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CLINICAL INVESTIGATION

Azotemia and extrarenal manifestations in old female Han:SPRD (*cy*/+) rats

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Azotemia and extrarenal manifestations in old female Han:SPRD (*cy*/+) rats. In humans suffering from polycystic kidney disease (PKD) a gender difference is seen with males exhibiting a faster rate of progression of chronic renal failure than females. The aim of this study was to examine renal function in female rats suffering from autosomal dominant PKD [Han:SPRD (*cy*/+)] and to look for the occurrence of extrarenal organ manifestations of PKD. In young (2 months) as well as in old female rats (21 months) relative kidney weight was greater in affected than unaffected animals. In contrast, only the old affected female rats developed azotemia (serum urea 95 ± 124 mg/dl) and severe cystic kidney transformation. Furthermore, old affected female rats exhibited liver cysts (affected 42%; unaffected 3%) and pancreatic cysts (affected 69%; unaffected 15%). Liver cyst epithelia stained positive for cytokeratin 19, a marker for bile duct epithelia. By immunohistochemistry liver cysts exhibited a similar extracellular matrix composition as observed in renal cysts of the same animals (staining positive for laminin, fibronectin and heparan sulfate proteoglycan, but not collagen I). This study proves PKD in the Han:SPRD (*cy*/+) rat model to be a truly multiorgan disease with a close resemblance of the human disease.

The autosomal dominant form of polycystic kidney disease (PKD) with its prevalence of 1:1000 is the most common hereditary renal disease in humans. Affected individuals present in early adulthood with renal dysfunction and hypertension, which often leads to renal failure during the sixth decade of life. The disease is characterized by the formation of large fluid-filled cysts in all segments of the nephron and pathological manifestations in other organs [1, 2].

Recently a rat strain with autosomal dominant polycystic kidney disease (PKD) was described by Kaspareit-Rittingshausen et al [3–7]. In their breeding colony the authors noted that rats from a Sprague-Dawley strain suffered from PKD, and developed uremia and hypertension. Azotemia was only observed in male rats. This observation was subsequently confirmed by a number of other groups [8–14]. Extrarenal organ manifestations of PKD, like cysts in liver or pancreas, have been observed in humans, but not in these rats.

In this paper we describe the occurrence of azotemia and the development of hepatic and pancreatic cysts in old female PKD rats. In addition, the liver cysts were further characterized as originating from bile duct epithelia and exhibiting similar extracellular matrix alterations as noted from renal cysts. Therefore, our findings provide additional evidence that this animal model resembles the human disease.

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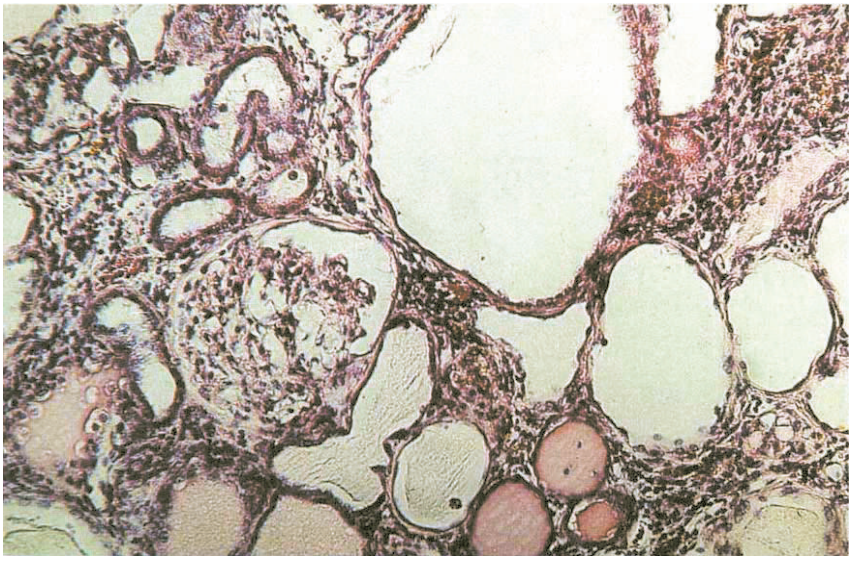
Methods

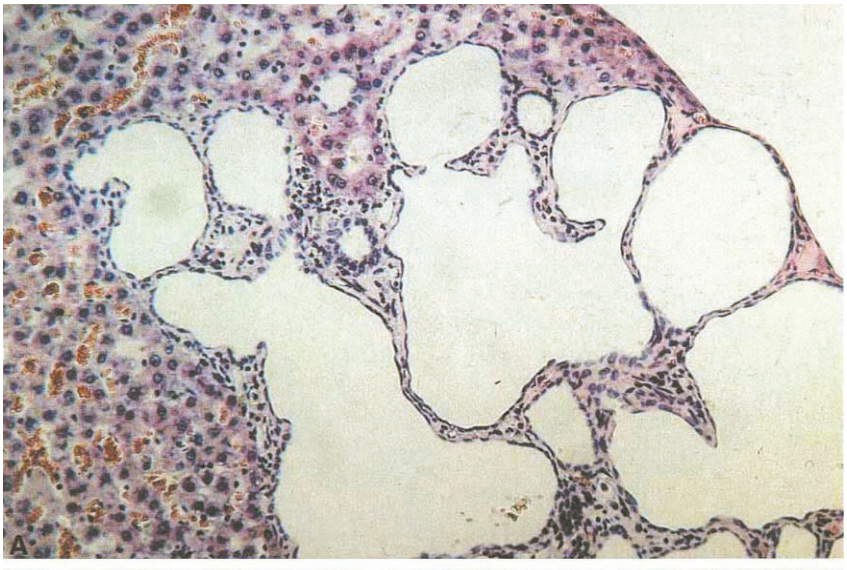
In this investigation heterozygous affected and homozygous unaffected female rats originating from the Han:SPRD [13, 14] strain originally obtained from Dr. Deerberg (Central Institute for Laboratory Animal Breeding, Hannover, Germany) were studied. All rats had free access to tap water and standard rat chow (1324 Altromin, Lage, Germany) containing 19% protein. The light cycle was 12 hours, humidity 55%, and room temperature 20°C. One group of animals was studied at the age of two months (N = 137) and the other at the age of 21.3 ± 2.6 months (N = 67).

Fourteen of the 67 rats died naturally and were necropsied, while 53 were sacrificed for the study under ketamine/xylazine anesthesia. Blood samples were taken from the aorta. Serum urea, serum creatinine, cholesterol and triglycerides were determined using a Hitachi automatic analyzer (Boehringer Mannheim). Body weight, wet weight of the kidneys and the heart was recorded. The carrier status of the affected animals was ascertained by the presence of renal cysts.

Kidneys, pancreas, spleen, lungs, heart and samples of the three largest lobes of the liver were fixed in 3% buffered formalin and embedded in paraffin. For light microscopy paraffin sections were stained with eosin-hematoxylin. In animals exhibiting liver cysts, small portions of cystic areas were prepared for transmission electron microscopy and frozen sections were used for immunohistochemical detection of extracellular matrix proteins and cytokeratin 19. Sections were preincubated with 5% goat serum or 2% bovine serum albumin to block nonspecific binding. After extensive washing with phosphate buffer sections were incubated with the primary antibody for 16 hours, extensively washed and then incubated again for 60 minutes with the FITC-conjugated secondary antibody.

For immunohistochemistry the following antibodies were used: mouse monoclonal IgG_{2b} anti-cytokeratin 19 (Amersham, Braunschweig, Germany), polyclonal rabbit anti-rat collagen I (Biodesign, distributed by Dunn, Asbach, Germany), polyclonal rabbit anti-rat fibronectin (Gibco, Eggenstein, Germany), IM403 (a monoclonal mouse anti-heparan sulfate proteoglycan directed against GAG [15]) and B-31, polyclonal goat anti-heparan sulfate proteoglycan core protein [16] (provided by Dr. Berden, Nijmegen, The Netherlands, and Prof. van der Woude, Mannheim, Germany), polyclonal rabbit anti-rat laminin (Biomol, Hamburg,





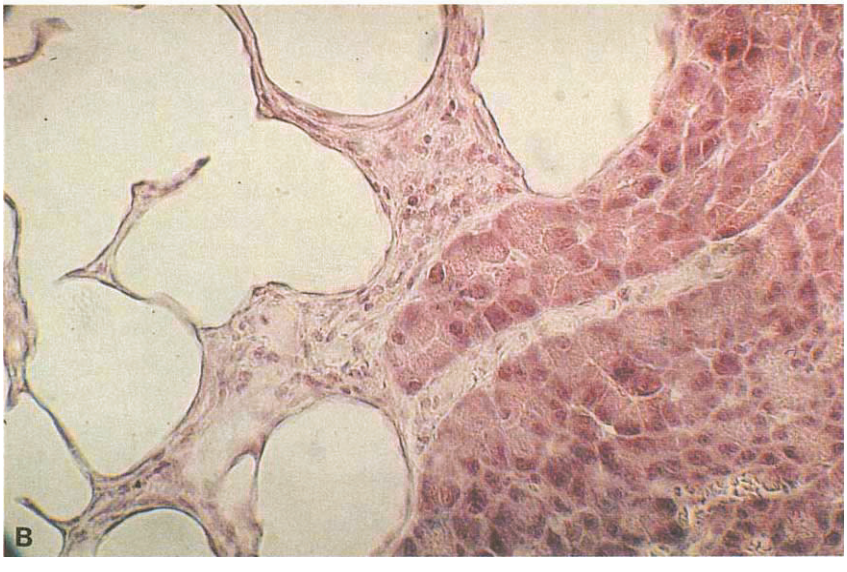


Fig. 1. *(top panel) Renal histology in a female Han:SPRD (cy/+) rat aged 25 months (HE-stain, magnification ×100).*

Fig. 2. *(bottom panels) Liver and pancreas histology in female Han:SPRD (cy/+) rats aged 25 months.* **A.** Hepatic cysts (HE-stain, magnification ×100). **B.** Pancreatic cysts (HE-stain, magnification ×100).

Table 1. Characterization of affected and unaffected female rats of the PKD strain

	Affected	Unaffected	*P*
Young age group (2 months)			
Body weight *g*	211 ± 15	209 ± 17	0.2216
Kidney weight *g*	2.28 ± 0.40	1.45 ± 0.26	0.0001
kw/bw *g/100 g*	1.10 ± 0.15	0.67 ± 0.06	0.0001
Heart weight *g*	0.86 ± 0.17	0.83 ± 0.12	0.1931
hw/bw *g/100 g*	0.41 ± 0.08	0.40 ± 0.06	0.4052
S_{urea} *mg/dl*	38 ± 5	37 ± 4	0.1704
S_{Cr} *mg/dl*	0.29 ± 0.04	0.29 ± 0.03	0.3515
Triglycerides *mg/dl*	150 ± 46	135 ± 45	0.0432
Cholesterol *mg/dl*	76 ± 9	76 ± 9	0.9831
Older age group			
Age *months*	21.5 ± 2.7	21.1 ± 2.6	0.5772
Body weight *g*	357 ± 63	344 ± 51	0.4118
Kidney weight *g*	4.29 ± 2.05	2.12 ± 0.41	0.0001
kw/bw *g/100 g*	1.26 ± 0.76	0.63 ± 0.15	0.0003
Heart weight *g*	1.67 ± 0.29	1.45 ± 0.18	0.0016
hw/bw *g/100 g*	0.49 ± 0.14	0.43 ± 0.07	0.0603
S_{urea} *mg/dl*	95.3 ± 124	43 ± 7.7	0.0416
S_{Cr} *mg/dl*	0.82 ± 1.1	0.45 ± 0.07	0.0962
Triglycerides *mg/dl*	231 ± 139	166 ± 84	0.0454
Cholesterol *mg/dl*	149 ± 63	123 ± 31	0.0645

Abbreviations are: kw/bw, kidney weight of both kidneys corrected for body weight; hw/bw, heart weight corrected for body weight; S_{urea}, serum urea concentration; S_{Cr}, serum creatinine concentration.

Germany), as secondary antibodies FITC-conjugated goat anti-rabbit IgG (Sigma, Munich, Germany), FITC-conjugated goat F(ab)2 fragment anti-mouse IgG (Cappel, distributed by Organon, Eppelheim, Germany), FITC-conjugated rabbit anti-goat IgG (Dako, Hamburg, Germany) and FITC-conjugated rabbit anti-mouse (Dako).

Data were analyzed using the SAS computer software package; the following procedures were applied: PROC TTEST and PROC UNIVARIATE [17, 18]. Data are given as mean ± SD.

Results

In the younger animals (2 months) no difference was noted (Table 1) between the affected ($N = 74$) and unaffected ($N = 63$) female rats with respect to body wt, heart weight, serum urea, serum creatinine and cholesterol concentration. Triglycerides were only marginally raised, while kidney weight was considerably higher in affected rats. No liver cysts were noted in this age group.

In the older age group the mean age of the heterozygous affected females was 21.5 ± 2.7 months (minimum 17.7 months, maximum 27.9 months) and did not differ significantly from the age of homozygous unaffected controls (mean 21.1 ± 2.6 months; minimum 17.1 month; maximum 28.1 month).

In the 53 older rats that were sacrificed for this study, blood samples could be obtained for biochemical analysis (Table 1). Significantly higher values for kidney weight, serum urea and triglycerides were noted in the affected in comparison to the unaffected rats. Serum creatinine and cholesterol levels revealed differences of only marginal significance. Analyzing the data of the affected rats according to the occurrence of liver cysts showed no significant differences (Table 2), although there seemed to be a trend towards a more pronounced azotemia in animals exhibiting liver cysts.

Renal histology of these old heterozygous females revealed

Table 2. Characterization of affected female rats of the older age group according to the occurrence of liver cysts

	Liver cysts ($N = 13$)	No liver cysts ($N = 13$)	*P*
Age *months*	22.2 ± 3.1	20.8 ± 2.0	0.2025
Body weight *g*	341 ± 72	374 ± 51	0.1923
Kidney weight *g*	4.02 ± 1.01	4.55 ± 2.8	0.5248
kw/bw *g/100 g*	1.21 ± 0.34	1.31 ± 1.04	0.7497
Heart weight *g*	1.69 ± 0.34	1.65 ± 0.25	0.6958
hw/bw *g/100 g*	0.52 ± 0.17	0.45 ± 0.09	0.2038
S_{urea} *mg/dl*	118 ± 164	72 ± 63	0.3589
S_{Cr} *mg/dl*	0.96 ± 1.45	0.69 ± 0.60	0.5361
Triglycerides *mg/dl*	227 ± 153	235 ± 130	0.8826
Cholesterol *mg/dl*	142 ± 46	157 ± 77	0.5637

Abbreviations are: kw/bw, kidney weight of both kidneys corrected for body weight; hw/bw, heart weight corrected for body weight; S_{urea}, serum urea concentration; S_{Cr}, serum creatinine concentration.

similar changes as in the previously described affected male animals (Fig. 1). The renal tissue was composed of irregular cysts of varying size, often filled with eosinophilic casts. Severe fibrosis and marked inflammatory infiltration could also be seen. No such changes were noted in control animals, in which occasionally slight interstitial mononuclear infiltrates and moderate glomerular changes were found.

Liver cysts were noted in 16 out of 38 heterozygous female PKD rats (42%), but only in one animal (3%) of the 29 homozygous unaffected rats of the same age range (Fisher's exact test, $P = 0.0002$). The liver cysts were localized predominantly under the serosa, varied in size from 1 to 7 mm and contained a clear watery fluid. In most of the cases multiple cysts built a network-like structure.

The cysts were lined by a monolayer of flattened or cuboidal epithelial cells (Fig. 2A). Small trabeculae composed of thin layers of connective tissue ran between the cysts. Adjacent liver tissue sometimes showed signs of vacuolating degenerative or fatty changes, especially when nests of hepatocytes were encircled and compressed by cysts. Localized cellular inflammatory infiltrations (lymphocytes and macrophages) were noted in the trabeculae. Yellow-brown intra- or extracellular pigments were often seen in the connective tissue. In the periphery of the cysts, connections to the cannalicular system were found that corresponded morphologically to bile ducts. The remaining liver tissue in all animals of the older age group exhibited moderate periportal fibrosis, marked bile duct proliferation and moderate inflammatory infiltrations, mainly in the connective tissue of the portal areas. Eosinophilic and basophilic foci were occasionally seen. Lesions like microhamartomas [19] were not noted in affected nor in unaffected rats.

No cysts could be detected in the spleen or the lungs of the animals, whereas the pancreas of 26 affected (69%) and of 4 unaffected animals (15%) showed microcystic changes, degenerative acini and in some cases larger cysts lined by a flattened epithelium (Fisher's exact test, $P < 0.0001$; Fig. 2B). These pancreatic cysts were more distinct and larger in the affected animals with liver cysts (10 of 12) than in affected animals without liver cysts (5 of 14; Fisher's exact test, $P = 0.0187$).

Transmission electron microscopy (Fig. 3) of the liver cysts revealed flattened cyst-lining epithelium, resting on a basal lamina supported by collagenous connective tissue. The cells showed

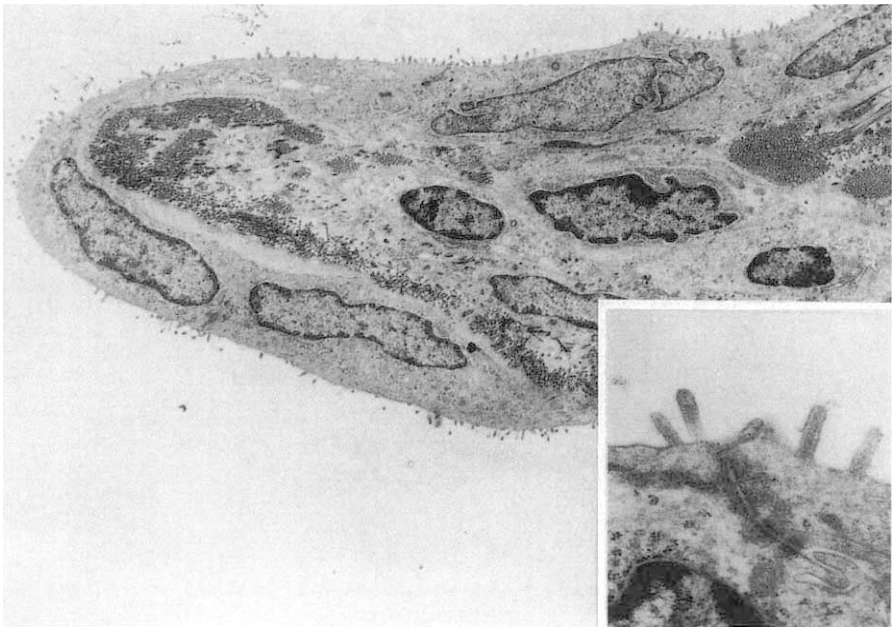


Fig. 3. *Electron microscopy of a liver cyst (magnification ×3400; inset ×15000).*

numerous apical microvilli with some folds and interdigitations on the lateral surfaces. They contained only sporadic organelles and were joined to one another by tight junctions, characteristic of bile duct epithelium.

As shown by immunohistochemistry, hepatic cyst epithelia stained strongly positive for cytokeratin 19, suggesting that the epithelium was derived from biliary epithelial cells (Fig. 4). Furthermore, cysts stained strongly positive for heparan sulfate proteoglycan, as observed with the antibody directed against the GAG chains as well as the one directed against the core protein. Heparan sulfate proteoglycan in normal liver was only expressed by biliary epithelium and in vessels. Laminin and fibronectin could also be detected in the cyst lining epithelia, while cysts stained negative for collagen I. In normal liver fibronectin was present in the Disse's space, while laminin could be noted in the basement membranes of bile ductules and large vessels.

The extracellular matrix composition of the liver cysts were in line with the one found in renal cysts of the same animals (Fig. 5): in kidney sections, two different antibodies against heparan sulfate proteoglycan (one directed against the core protein, the other one against heparan sulfate) stained the cyst lining epithelia, the glomerular and tubular basement membranes and vessels (Fig. 5A). Laminin was noted in the epithelial lining of cysts, the glomerular and tubular basement membrane as well as in the intima of arterial vessels (Fig. 5B). Fibronectin could be localized in renal cyst lining epithelia, mesangium and in the interstitium. Interstitial fibronectin staining appeared to be even stronger in direct neighborhood of cysts (Fig. 5C). Collagen I was present in the interstitium and appeared enhanced in the cyst lining epithelia of only a few cysts or in close neighborhood to the cysts (Fig. 5D).

Discussion

Our data clearly reveal that azotemia occurs in female Han:SPRD (*cy*/+) rats, if the animals are allowed to age. In this rat strain suffering from PKD no extrarenal manifestations of PKD had been noted so far. We describe and characterize hepatic and pancreatic cysts as extrarenal organ manifestation in these animals.

In previous descriptions of the course of PKD in the Han:SPRD (*cy*/+) rat, female animals were observed up to an age of 14 months [10]. During this observation period no signs of renal functional impairment occurred. In our colony, however, we noted increased serum urea and serum creatinine values in affected female rats aged 17 months or older. Furthermore, an increased death rate became evident. Testing systematically for serum urea and creatinine revealed azotemia in this age group. Compatible with these functional findings are the changes in renal histology (Fig. 1), where numerous small, medium and large cysts, marked fibrosis and inflammatory infiltration were found. Thus from our data it becomes obvious that both affected male and female PKD rats develop azotemia due to cystic disease with females developing azotemia at a much older age than males.

Mean observation period in this study was 22.3 ± 2.6 months ($N = 67$), which is significantly longer than the median survival of affected male rats (median 17.0 months) [13]. Thus, as also noted in humans [20, 21], a significant gender difference seems to exist with respect to the occurrence of renal death. A similar difference was observed in another rat model of renal disease, the fawn hooded rat [22–24]. From our data survival time cannot be calculated in the female group, as only a cross-sectional study was performed. This notion is calling for a formal study to establish survival rates/survival curves in female Han:SPRD (*cy*/+) rats as has already been done in their male counterparts [13].

Extrarenal manifestations of human PKD include cardiac valvular abnormalities, colonic diverticuli, cerebrovascular aneurysms and the occurrence of cysts in various organs [1, 2, 25]. About 70% of all patients develop liver cysts by the age of 60 years while cysts in other organs (pancreas, ovary, testis, spleen and meninges) are very rare [1]. Massive hepatic cystic disease mainly occurs in women and is influenced by the number of pregnancies and the use of oral contraceptives. In old female PKD rats (> 17 months) we noted the occurrence of liver cysts in 42% of all cases.

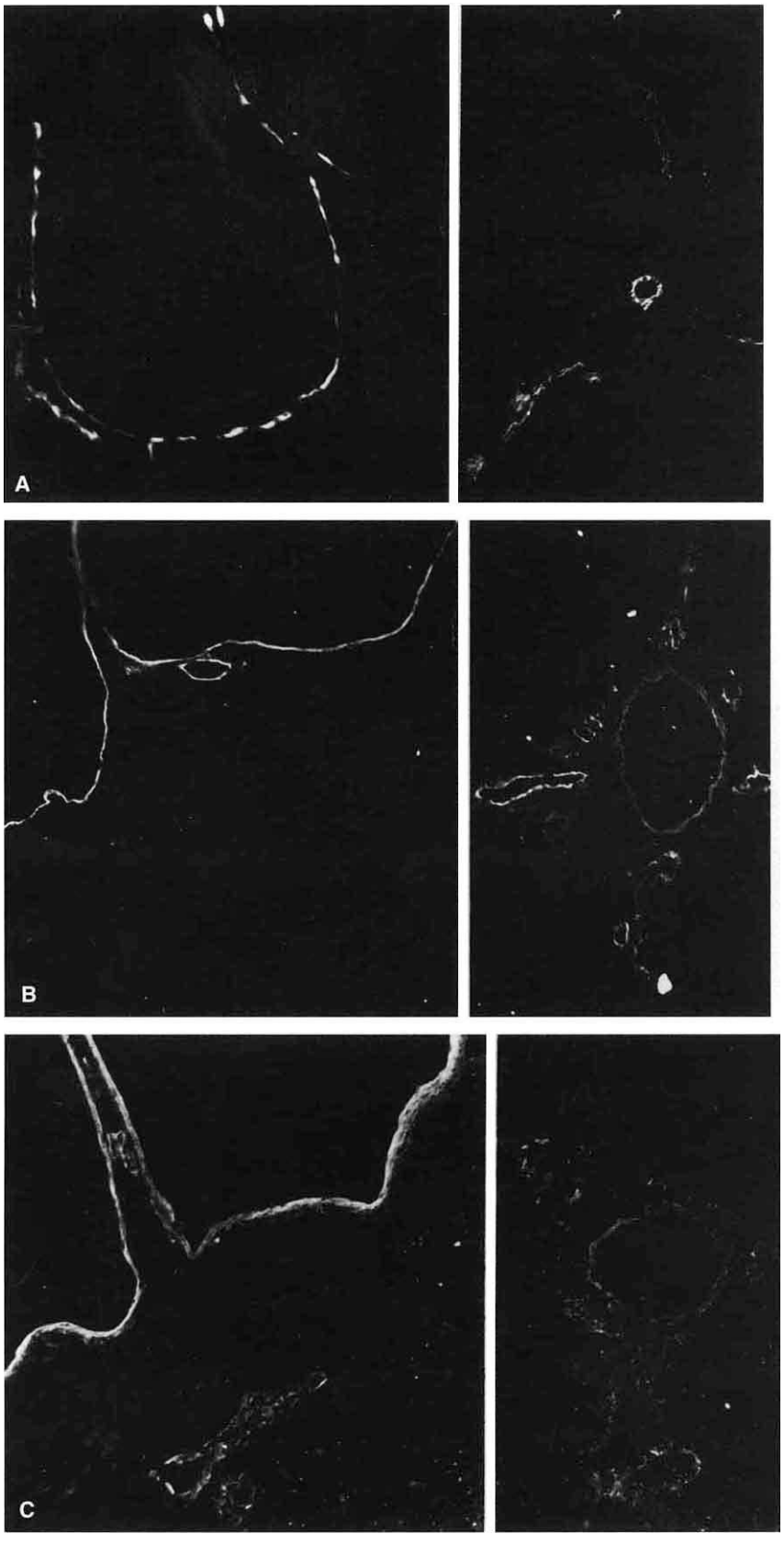


Fig. 4. *Liver cyst epithelium (left) and portal tract (right) in heterozygous affected female PKD rats aged 25 months showed strong immunoreactivity for* (**A**) *cytokeratin 19 (FITC-immunostaining, magnification ×100),* (**B**) *heparan sulfate proteoglycan (FITC-immunostaining, ×100,* (**C**) *laminin (FITC-immunostaining, ×100),* (**D**) *fibronectin (FITC-immunostaining, ×100),* (**E**) *but not for collagen I (FITC-immunostaining, ×100).*

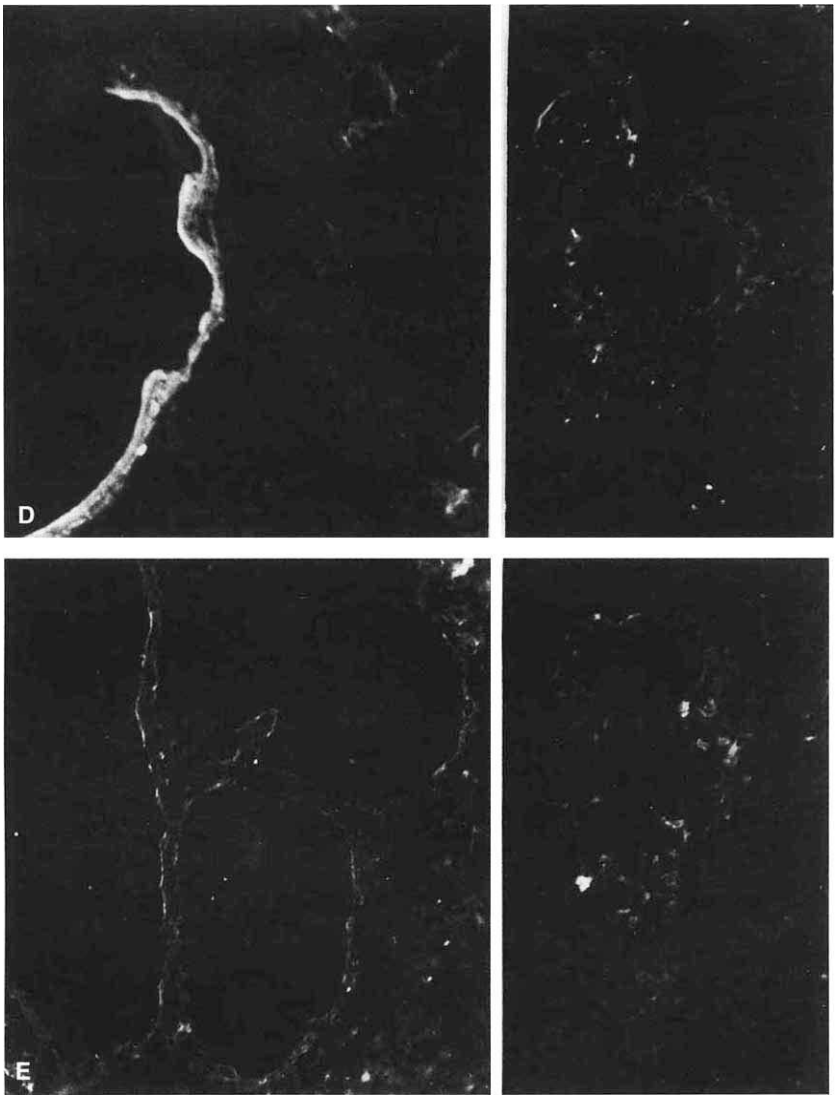


Fig. 4. *Continued.*

No correlation between the number of pregnancies and the development of liver cysts could be detected (data not shown). In younger female heterozygous rats up to the age of one year, no renal functional impairment and also no extrarenal manifestations, like liver cysts, were noted (data not shown). Admittedly, however, no large scale study was performed by us and others. In addition, it is of note that in male heterozygous rats dying of uremia [13] no liver cysts were observed. Thus, the extent of renal functional impairment and extrarenal manifestations seems to be a function of age in this rat model. Interestingly, the female rats exhibiting liver cysts seemed to exhibit a higher degree of azotemia, though this was statistically not significant. This trend, however, seems to be associated with a slightly older age in this group of rats.

In rats hepatic cysts and proliferative lesions of the biliary epithelium are often described following liver injury or treatment with carcinogens [26–28]. Single or multiple cysts, however, may also occur in untreated rats [29, 30]. The incidence of spontaneous liver cysts in rats differs with respect to the strain and normally increases with age. In two different strains (WAG/Rij (Wistar) and BN/Bj) liver cysts developed in not more than 10% of all animals up to an age of 30 months. Older female rats (WAG/Rij > 36 months; BN/Bj 30 to 54 months) exhibited liver cysts in 30% or 55% of all cases [31]. In Sprague-Dawley (SD) rats a low incidence of 2.3% in male and 1% in female rats older than 18 months was described [32]. As our PKD strain originates from SD rats, one would expect a low incidence of liver cysts. As we observed cysts only once in an unaffected rat (3%) of the same age, the high incidence (42%) seems to be a specific feature of affected old female PKD rats.

The hepatic cysts seen in PKD rats resembled the cysts in humans in two ways (Figs. 2A and 3): they could only be seen at an older age (> 17 months) and the cyst epithelium appeared to be similar to biliary duct epithelium (light and transmission electron microscopy). Histomorphometric and microdissection studies of liver cysts of patients with autosomal dominant PKD indicated that cysts begin as focal dilations of the intrahepatic bile ducts [33]. Furthermore, hepatic cyst epithelium has functional characteristics of biliary epithelium [34, 35]. In addition liver cyst-derived epithelial cells in culture from female patients with

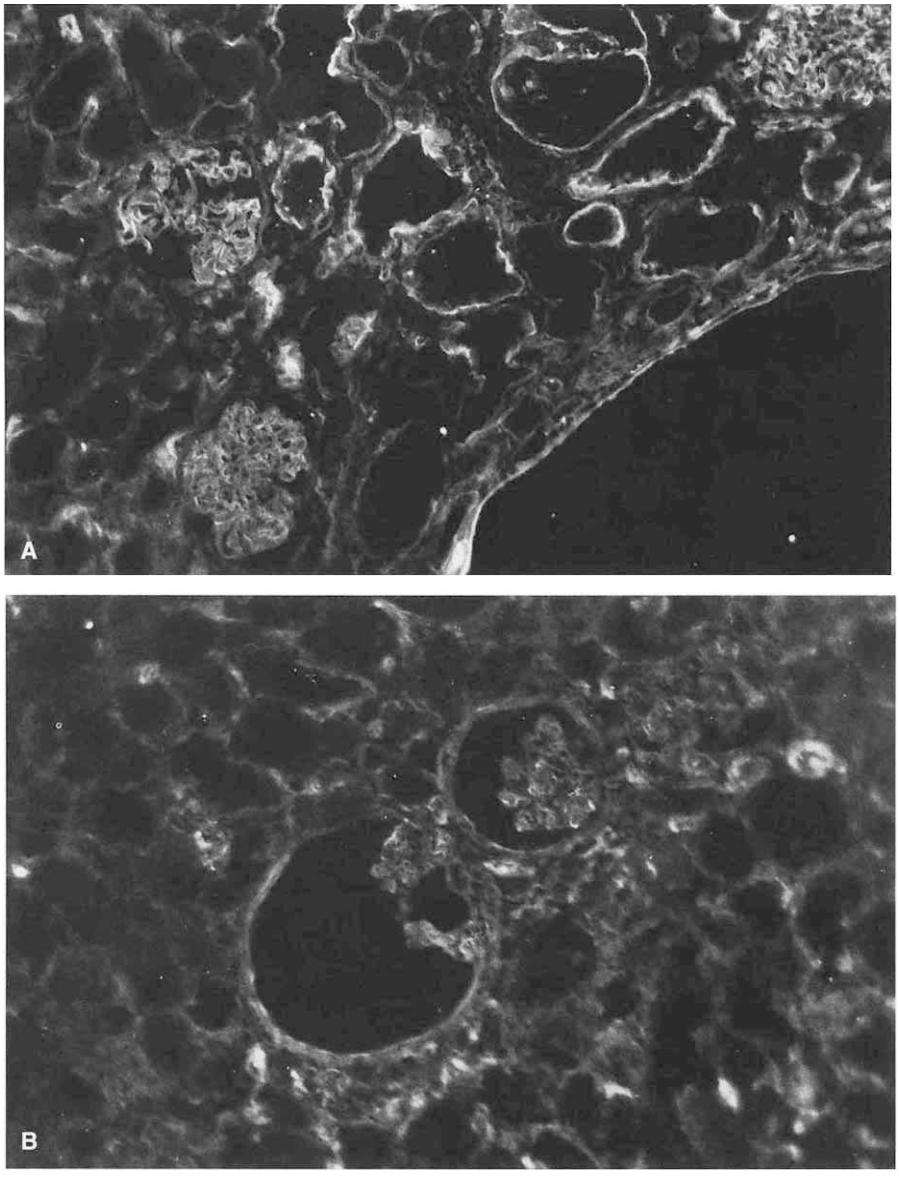


Fig. 5. *Extracellular matrix protein expression in PKD rat kidneys.* **A.** Heparan sulfate proteoglycan in the glomerular basement membrane, the mesangium and in larger vessels as well as in cyst-lining epithelia (FITC-immunostaining, magnification ×100). **B.** Anti-laminin reactivity in glomerular and tubular basement membrane, larger vessels as well as cyst-lining epithelia (FITC-immunostaining, ×100). **C.** Positive fibronectin staining in the cyst-lining epithelia, the mesangium, large vessels as well as in the interstitium (FITC-immunostaining, ×100). **D.** Collagen I in the interstitium and in cyst-lining epithelia of few cysts (FITC-immunostaining, magnification ×100).

PKD exhibit characteristics of intrahepatic biliary epithelium [36]. Similarly, rat liver cyst epithelium showed strong immunoreactivity for cytokeratin 19, a specific marker for biliary epithelium (Fig. 4A) [37], and for heparan sulfate proteoglycan, the latter normally only found with biliary epithelium and vessels [38]. Therefore, the epithelial lining could be classified as a proliferative cystic lesion of biliary epithelium.

Pancreatic cysts were found in human PKD in 9% [1]. In the pancreas of all our old female rats (> 17 months), either affected and unaffected animals, we noted the occurrence of microcystic changes (Fig. 2B) in 42%. Since these changes are also a phenomenon related to age [30] care has to be taken when interpreting the results. We found a relationship between the frequency of pancreatic cysts and PKD: 69% of the affected, but only 15% of the unaffected animals showed cystic changes. Furthermore, the cystic changes in the pancreas of affected animals with liver cysts were more marked and the rats exhibited larger cysts (10 of 12 animals) than affected animals without liver cysts (5 of 14 animals).

In the liver several extracellular matrix proteins have been described and localized [38]. In normal histological staining, a basement membrane cannot be detected except around the biliary ductules and major vessels [39]. Collagen IV, laminin and heparan sulfate proteoglycans have been shown to be its major constituents, while fibronectin is mostly localized in the space of Disse. Collagen I is sparsely distributed in bundles across the lobule [38]. Similarly in the kidney, collagen I and fibronectin are mostly found in the interstitium, the latter also representing the main extracellular matrix component of the mesangium. Laminin and collagen IV are the main constituents of basement membranes.

In different models of PKD an early change in renal extracellular matrix composition has been described [40–43] with dramatic changes in the expression of fibronectin and collagen I. An early increase in mRNA levels of fibronectin, collagen I, collagen

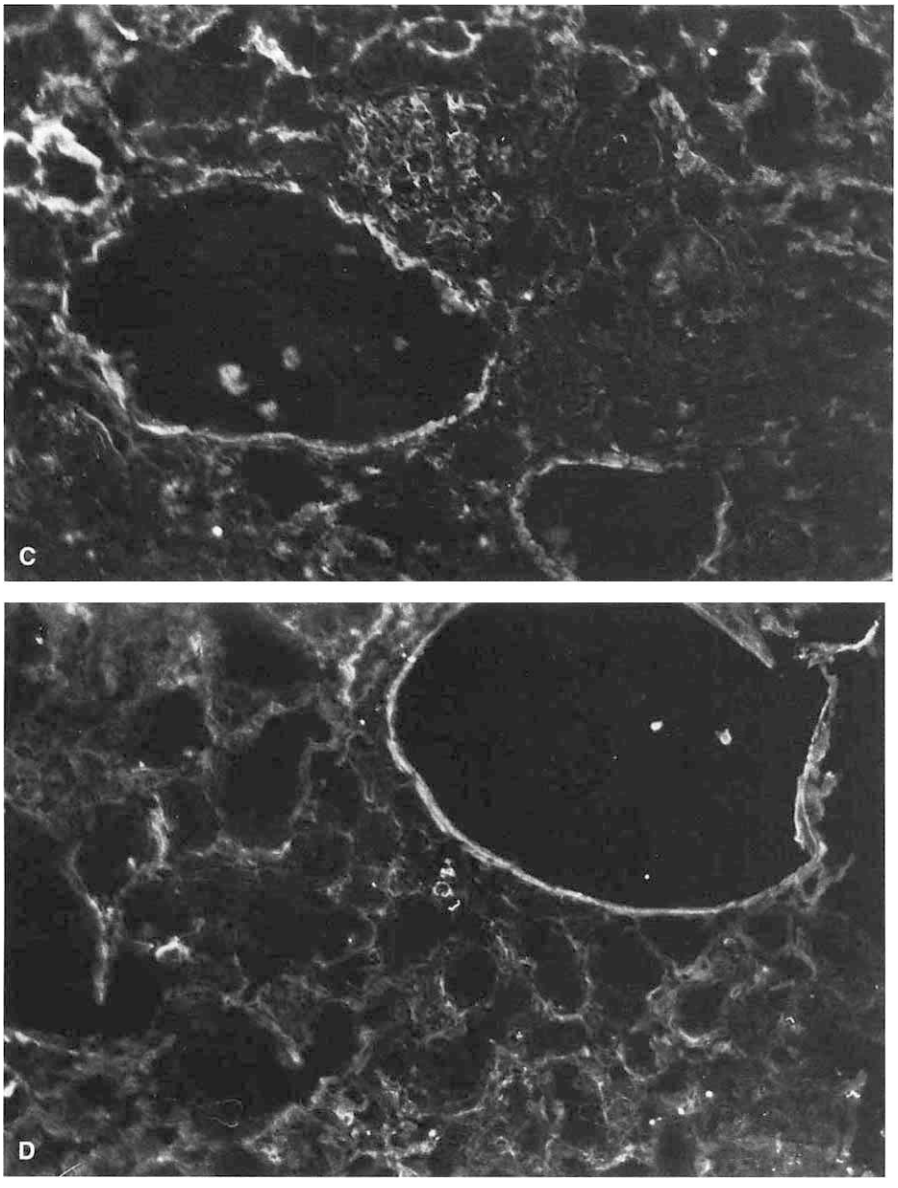


Fig. 5. *Continued.*

IV and laminin in the *pcy* mouse model was noted [40]. In DPT induced PKD in the rat, Carone et al [41] detected intense staining for fibronectin in the basement membrane of cystic tubular epithelia and a decrease in anti-heparan sulfate proteoglycan core protein reactivity. These findings were in line with data observed in human PKD [42], where an increase in fibronectin staining and a loss of anti-heparan sulfate proteoglycan core protein staining was described. In a corticoid induced model of cystic disease in rabbits, Ojeda et al [43] reported an increase in fibronectin staining of cystic tubuli and an unchanged or slightly decreased reactivity of cystic basement membrane with anti-laminin and anti-collagen IV. In our study extracellular matrix composition of renal cysts in the older female PKD rats resembled published data, except for an increase in heparan sulfate proteoglycan staining of the cyst lining epithelia.

An overall increase in the concentration of low molecular weight glycoproteins has been shown by biochemical analysis [44]. The difference in heparan sulfate proteoglycan protein expression, as found by immunohistochemistry, might be due either to a different recognition site of the antibody or to the fact that a different, inherited model of PKD was studied. It might also be explained by the different stages of the disease, which were studied. While Carone et al [41] examined early changes in the DPT rat model, two-year-old female rats were studied in the present study. Our findings are corroborated by the fact that two different antibodies against heparan sulfate proteoglycan were used exhibiting similar results. Furthermore, the general tissue distribution found in liver and kidney is in line with the localization described by others [15, 16].

Similarly to the extracellular matrix composition found in renal cysts (Fig. 5), the cyst-lining epithelia in PKD rat liver stained strongly positive for fibronectin, heparan sulfate proteoglycan and laminin, while no increase in anti-collagen I reactivity was observed (Fig. 4). As the cystic epithelia stained positive for cytokeratin 19

and heparan sulfate proteoglycan, it seems to be derived from biliary duct epithelium. The resemblance of extracellular matrix changes between renal and liver cysts suggests a similar process of cystogenesis to take place in liver and kidney, adding to the notion that the Han:SPRD (*cy*/+) rat model represents a multi-organ disease.

In summary, we could demonstrate that older affected female PKD rats are suffering from azotemia and that these rats develop hepatic and pancreatic cysts as signs of an extrarenal organ manifestation. These features add to the resemblance of the human disease. Furthermore, liver cysts were not only derived from biliary duct epithelia like in human PKD, but also exhibited similar changes in extracellular matrix composition as described for renal cysts, indicating a similar process of cystogenesis taking place in the liver of old female rats as observed in the kidney. Thus, our findings underline that the Han:SPRD (*cy*/+) rat model could be a useful tool to study the pathogenesis of human PKD as a multiorgan disease.

Acknowledgments

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