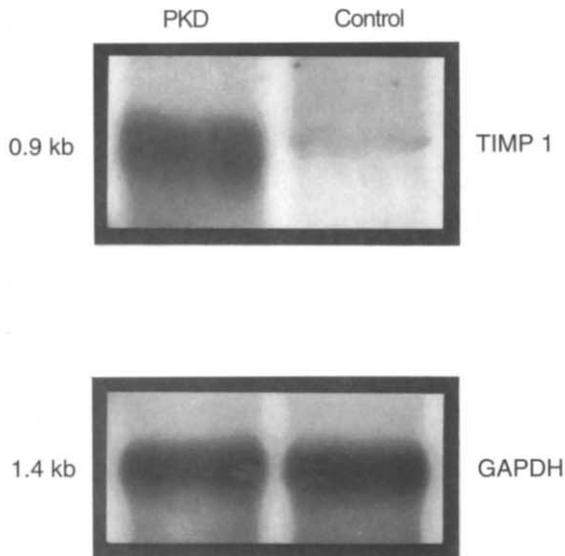
Fig. 3. *Northern blot analysis of tubular TIMP-1 mRNA of PKD and control rats.* The levels of mRNA coding for TIMP-1, measured by densitometry, were 9.0 ± 1.1-fold higher in PKD (N = 4; P < 0.05) as compared to control animals. Expression of GAPDH mRNA in the same membrane is shown on the lower panel.
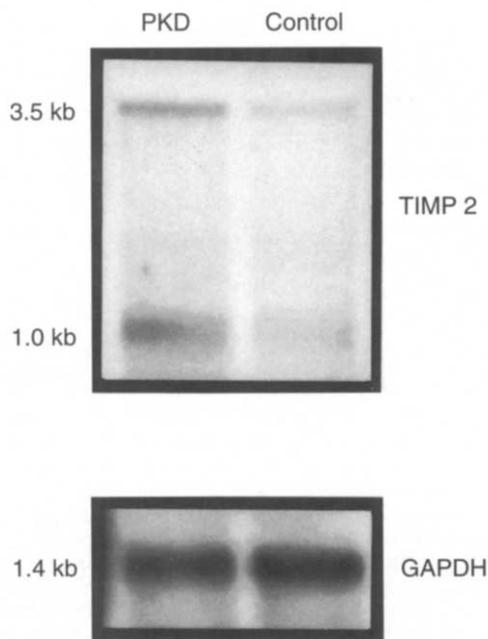

Fig. 5. *Zymography of cyst fluid MMP-2, yielding a triplet of gelatinolytic activity (72, 68 and 62 kD; lane A).* In the presence of 20 mM EDTA MMP-2 is completely inhibited (lane B). Using a polyclonal antibody to human MMP-2, Western blotting revealed a 68 kD and 62 kD form of cyst fluid MMP-2 (lane C).

Fig. 4. *Northern blot analysis of tubular TIMP-2 mRNA of PKD and control rats.* The levels of mRNA coding for TIMP-2, measured by densitometry, were 3.8 ± 0.7-fold higher in PKD (N = 4; P < 0.05) as compared to control animals. Expression of GAPDH mRNA in the same membrane is shown on the lower panel.

inhibited these activities (Fig. 5, lane B). Western blotting with a polyclonal antibody against MMP-2 recognized the 68 and 62 kD protein bands (Fig. 5, lane C), confirming that these activities were due to MMP-2. No other bands at 92 kD were detected in the cyst fluid by zymography. Samples were also subjected to casein zymography, but no activity bands of MMP-3 were detected.
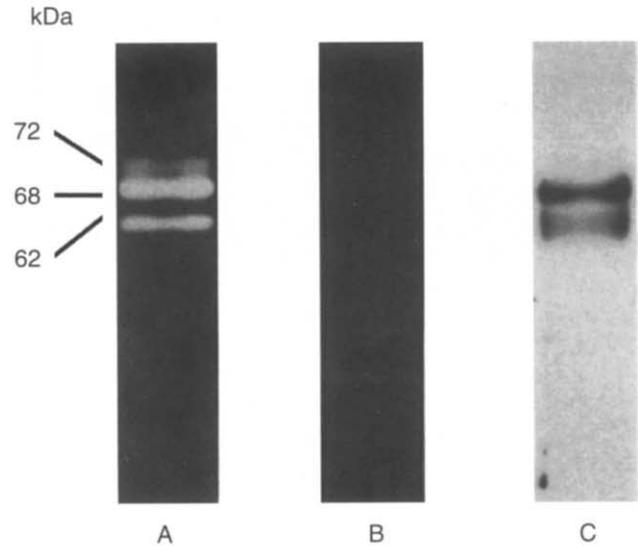
Discussion

Our findings provide evidence for the presence of a 72 kD gelatinase (MMP-2) as well as TIMP-1 and TIMP-2 in proximal tubules from normal rat kidneys. In contrast, we were unable to detect 92 kD gelatinase (MMP-9) and stromelysin (MMP-3) in the same preparations. In comparison to controls, tubular preparations from PKD animals displayed significantly reduced gelatinolytic activity. The likely reason for this was probably a down-regulation of tubular MMP-2 and an up-regulation of TIMP-1 and TIMP-2, which we demonstrated in terms of gene expression. As cyst fluid contained MMP-2 activity and protein, aberrant secretion of the enzyme into the luminal space might have also contributed to the reduced gelatinase activity in tubular preparations from PKD animals.

Physiologically matrix metalloproteinases are secreted into the extracellular space [13], and many investigators have measured these enzymes in culture supernatants from various renal cells [10, 11, 34, 35]. In the present study, however, we were able to demonstrate both MMP-2 activity and protein within isolated proximal tubules. These findings are in agreement with the immunohistochemistry data from Norman et al [36] who showed that proximal human tubules were stained positively for MMP-2.

It is of note that MMP-2 is unique among all other MMPs in that it cannot be activated by proteinases such as kallikrein, neutrophil elastase or cathepsin G [37], which have been identified as putative activators of the MMP family. Instead pro-MMP-2 appears to be activated after secretion by binding to an as yet unidentified pro-MMP-2-receptor on the cell surface, and then proteolytically cleaved to the active enzyme by a membrane-type MMP [38]. Thus, binding of MMP-2 to the cell surface, which has been shown in other cells systems [39] might explain why we found

MMP-2 activity in the tubular preparation which is free of interstitial matrix.

In general, there are only a few reports that address the potential involvement of tubular metalloproteinases in the evolution of PKD. In advanced stages of human ADPKD Norman et al [36] observed reduced MMP-2, MMP-3 and MMP-9 proteins as determined by immunohistochemistry, while the expression of TIMP protein was simultaneously increased. This fits quite well to our findings in isolated tubules from the heterozygous Han:SPRD rat, which displayed reduced MMP-2 mRNA, protein and activity, while mRNAs for TIMP-1 and TIMP-2 were markedly overexpressed.

In addition, a number of preliminary reports has also addressed MMP-2 in conditioned media of cultured tubular epithelia from the *cpk* mouse, a model of the autosomal recessive form of PKD. While Norman et al [22] observed lower MMP-2 activity (determined by zymography), van Adelsberg [40] reported that cystic and normal tubular cells from *cpk/cpk* mice secreted the same amount of MMP-2 protein, and Rankin et al [23] found increased activities of MMP-2 by zymography. These preliminary *in vitro* data are difficult to interpret and some of the differences are most likely due to different culture conditions and cell lines. Van Adelsberg [41] used immortalized cells, while Norman et al [22] and Rankin et al [23] used primary tubular cell cultures from the *cpk* mouse.

In terms of MMP-2 and TIMP, our *in vivo* findings in the heterozygous Han:SPRD rat correspond quite well with the observations of Norman et al both in human ADPK [36] as well as in the *cpk* mouse model [22]. However, with respect to MMP-3 (stromelysin) we found neither enzyme activity by casein zymography, nor did we demonstrate mRNA for this enzyme both in tubules from normal and PKD animals. This contrasts with findings in human ADPKD, where Norman et al [36] and Gardener et al [42] demonstrated MMP-3 protein to be present either in tubular epithelia or in the cyst fluid. We are well aware that the casein zymography is less sensitive than the zymography using gelatin for the detetcion of MMP-2 and MMP-9. Similarily, Northern blot analysis of total RNA may not detect very small amounts of MMP-3 mRNA. Thus, we cannot rule out that minute amounts of MMP-3 mRNA were present which might would have been detected by amplification using RT-PCR techniques.

In contrast to Norman et al [22, 36] who found MMP-9 in cyst epithelia from human and *cpk* mouse kidneys, we could not detect this enzyme in proximal tubules from the Han:SPRD rat. We do not think that this was due to technical problems, because we readily detected MMP-9 mRNA in isolated glomeruli from the same kidneys (data not shown).

These obvious discrepancies may be due to the fact that MMP-2 and MMP-9 are independently regulated, with the MMP-2 gene possessing an AP2 promotor site [43], while transcription of the MMP-9 gene is being controlled through the gene promotor sequence AP1 [44]. In many tissues MMP-2 is expressed constitutively and is linked to the normal turnover of the extracellular matrix, while MMP-9 is only expressed upon stimulation with cytokines such as interleukin-1 through the AP1 promotor site [10]. Therefore, it may be conceivable that we could not demonstrate tubular MMP-9 at an early stage of renal cyst formation, while others have found this enzyme at more advanced stages of PKD or under cell culture conditions, where changes in the

expression of metalloproteinases have been reported with increasing passage numbers [10].

Our results regarding TIMP-1 and TIMP-2 are based only on the mRNA levels for these inhibitors. Therefore, we cannot exclude translational modifications of TIMP expression. However, a number of reports indicate that mRNA and protein levels of TIMPs in the kidney are tightly coupled. Thus, Jones et al [45] found increased TIMP-1 mRNA and protein in the kidneys of rats treated with aminonucleoside. Simulataneous expression of TIMP-2 mRNA and protein was demonstrated by Carome et al [46] in glomerulosclerosis. What is more, Tang et al [47] demonstrated elevated protein and mRNA levels of TIMP-1 in an immune-mediated model of interstitial injury.

The intriguing question whether reduced MMP-2 activity does in fact result in accumulation of extracellular matrix in PKD remains. While there is no direct experimental evidence presently available, several observations suggest a close relationship. Abnormalities in the turnover of extracellular matrix with accumulation of collagen IV, laminin and fibronectin occur early during the course of human PKD and in the Han:SPRD rat model [6, 26]. Given the substrate specificity of MMP-2 which primarily degrades collagen IV and fibronectin [11], a reduction in the activity of this proteinase may readily result in accumulation of matrix components. Therefore, it appears likely that reduced tubular MMP-2 activity may play a role in the evolution of cystogenesis.

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