Pathophysiology of Polycystic Kidney Disease

Experimental studies

Jeroen Nauta

Nauta, Jeroen
Pathophysiology of polycystic kidney disease. Experimental studies.
Thesis, Erasmus University Rotterdam. With summary in Dutch.
Cover: "Horizon", photography by: Louisette J. Nauta-Borgerhoff Mulder
ISBN 90-9013489-1

Publication of this thesis was financially supported by The Dutch Kidney Foundation,
Novo Nordisk Farma B.V. and Baxter B.V.

Printed by Ridderprint B.V., Ridderkerk, The Netherlands

Pathophysiology of Polycystic Kidney Disease

Experimental studies

Ontstaanswijze van polycysteuze nierziekten
Experimenteel onderzoek

Proefschrift

Ter verkrijging van de graad van doctor
Aan de Erasmus Universiteit Rotterdam
Op gezag van de Rector Magnificus

Prof. dr. P.W.C. Akkermans M.A.

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 8 maart 2000 om 15.45 uur

door

JEROEN NAUTA
Geboren te Leiden

Promotie commissie

Promotor:	Prof. dr. H.A. Büller
Overige leden:	Prof. dr. S.L.S. Drop
	Prof. dr. A.J. van der Heijden
	Dr. A.P. Provoost

Aan Louisette

Contents

Preface

Polycystic kidney disease (PKD) is a heritable disorder with diffuse cystic destruction of both kidneys, leading to renal failure in many patients. Two entities can be identified: autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD). ADPKD is one of the most common inherited monogenic disorders, affecting approximately 1:1.000 individuals; ARPKD affects 1:40.000. The severity of disease in affected patients is highly variable in both ADPKD and ARPKD, even within families. ADPKD progresses to end stage renal failure in about half the patients, usually after the 4th decade. ARPKD leads to renal failure during infancy or early childhood in all cases and is associated with abnormalities of the biliary system in the liver, leading to severe liver disease in many children. No direct treatment of the formation and subsequent expansion of the cysts is currently available.

Recent genetic studies characterized the principle genes involved in ADPKD (PKD1 and PKD2) and localized the gene for ARPKD. Intensive investigations were undertaken into the role of these genes during normal life and on how mutations of these genes eventually lead to the formation of cysts. It has now become apparent that this cystic process involves many different proteins, encoded by many different genes.

The cellular mechanisms involved in cyst formation include abnormal proliferation and abnormal survival of the involved cells, altered transport of fluid into the cysts and altered regulation of the extracellular matrix composition. These mechanisms have been explored in renal tissues from patients as well as in various experimental models of PKD.

Murine (mouse and rat) models of PKD have contributed significantly to the current understanding of PKD. In addition to allowing the systematic analysis of cystic tissues and renal physiology during cyst formation, these models have revealed genes and proteins that are potentially involved in the molecular pathogenesis of cyst formation, including genetic factors that modulate the severity of disease. Moreover, animal models have and will be used to explore therapeutic strategies for patients with PKD. However, none of the available murine models of PKD completely parrallels either human ARPKD or ADPKD. They differ from human disease states by the clinical expression of disease, the underlying genetic defect, or both.

In the studies described in this manuscript we set out to characterize new murine models of PKD and opted in particular to develop tools for the study of polycystin-1, the gene product of PKD1. In addition we set out to determine whether 'programmed cell death' is part of the cellular phenotype of cystic tissues.

1

Introduction

Introduction

The development of renal cysts is a feature of several genetic and non-genetic disorders. The most common and best understood causes of renal cystic disease in humans are genetically determined and include autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD) and the juvenile nephronophthisis / medullary cystic disease complex, for which the genes have been identified and/or chromosomal locations have been mapped. Other causes of renal cyst formation include the genetic disorders tuberous sclerosis and von Hippel-Lindau syndrome as well as a large number of genetic malformation syndromes associated with renal cyst formation. Non-genetic disorders include multicystic dysplasia, simple cysts, medullary sponge kidneys, acquired cysts (associated with uremia), Wilms tumor and renal carcinoma.

The current understanding of renal cyst formation is largely based on both clinical and experimental observations of Polycystic Kidney Disease (PKD). Here, we will first describe the clinical aspects of the two different entities of PKD, including the clinical differentiation from each other. Secondly we will summarize what is known about the pathophysiology of cyst formation, using data from both human PKD as well as from experimental models of PKD. This includes an extensive summary of the characteristics of the various animal and cell models of cyst formation that are currently available. Finally we will present a theoretical framework that outlines the relationship of the various components of cyst formation.

1.1 Polycystic kidney disease, the clinical entities

Polycystic kidney disease (PKD) is a subset of renal cystic disorders in which cysts are distributed throughout the cortex and medulla of both kidneys. It is the most frequent inherited cause of renal failure in man. The most severe forms are seen in children, but the most prevelent forms typically become manifest during adulthood. Recent progress in molecular genetics has improved the understanding and classification of these diseases and consequently has made genetic counseling more accurate. The genetic classification of PKD into an autosomal dominant and an autosomal recessive form has replaced the previous classification, which was based on age at clinical presentation: infantile and adult type PKD. This terminology is now obsolete.

Autosomal dominant polycystic kidney disease (ADPKD)

ADPKD is a systemic disorder characterized by the development of both small and large renal cysts and is associated with liver, cardiovascular, gastrointestinal and genital

abnormalities. It is one of the most common monogenic disorders. Approximately half of the patients will eventually need renal replacement therapy, typically starting after the fourth decade. ADPKD is often referred to as adult PKD, but this is a misnomer because it has been diagnosed in the fetus, newborn and older child. The disease is genetically heterogenous and phenotypic heterogeneity has been demonstrated in different individuals within the same family.

Genetics

ADPKD has a worldwide distribution, affects all races and affects males and females equally. The disease is caused by a mutation in one of at least three distinct genetic loci. Approximately 85% of the patients have a mutation in the PKD1 gene on chromosome 16, while most of the remaining patients have a mutation of the PKD2 gene on chromosome 4 (Reeders '85, Romeo '88). The possibility of a third locus, unlinked to PKD1 and PKD2 has been suggested (Daoust '95). ADPKD is inherited as an autosomal dominant trait with complete penetrance, but variable expression (Dalgaard '57). Approximately 30% of patients is unable to give a family history consistent with ADPKD, suggesting the possibility of a high spontaneous mutation rate or, alternatively, the possibility that environmental and/or epidemiologic factors strongly affect the expression of ADPKD.

With the recent isolation of the PKD1 and PKD2 genes, direct mutational screening is possible. Although mutations of PKD1 and PKD2 lead to the same clinical phenotype, members of PKD1 families appear to have on average an earlier disease onset with renal cyst development, hypertension and renal failure at a younger age than do members of PKD2 families (Ravine '92). Evidence is accumulating that germline PKD1 mutations are inactivating. Although relatively few mutations have been described, those characterised are mainly predicted to terminate the protein prematurely due to the introduction of a stop or frameshifting change (Peral '96, Peral '97, Roelfsema '97). In addition, an analysis of patients with deletions disrupting PKD1 and the adjacent tuberous sclerosis gene TSC2 shows that complete PKD1 deletion is associated with severe early onset polycystic kidneys (Sampson '97). Germline mutations identified in PKD2 families were similarly predicted to code for truncated polycystin-2 (Mochizucki '96).

Although the genetic defect is the primary cause of PKD, it does not predict the precize clinical course in individual patients. This clinical course is highly variable and differs between different families, and also between affected members of the same family (Churchill '84, Peral '96). It is generally agreed that mutation analysis does not allow the prediction of when and whether renal failure will develop in a given patient. The intrafamiliar variability suggests an additional role for other genetic or environmental factors as well.

Prenatal diagnosis of ADPKD

Antenatal ultrasound abnormalities have been reported in ADPKD families, but are usually not evident until the third trimester (Pretorius '87). Prenatal diagnosis sometimes leads to the diagnosis of ADPKD in other family members. If ADPKD is suspected, family members should be considered for ultrasound evaluation. This includes the grandparents if the parents are younger than 30.

DNA analysis enables carrier detection and prenatal genetic diagnosis in 'informative' PKD1 or PKD2 families with suitable pedigrees. Such analysis may provide the diagnosis during early pregnancy (Hodgkinson '90). However, given the wide clinical spectrum and the treatable nature of renal failure, the question arises whether termination of an affected pregnancy is an acceptable option. For that reason is prenatal genetic linkage analysis not routinely performed in the USA or Europe (Zerres '92).

Pathology

Nephron reconstruction studies revealed that cysts develop as dilations of pre-existing renal tubules. With enlargement beyond a few millimeters in diameter, it appears that the majority of cysts become completely detached from the tubule of origin (Grantham '87b, '93). Cysts may arise in any nephron segment, including glomeruli, proximal tubules, distal tubules and collecting tubules. This has been indicated by microscopic and electron microscopic studies, as well as by the study of cyst fluid compositions (Grantham '93, Huseman '80). In advanced disease, the kidneys are usually diffusely cystic en enlarged. Reported kidney weights in adults ranges from normal to 4 kilogram. Both the outer and the cut surfaces show numerous cysts ranging in size from microscopical to several centimeters in diameter. The cysts appear throughout the kidney in both the cortex and the medulla.

Microscopically, most cysts are lined by a nondiscript cuboidal epithelium (Gregoire '87). Microscopic polyps are common in smaller cysts. In between the cysts normal nephron structures can always be found by microscopic examination. In advanced disease the kidneys often show secondary changes like tubular atrophy, glomerular sclerosis and interstitial fibrosis. Ultrastructurally, cyst walls have a generally single layered simple epithelium lying on a basement membrane that varies from normal to thickened or extensively laminated (Grantham '87b, Cuppage '80). The epithelial cells are of various types, some of which resemble those lining normal nephron segments. However, the majority appears to be lined by a primitive epithelium that does not resemble normal tubule segments.

ADPKD is associated with hepatic cysts in the majority of adult patients. These contain a viscid fluid that resembles serum and are lined by a single layer of columnar epithelium, resembling biliary epithelium. In children and adults, about half the patients have also increased connective tissue in portal areas and increased numbers of bile ducts (Potter '72).

Regardless of these abnormalities it is only rarely that patients with ADPKD present with liver dysfunction and/or portal hypertension. This is unlike the situation in patients with congenital hepatic fibrosis and autosomal recessive polycystic kidney disease.

Clinical presentation and course

Advanced states of ADPKD are easy to diagnose by ultrasound and in less obvious cases by CT scan. The patients present with abdominal pain, hematuria, urinary tract infections, nephrolithiasis or symptoms of hypertension and/or renal failure and have a positive family history in at least 70 % of new cases. Most kidneys are bilaterally enlarged and have irregular surfaces that can be felt on carefull palpation. An enlarged liver with palpable cysts adds further to the diagnosis. Massive proteinuria is rare. The hematocrit may be increased above normal, possibly resulting from increased production of erythropoetin by the cystic kidneys. Hypertension is a common complication, also in children, even in the absence of renal failure. Hypertension appears to be mediated by activation of the renin-angiotensin system and sodium retention, possibly as a result of locally reduced renal blood flow (Harrap '91). Urinary tract infection are common complications in adults with ADPKD and are less manifest in childhood. These can be very difficult to treat and may lead to chronic pyelonephritis, perinephric absces, sepsis and death.

The diagnosis of earlier cases of ADPKD can be more difficult. Children and adolescents are often asymptomatic and may have unilateral cysts or no cysts at all. In PKD1 only 10% of children younger than 10 years old have cysts that can be demonstrated by ultrasound, whereas 95% has cysts at the age of 20 years. The probability of demonstrable cysts increases to 100% at the age of 30. In less obvious cases, when only small cysts are present, diffuse bilateral cystic involvement may only be revealed by CT scan. The demonstration of cysts in liver, pancreas and spleen can be of diagnostic help. In some patients, analysis of genetic linkage may be useful to identify those predisposed to develop ADPKD, provided that at least two affected family members are available for DNA testing.

The clinical presentation in early childhood and in neonates ranges from severe neonatal disease indistinguishable from ARPKD to renal cysts noted on ultrasound in asymptomatic children. The diagnosis has occasionally been made in utero by ultrasound and affected newbornes can present with very severe disease leading to renal failure and neonatal death from pulmonary hypoplasia. However, in the large majority of cases, the renal function is well maintained during childhood.

Co-morbidity from extrarenal manifestations is largely confined to adult patients. Hepatic cysts develop later than renal cysts and are rarely found in children. Their prevalence reaches 80% after 60 years of age (Grunfeld '84). Most patients remain asymptomatic with preserved liver function. Persistent and severe pain may require cyst decompression. Infection of hepatic cysts is rare and requires antibiotics and sometimes

drainage. Epithelial cysts in other organs are infrequently seen. These include pancreas, ovaria, spleen, ovary, thyroid, endometrium, seminal vesicle and epidydimis. Along with the progressive cyst formation in the kidney and other organs, patients with ADPKD are at increased risk for a variety of vascular abnormalities. Intracranial aneurysms have been found in 8% of patients, compared to 1.2% in the general population, and appear to be clustered in families (Iglesias '83, Dalgaard '57, Kaehny '87, Chapman '92). Ruptures of aneurysms are the most serious complication in ADPKD and may account for 7% to 13% of deaths in ADPKD. Aneurysms of the aorta and cardiac valve abnormalities have also been reported (Hossack '87, '88). It is uncertain whether vascular complications result directly from the genetic defect or merely as a consequence of hypertension and renal failure in these patients. In young children extrarenal manifestations have only rarely been noted.

Clinical management

Direct treatment of the cystic process is not possible. The clinical management is largely focussed on the symptomatic manifestations of the disease, such as hypertension, infections, hematurie, pain, renal failure and cerebral hemorrhage.

Hypertension occurs in more than half the patients and often antedates the development of renal failure and may also affect children with ADPKD (Zeier '93, Iglesias '83, Gabow '92). It has been associated with an increase of left ventricular mass and is considered to be a risk factor for cardiovascular morbidity as well as for the devevelopement of renal failure. Salt restriction is generally recommended and ace-inhibitors are effective first choice antihypertensives, with calcium entry blockers as a good alternative. The use of diuretics is considered to be a problem. Allthough helpful in controling hypertension, diuretics have the potential to adversely alter the movement of fluid across the cystwalls. In addition, hypokalaemia, a common complication of diuretic use, has been associated with the development of renal cysts in normal individuals and may therefore increase the growth of cysts in ADPKD.

Urinary tract infections may lead to cyst infection, renal absces and sepsis and are considered to be risk factors in progression of renal disease (Gabow '92). These infections may be difficult to treat. Adequate treatment with antibiotics that can penetrate cyst walls is critical (Schwab '87). Macroscopic and microscopic hematuria may result from a rupturing cyst and is usually self limiting. Reduced physical activity may be recommended temporarely in cases of protracted bleeding.

Pain from ADPKD, sometimes associates with perinephric hemorrhage, can ususally be treated with analgesics and sometimes bedrest. When the pain persists for more than a few days one must consider the possibility of renal infection, stones or tumor. Pain may also be associated with enlargement of one or more cysts. In such cases some relief may be

obtained from percutaneous aspiration or surgical reduction of cysts (Uemasu '93, Elzinga '92).

ADPKD progresses to end stage renal failure in approximately 45% of patients at 60 years of age (Parfrey '90). Progression appears to be faster in those who have the PKD1 as opposed to the PKD2 genotype (Gabow '92). Hypertension and renal infections are considered risk factors for the development of renal failure and should therefore be adequately treated. A slow down of the progression to renal failure by early treatment of normotensive patients with angiotensin converting enzyme inhibitors may be hypothesized, but has not yet been established. Reduction in dietary protein intake has shown disappointing results on slowing progression of renal insufficiency (Klahr '95). Earlier onset of renal failure has been related to a youger age at diagnosis, larger kidneys, episodes of hematuria, moderate to severe proteinuria and multiple pregnancies (Gabow '92). ADPKD patients with end stage renal failure can be dialysed and receive renal tranplants equally well as patients with most other renal disorders.

Patients at-risk for the development of cerebral aneurysms, including those with a positive family history, should be screened by cerebral CT and/or MRI scanning (Torres '90). Asymptomatic at-risk children in ADPKD families are usually followed annually for the development of hypertension, hematuria and urinary tract infections. Hypertension and urinary tract infections need prompt and adequate treatment, in particular also because these may enhance progression of the renal lesions.

Renal prognosis

For patients with ADPKD, the probability of being alive and not having end stage renal disease is about 77% at 50, 57% at 58 and 52% at 73 years of age (Bear '84). The prognosis of ADPKD presenting in childhood is variable. Neonatal ADPKD usually manifests severe symtoms (McDonald '91), while ADPKD presenting in older children generally has a better prognosis. More than 80% of children diagnosed by ultrasound maintained a normal renal function throughout childhood (Sedman '87).

Autosomal recessive polycystic kidney disease (ARPKD)

ARPKD is characterized by the development of many small cysts in both kidneys and is associated with biliary dysgenesis and hepatic fibrosis in all. The severity of these severe renal and hepatic manifestations appears to be inversily correlated. ARPKD and congenital hepatic fibrosis (CHF) may be different phenotypic manifestations of the same genetic abnormality. Many patients die at birth from renal failure and associated pulmonary insufficiency. The vast majority of patients surviving the neonatal period develop renal failure during childhood. ARPKD has been refered to as 'infantile' PKD, but this is

incorrect since some patients present later during childhood. Moreover, other forms of PKD may present during infancy.

Genetics

The incidence of ARPKD is 1:10.000 to 1:40.000 (Cole '90). The disease is inherited as an autosomal recessive trait and thus may be seen in siblings, but not in successive generations. The sexes are equally affected, heterozygotes are not affected, and the recurrence rate in successive pregnancies is 25%. The ARPKD locus has been mapped to chromosome 6p21.1-p12, but has not yet been characterized (Zerres '94, Guay-Woodford '95). Linkage analysis suggests that ARPKD involves a single defective gene.

Prenatal diagnosis

Prenatal ultrasound findings of enlarged kidneys, oligohydramnios and an empty bladder can suggets ARPKD, but are not diagnostic (Romero '84). These abnormalities may be found in the second trimester but usually are not apparent before 30 weeks gestation (Zerres '88). Prenatal genetic diagnosis in at-risk families can be performed by haplotype based linkage analysis, provided that DNA of an index patient is available for testing. The correct and accurate phenotypic diagnosis of previously affected children is absolutely essential for reliable testing.

Pathology

The enlarged kidneys maintain their reniform shape remarkably well. Cysts are diffusely distributed over the cortical and medullary zones, in a radially oriented pattern. In young patients the cysts are usually less than 2 mm in size and were shown to be dilated collecting ducts by both microdissection studies and immuno-histochemical and electron microscopical analysis (Osathanondh '64, Verani '89, Faraggiana '85). In addition, proximal tubular cystic lesions have rarely been identified in fetal and neonatal kidneys. Microdissection studies and scanning electron microscopy demonstrated that obstruction of urinary flow is not a component of ARPKD (Kissane '90). During disease progression, a pattern more like ADPKD may develop, including the appearance of larger cysts and interstitial fibrosis and inflammation.

In addition to the renal lesions, all patients with ARPKD exhibit hepatobiliary abnormalities. These consist of biliary hyperplasia and periportal fibrosis and are often associated with dilation of the intra- and extrahepatic biliary tract (Bernstein '87, Alvarez '81). The lesions are limited to the portal areas, while the parenchyma is unremarkable. Although macroscopic hepatic cysts are uncommon, choledochal cysts have been reported. During disease progression many patients develop hepatomegaly and portal hypertension.

Clinical presentation and course

ARPKD affects both the kidneys and the liver and its expression pattern is variable. The disease may be viewed as a spectrum ranging from severe renal involvement and mild liver lesions at one end to mild kidney damage and severe liver lesions at the other. The form with severe renal disease is the more common. It is well recognized that siblings may present at different ages and with different forms of the disease.

Most patients present in infancy (Cole '90). Some die soon after birth from pulmonary hypoplasia. Others present with huge flank masses, severe hypertension, urinary tract infections and / or renal failure. If the respiratory status and hypertension can be controled, most patients stabilize for several years, then progressively decline. ARPKD may also present in older children up to 10 years of age, with abdominal enlargement or hepatosplenomegaly (Gagnadoux '89).

The clinical course is often complicated by hypertension, polyuria, hepatic fibrosis and portal hypertension (McDonald '99). Hypertension can be a very severe complication of ARPKD, in both infants and children. Its pathophysiology is not well understood. Plasma renin levels are usually not elevated (Kaplan '89). If not well managed, hypertension may lead to cardiac hypertrophy and congestive heart failure. As expected in children with collecting tubular lesions, almost all have a reduced capacity to concentrate urine, leading to polydipsia and polyuria (McDonald '99). The serum electrolytes are usually normal.

In children with hepatobiliary involvement, portal hypertension may lead to very severe complications, such as bleeding from esophageal varices, portal thrombosis and hypersplenism causing thrombocytopenia and anemia. Children with biliary tract lesions are also at risk for bacterial cholangitis, which has been noted as early as a few weeks of age (Kääriääinen '88). Cholangitis is often a difficult diagnosis and should be considered in any ARPKD patient with fever of unknown cause.

Clinical management

In neonates with ARPKD severe pulmonary distress may require artificial ventilation. The potential reversibility of this condition may be difficult to asses in early stages because underlying irreversible pulmonary hypoplasia may be complicated by fluid overload and restricted diaphragmatic motion secondary to massively enlarged kidneys.

Infants and young children surviving the early stages must be followed closely. The urinary concentrating defect is a risk factor for dehydration during a feverish illness. Patients with severe polyuria may benefit from thiazide diuretics, which reduce solute and free water delivery to the distal nephron. Hypertension can be treated with calcium channel blockers, diuretics and, in the absence of chronic pulmonary disease, with β-blockers. In addition many patients respond well to ace-inhibitors, despite the fact that high renin levels are not usually associated with ARPKD induced hypertension.

As in other renal disorders, chronic renal failure needs aggressive management of its complications: renal osteodystrophy, anemia, growth disturbancy, as well as monitoring and support of adequate nutrition. Patients with end stage renal disease will require dialysis. Peritoneal dialysis is often succesful even in the face of large kidneys and hepatosplenomegaly. Renal transplantation may require the removal of the cystic kidneys to control hypertension or to allow space for transplant placement.

In children and infants with predominant hepatic involvement close monitoring for complications of portal hypertension and for cholangitis is mandated. Cholangitis may be a particularly difficult problem in immunocompromised ARPKD transplant patients.

Renal prognosis

Improvements of respiratory and renal supportive treatments during recent years have dramatically improved the outcome of patients with ARPKD. Survival of all but the most severely affected neonates with pulmonary hypoplasia, is now possible. In one series actuarial survival rates revealed that of those surviving the neonatal period, approximately 80% were alive at 1 year and 50% at 10 years (Kaplan '89). Systematic data on the deterioration rate of renal function are not available. The increased survival of even very young patients undergoing treatment for end-stage renal disease, in concert with the progressive nature of the hepatic abnormalities in ARPKD, leads to the expectation that many of these patients may eventually develop portal hypertension and progressive liver fibrosis. Therefore hepatic lesions are not only inherent to human ARPKD but also of increasing clinical importance.

Clinical differentiation between ADPKD and ARPKD in childhood

Enlarged or cystic kidneys can result from a large number of different renal or systemic disorders (McDonald '99). The differentiation between ARPKD and ADPKD can be particularly difficult in the pediatric age group, while it is not an issue in adult patients because the vast majority, if not all, patients with ARPKD present during childhood. In children, polycystic kidney disease can ususally be differentiated from other inherited or syndromic disorders by associated clinical features and other investigations, such as ultrasound. The subsequent differential diagnosis of PKD into ADPKD or ARPKD is easy in some, but may be very difficult in other pediatric patients (Kääriääinen '88). This differentiation is critical because these entities have a very different prognosis in terms of renal outcome and complications and will require a different follow up and clinical management. Moreover an accurate diagnosis is crucial for genetic counseling.

A staged evaluation of difficult cases would include a complete family history, a careful clinical history, imaging by ultrasound and intravenous pyelogram, a renal and/or

liver biopsy and, in the near future, molecular analysis of the involved genes. The family history should be complemented by renal and liver ultrasonography of the parents and siblings. If the parents have normal kidneys and are less than 30 years of age, the grandparents should also be investigated. Ultrasound indications of ADPKD are macrocysts in the kidneys and cysts in liver, spleen, ovary or pancreas. ARPKD kidneys are typically echogenic with loss of corticomedullary demarcation and the microcysts are usually less than 0.5 mm, with only occasionally macrocysts more than 2 cm in diameter. Intravenous pyelogram may show splaying of calices around macrocysts in ADPKD and delayed filling with a cortical brush pattern and tubular striation in ARPKD. Histologic examination may reveal cysts in all nephron segments (lectin staining) including glomeruli and a generally normal liver histology in ADPKD. The cystic lesions in ARPKD are largely located in a radially oriented pattern in collecting ducts. The liver may show biliary hyperplasia and ectasia, associated with periportal fibrosis. The differentiation is thus based in most cases on clinical and family history, age of onset, ultrasound findings and occasionally on linkage analysis or histological examination of liver or kidney.

1.2 Pathophysiology of Polycystic Kidney Disease

The current understanding of how cysts are formed is largely based on two fields of research: genetics and cell biology. Genetic research has localized the genetic defects responsible for most cases of ADPKD and ARPKD. The primary disease genes PKD1 and PKD2 were subsequently cloned and sequenced. The identified gene sequences allowed predictions of the structure and potential function of the corresponding protein products. In addition to this genetic approach, cell biological research has identified a unique cellular phenotype of cyst-lining epithelia.

The mechanisms that lead from the genetic defects to the cellular pathophysiology of cyst formation and ultimately to disease, are largely unknown and represent one of the main challenges to PKD research to date.

Human PKD

Molecular genetics

The gene defects responsible for the vast majority of PKD patients have recently been localized. The ADPKD genes, PKD1 and PKD2, were subsequently cloned and characterized. The gene for ARPKD has been mapped to chromosome 6p21.1-p12, but has not yet been cloned (Mucher '98).

ADPKD is caused by a germline mutation in one of at least 3 unlinked genes. Responsible for most cases are two unlinked genes, PKD1 and PKD2, which code for the

predicted proteins polycystin-1 and polycystin-2. Mutations in the PKD1 gene account for 85-90% of cases. Based on sequence and motif analysis polycystin-1 is thought to be a very large transmembrane glycoprotein, 450 kD in size (Hughes '95). Although full length PKD1-cDNA has been synthesized, polycystin-1 has not yet been stably expressed in eukaryote cells in vitro (Ibraghimov '97). At least three other transcripts, that are approximately 97% identical to PKD1, are expressed from multiple copies of the genomic region containing PKD1 exons 1 - 31. It is not known whether these transcripts code for protein (The Eur Cons '94).

The large extracellular region of polycystin-1 contains several different protein motifs, suggestive of protein-protein and protein-carbohydrate interactions (Sandford '97). The cytosolic region includes phosphorylation sites and other putative signaling motifs. Polycystin-1 is thought to function as a plasma membrane receptor.

Polycystin-2 is also predicted to be an integral membrane protein and may be part of a Ca^{2+} ion channel family (Tsiokas '97). In vitro data indicate that polycystin-1 and polycystin-2 can interact to form heterodimers and that polycystin-2 can also form homodimers in a domain separate from its heterodimeric interaction with polycystin -1 (Qian '97, Tsiokas '97). Wilson et al demonstrated by yeast 2-hybrid and functional studies co-localisation, co-association and potential interaction of polycystin -1 with cell-cell adhesion and focal adhesion molecules (Wilson '99b, '99c).

The proposed role of polycystin -1 as a membrane receptor has led to the suggestion that the C-terminal domain may directly interact with cellular signaling proteins. This idea was supported by in vitro studies, which has turned up a number of potentially interacting proteins in addition to polycystin-2, including G-proteins (Parnell '98) and regulators of G-protein signaling (Kim '99), suggesting that polycystin -1 may function as a G-protein coupled receptor. Expression studies indicated that polycystin-2 can mediate the activation of AP-1, a transcription factor that regulates different cellular programs such as proliferation, differentiation and apoptosis (Arnould '99). Co-expression of polycystin-2 with the C-terminus of polycystin -1 augmented this polycystin-2 mediated activation. Thus, polycystin -1 is thought to be involved in cell-cell and/or cell- matrix interactions through its participation in multiprotein signaling complexes.

The PKD1 mRNA is expressed in most, if not all tissues and cell lines examined (The Europ Cons '94, Ward '96). To investigate the in vivo expression of polycystin-1 itself, many groups have developed antibodies against fragments of the predicted protein. In each report the renal expression of polycystin-1 was predominantly localized to tubular epithelia of fetal, adult and cystic kidneys. The most intense staining was usually observed in the ureteric bud of fetal kidneys and in cyst-lining epithelia from ADPKD patients. To our opinion, the data published so far have not been completely consistent and clear, as further addressed in Chapter 3.

The mechanism by which a mutation of the PKD1 gene leads to the formation of cysts is not clear. Recent evidence indicates that cysts are lined by cells that underwent a 'second hit' mutation of the wild type (normal) allele, suggesting that cyst formation is caused by the absence of functional polycystin-1, rather than the presence of abnormal polycystin-1. Loss of heterozygosity (LOH) by a second hit somatic mutation, has been demonstrated in cyst-lining epithelia of both PKD1 and PKD2 (Brasier '97, Watnick '98, Koptides '99, Wu '98). However, the precize role of LOH needs to be further clarified because in these studies, LOH was demonstrated in only a minority of the cysts.

Cellular pathophysiology

In general, renal cysts are epithelial fluid filled cavities that initially arose as progressive dilations of normally developed renal tubules. These early cysts have an open connection to the tubular lumen. However at later stages many cysts become disconnected and may enlarge progressively. This process of cyst formation does not affect all nephrons equally. It was estimated that, cysts in ADPKD develop in only 1-10% of the nephrons, suggesting that factors other than the initial mutation (present in all cells) are also involved in the development of the lesions.

Cellular abnormalities of ADPKD cystic tissues have been reported by many investigators. It should be noted however, that the vast majority of data is based on studies using end-stage kidneys and therefore may not represent earlier stages of cyst formation.

This summary will focus on the three key features of cyst formation and cyst expansion: Proliferation, secretion and extracellular matrix abnormalities.

Abnormal proliferation of the cyst lining cells plays a crucial role in the development and subsequent expansion of the cysts in all described human renal cystic diseases (Gabow '91). An increased number of proliferating cells has been documented in both cystic and non-cystic tubules of ARPKD as well as ADPKD, suggesting the possibility that proliferation may be a primary event in cyst formation (Nadasdy '95). Proliferation has been associated in cystic epithelia with a high level of 'programmed cell death' (apoptosis) and upregulation of apoptosis related genes, such as bcl-2 (Woo '95, Lanoix '96). This suggests that pathways of proliferation and apoptosis are abnormally regulated in ADPKD. Evidence from both human and rodent PKD indicates that the epidermal growth factor receptor (EGFR) axis is particularly involved in the pathophysiology of abnormal cell proliferation. It was found that the EGFR is overexpressed and mislocalized to the apical cell membrane, as opposed to the basolateral membranes, in human ADPKD and ARPKD as well as in early and late stages of murine PKD (Wilson '93, Lee '98, Richards '98). This apical EGFR is functionally active in vitro (Sweeney '98) and can be stimulated by EGF, which has been demonstrated in mitogenic concentrations in ADPKD and ARPKD

cystfluid (Wilson '93). Moreover, inhibition of the EGFR has been associated with a reduction of the cystic process in experimental models (Richards 98, Sweeney 99).

The secretion of fluid is another key feature of cyst formation, requiring the transformation of normal absorptive epithelium into secretory cystic epithelium. In ADPKD, most cysts eventually become disconnected from the nephron of origin as well as from the influx of glomerular filtrate. The cysts therefore require transepithelial secretion to prevent a collapse of the cystwall. This secretion appears to be driven by the cystfluid itself: Intact isolated cysts in culture can secrete fluid in the presence of either cystfluid or by activation of the cAMP signal transduction pathway. In the absence of stimuli however, these epithelia absorb similar to wild type tubules. These data indicate an important role for cystfluid secretagogues (Sullivan '96). Such agents, including ATP and specific lipidfractions, have indeed been demonstrated in cystfluid from both human and murine PKD (Wilson '99a, Grantham '97).

The extracellular matrix and basement membranes play an important role in the maintenance of tissue organization and integrity. It can be hypothesized that in PKD abnormal regulation of the extracellular matrix and basement membranes is required to give way to the expanding cysts. Several abnormalities of the extracellular matrix have been reported in human and rodent renal cystic diseases. Thickened basement membranes as well as altered biosynthesis of several basement membrane and cell adhesion macromolecules have been reported in advanced ADPKD (Granot '90,Carone '93, Wilson '96, '99b). In addition, in vitro studies suggest that increased degradation of basement membrane components by metalloproteases may contribute to the lesions by permitting the cysts to grow (Rankin '99). Abnormal matrix and cell functions may be interrelated since the interactions between cells and extracellular matrix are reciprocal: the cell has a major role in matrix synthesis and degradation, whereas matrix composition and signaling affects cell shape, division, differentiation and gene expression. It was suggested that abnormal matrix-receptor interactions may contribute to cyst formation by interfering with fundamental cell processes, such as cell differentiation, polarity and proliferation (Wilson '99b).

Many of these cellular phenomena are also present – and some were initially observed - in either cellular or whole animal models of cyst formation. The detailed analysis allowed by these models has and will have an important role in the study of the cellular and molecular pathophysiology of human PKD. In the following we will summarize the characteristics of the various experimental models, in the context of their contribution to the current understanding of PKD.

Models of PKD

The systematic study of the pathophysiology and potential therapeutic options of human renal cystic diseases is hampered by the lack of human tissues from early disease stages, as well as by the variable clinical expression of disease. In order to better understand the factors involved in the development of cysts and in the process of disease progression, many investigators have focussed on either animal models, whole organ models or cell models of renal cyst formation. In this Chapter we will summarize the currently available models, in view of their contribution to the understanding of the pathofysiology of renal cystic diseases.

Animal models of PKD

The study of animal models has the potential of identifying genes and gene products involved in the cystogenic process. They also provide the opportunity to study factors that determine the severity and progression of renal cystic diseases. In addition, these models may be crucial for the development of therapeutic interventions. Such models are usually genetically transmitted, resulting from either a spontaneous or an artificially induced mutation. In the following summary we categorized them as spontaneous mutations, transgenic models and chemically induced models.

Spontaneous mutations

This summary is limited to those rodent models of PKD that have been reported adequately in the recent literature (Table. 1). For rodent models less well described and/or not currently available for experimental investigations, we refer to the literature (Gorer '40, Fox '70, Ribacchi '77, Rupple '55, Lozzio '67). Cystic diseases have also been reported in many other species including cat, dog, pig, sheep, horse, springbok, rabbit, raccoon and pigeon. (Jones '90, Scott '86, Crowell '79, Fox '71, Iverson '82, McKenna '80, Wijeratne '80, Eaton '97, McAloose '98, Hamir '96, Van Alstine '84). However, none of these non-rodent species are used for systematic investigations.

The CBA/Ca-KD mouse

Autosomal recessive disease of the renal interstitium was identified in 1971 in an inbred CBA/Ca mouse strain (Lyon '71). Affected homozygotes were called '*kdkd*' for 'kidney disease'. They were normal at birth, but at 10 weeks the kidneys of affected mice developed focal peritubular mononuclear cell infiltrates and cystic dilations of both medullary and cortical tubules. At 4 weeks of age, tubulointerstitial fibrosis developed and the glomeruli became sclerotic. An urinary concentrating defect and progressive renal

failure were demonstrated in later disease stages. Lyon proposed that these mice were a model of the juvenile nephronophthisis – medullary cystic disease - complex (JN/MCD).

Later it was reported that kdkd mice inherited a T-cell-mediated autoimmune interstitial nephritis, in which tubulointerstitial antigens in association with class I major histocompatibilty antigens are targeted by T cells (Kelly '87). The model may be used to study the mechanisms by which immune cells in the renal interstitium and their cytokines contribute to the formation of cysts.

Table. 1

Model	Inherit.	Proposed model of	Chrom.	Human Chrom.	Refs.
Mouse					
CBA/Ca-KD	AR	JN-MCD	-	-	Lyon71, Kelly87
PCY	AR	ADPKD	9	3	Takahashi86, Nagao95
CPK	AR	ARPKD	12	2	Russel77, Simon 94
Xpl	XD	OFD1	X	X	Sweet 80, Feather 97
CFWwd	AD	ADPKD	-	-	Werder84
CBA/N	XR	-	X	-	Rahilly92
JCK	AR	?	11	-	Atala93, Iakoubova 95
BPK	AR	ARPKD	10	-	Nauta 93,95, Guay-W.96
KAT2J	AR ?	?	8	19p	Janaswami97
Rat					
Chi	AR	JN-MCD	-	-	Ohno89, Inage93
CY	AD	ADPKD	5		Kaspareit91,
WPK	AR	ARPKD	5[a]		Nauta Y2K

a. not allelic to CY

The PCY mouse

In 1973, polycystic kidney disease was recognised in a KK mouse strain (Takahashi '86). Due to poor reproduction of the original strain, a congenic pcy strain was developed in DBA/2 mice, DBA/2FG-pcy/pcy. The *pcy* locus has been mapped to a locus on chromosome 9, homologous to human chromosome 3 (Takahashi '91, Nagao '95). Two modifying loci were localized to chromosomes 4 and 16 resp.(Woo '97b).

DBA/2FG-pcy/pcy mice express an autosomal recessive disease with clinical and morphological aspects of human ADPKD. A slowly progressive disease leads to end stage renal failure at the age of 8 months. The progressively enlarging cysts develop in all nephron segments and are lined by a single layer of hyperplastic epithelia. At later stages of disease interstitial inflammation was noted and 10 % developed cerebral aneurysms. On another genetic background, the C57/FB/6 mouse, the histological lesions and the clinical phenotype progressed much slower (Nagao '91).

Progression of the cystic lesions has been associated with increased mRNA levels of a number of growth related proteins, including PCNA, TGF-β, PDGF-α, PDGF-β, IGF-I and

bFGF (Nakamura '93a). In contrast, but consistent with other models (Gattone '90), EGF mRNA levels decreased with age in affected *pcy* animals. Further, abnormalities of phosphoinositide metabolism and abnormal ratios of membrane lipids have been noted in cystic kidneys (Aukema '92a, '92b). In addition, the *pcy* model was used to explore the effect of a number of therapeutic interventions and dietary restrictions: Dietary protein restriction (Aukema '92c, '99), soy protein diets (Tomobe '98), n-3 fatty acid enriched diets (Yamaguchi '90) and methylprednisolone (Gattone '95a) were shown to retard the disease progression in this model.

The CPK mouse

In 1977, autosomal recessive PKD was detected in an otherwise healthy C57/BL/6J strain of mice at the Jackson Laboratories (Russel ''77). The *cpk* gene was localized to chromosome 12 (Davisson '91, Simon '94) and the human *cpk* homologue is predicted to be on 2p23-25 (Guay-Woodford '93). Affected homozygotes of the *cpk* mutation, which appear normal at birth, develop massive cystic enlargement of the kidneys and die in renal failure at 4 weeks of age (Preminger '82). The ontogeny and morphology of the cyst formation was extensively studied by a number of investigators, using light and electron microscopy and microdissection of intact nephrons (Preminger '82, Nidess '84, Fry '85, Avner '87a, Gattone '88). The earliest cysts can be detected in 17 day old fetuses and consist of dilations of proximal nephron segments (Avner '87a). During disease progression, the site of the cystic lesions shifts from juxtamedullary proximal tubules to cortical and medullary collecting tubules. The expansion of the cysts is associated with prominent hyperplasia of the cyst-lining epithelia.(Avner '87a, Gattone '88). The abnormalities in affected mice are limited to the kidney. However, the cystic *cpk* gene has produced hepatic fibrosis and ductal dilatations, similar to human ARPKD, when bred into DBA/2J mice (Fry '85) and dilatations of the biliary and hepatic ducts in CD-1 mice (Gattone '96).

Early stages of proximal tubular cyst formation were correlated with increased Na-K ATPase activity in proximal tubules (Avner '89). This occurred before significant epithelial hyperplasia was present, suggesting that increased Na-K pump activity and cellular hyperplasia were early components of cystogenesis. Subsequent studies indicated abnormal cellular localization of the Na-K ATPase (Avner '92). In proximal tubules the enzyme (α -1 and β-1 subunits) were localized to the basolateral cell membranes in both control and cystic tubules. Although apical Na-K ATPase expression is a transient feature of early development in normal collecting ducts, the percentage of cystic collecting tubules with apical Na-K ATPase remained significantly greater than in controls. The persistence of apical expression may be a manifestation of the relatively undifferentiated phenotype of the cyst-lining cells and if the enzym is functional, this may play a role in abnormal transcellular fluidtransport in PKD.

Abnormalities of the EGF-EGFR axis (epidermal growth factor and its receptor) have been studied extensively in the *cpk* model. This axis is thought to be important in regulating cell growth and maturation in the mature kidney (Uchida '88) and may modulate solute and fluid transport in the collecting duct (Breyer '88). A number of investigators reported decreased expression of EGF precursor genes in cystic *cpk* kidneys (Gattone '90, Lakshmanan '93, Horikoshi '91). Horikoshi et al. found decreased EGF concentrations and activity in serum and urine obtained from *cpk/cpk* animals. Gattone et al. observed that subcuteneous EGF administered to neonatal *cpk/cpk* mice ameliorates the disease process (Gattone '95b). In cystfluid however, both the activity and concentration were increased, when compared to urine (Horikoshi '91). Lakshmanan et al. described a number of immunreactive EGF-like substances in cystfluid in mitogenic concentrations (Lakshmanan '93). In addition, the receptor for EGF (EGFR) was shown to be overexpressed and mislocated in cystic kidneys (Orellana '95). In collecting duct cysts EGFR was localized to the apical instead of the basolateral cell membranes. This raises the possibility that EGFR mislocalization and overexpression may be mechanisms whereby EGF-like proteins stimulate hyperplasia of the cystic epithelia.

A relatively undifferentiated cellular phenotype of cyst-lining epithelia has been suggested by a number of observations in affected mice. Proliferation of cystic epithelia was linked to overexpression of the oncogenes c-myc, c-fos, c-Ki-ras, consistent with an increased rate of cellproliferation and an altered state of differentiation (Cowley '91, Harding '92). A delay in terminal differentiation is suggested by persistent postnatal expression in cystic kidneys of the Cux-1 gene. This gene is essential for normal development of Malpighian tubules in Drosophila and is also expressed in embryonic mouse kidneys, but downregulated in postnatal mouse kidneys (Vanden Heuvel '96). An abnormally high expression was also noted for the sulfated glycoprotein-2 (SGP-2) mRNA in cystic epithelia (Harding '91). SGP-2 is secreted by various epithelial cell types and is progressively down regulated during normal development in the mouse kidney. This protein may be involved in the promotion of cell-cell interactions and apoptosis. A correction of this relative overexpressed SGP-2 was observed in parallel with the benificial effect of EGF administration to affected pups (Gattone '95b). An additional indication of delayed differentiation is the postnatal persistence of apical localization of Na-K-ATPase and of the EGFR (see earlier sections). This polarization defect may relate to the observed decreased expression of the epithelial cell adhesion molecule E-cadherin (Rocco '92), since this molecule is believed to be involved in guiding the sequential differentiation and polarization of renal epithelia.

The basement membrane of *cpk* cysts is ultrastructurally unremarkable (Avner '88a). The components collagen type-IV and laminin were normally distributed in early cystic tissue (Ebihara '88). However, in more advanced stages increased mRNA levels of both were associated with focal decreases of immunoreactive protein. Despite these alterations,

the viscoelastic properties of the cystic tubular wall was not different from controls (Grantham '87a).

Additional observations in *cpk* mice includes the increased expression of a number of different molecules, including: endothelin (ET-1) and its receptor, proliferating cell nuclear antigen (PCNA), TGF-β and TNF-α (Nakamura '93a, '93b), cAMP (Yamaguchi '97). Further studies will be required to link these observations to specific cystogenic processes.

Abnormalities of the glucocorticoid metabolism were reported by Crocker et al. and were suggested to play a role in the pathophysiology of the cystic process (Crocker '87). This may relate to a significant downregulation of the Ke 6 gene, as demonstrated in a number of different mouse models of PKD, including *cpk* (Aziz '93). The Ke 6 gene encodes a 17β-hydroxysteroid dehydrogenase that can regulate the concentration of biologically active estrogens and androgens (Fomitcheva '98, Ramirez '98. Glucocorticoids increase renal Na-K ATPase activity and stimulate intracellular alkalinization (Igarashi '83). Elevation of the intracellular pH is a proven stimulus to cellular proliferation and may therefore contribute to hyperplasia of cystic epithelia. Blockade of the glucocorticoid action in affected *cpk* mice, prolonged the survival of these animals (Ogborn '87).

Several experimental interventions of the cystic process in *cpk/cpk* mice have provided insight into the mechanisms of cyst formation and may suggest targets for future treatment strategies in human disease states. The weekly subcutaneous administration of taxane (Taxol) in 10-day old *cpk/cpk* mice prolonged the life span and slowed the progression of the cystic lesions (Woo '94). This therapeutic effect was related to the ability of taxanes to promote microtubule assembly (Woo '97a). The beneficial effect of EGF administration to cystic animals (see earlier) indicates the role of the EGF/EGFR axis for normal renal development. A recent report by Gattone et al. suggests that stimulation of the collecting duct cell by the vasopressin receptor contributes to the cystogenic process (Gattone '99). They found increased expression of this receptor in cystic *cpk* kidneys and noted amelioration of cyst enlargement and of renal failure following blockade of the receptor with experimental drugs. Activation of this arginine vasopressin type-2 receptor is known to stimulate the production of cAMP, a substance known to promote cyst enlargement.

The CFWwd mouse

In 1984, Werder et al. described a genetically determined renal cystic disease in a Carworth Farm White (CFW) mouse colony and suggested this to be a model of human ADPKD, based on its clinical, genetical and histological characteristics (Werder '84). Affected *CFWwd* animals develop progressive cystic destruction of the kidneys and die in renal failure at the age of 12-18 months. The cysts were localized in both the renal cortex and medulla and included also glomerular cysts. The lesions were accompanied by interstitial fibrosis and infiltration of the renal tissue by immune cells. Interestingly, the

expression of disease was significantly modulated by the microbiological environment. Under standard conditions over 90% of affected mice died of renal failure at 2 years of age, whereas only 4 % of the mice were affected if raised under germfree conditions. The renal disease was associated with hepatic cysts in 15% of cases and occasionally with aneurysms of the thoracic aorta. The genetic basis of this model is not clear. The study of an F2 intercross with AKR mice, suggested the possibilty of an autosomal dominant mode of inheritance with incomplete penetrance (Werder '84).

The *CFWwd* model illustrates that genetically determined renal cyst formation can be profoundly modulated by environmental factors. Unfortunately, further studies into the mechanism of this modulation have not been reported.

The CBA/N mouse

In CBA/N polycystic kidney disease is associated with an X-linked recessive B cell immunodeficiency syndrome (Rahilly '92). All mice more than 3 months old exhibit PKD. At birth the kidneys appeared normal, but postnatally they progressively enlarged and formed cysts up to 1mm in diameter throughout the parenchyma. The cysts were localized to glomeruli and all tubular segments, except collecting ducts. Cyst formation was associated with basement membrane thickening of the tubular wall, and in older animals with interstitial fibrosis, mononuclear cell infiltrates and glomerulosclerosis. The liver is unaffected in these animals. This model, as well as the *CBA/Ca-KD* model (see earlier section) offers an opportunity to study the relationship between the immune system and the formation of renal cysts. To our knowledge no further studies on the renal lesions in this model were reported.

The JCK mouse

Autosomal recessive PKD was identified in a *c-myc* transgenic line of mice (Atala '93). The *jck* mutation (juvenile cystic kidneys) appears unrelated to the *c-myc* transgene since it segregates freely from it. The animals develop early cystic lesions in the renal cortex at 3 days and subsequently a slowly progressive cystic destruction of the cortical and medullary parenchyma. The localization of the cysts to specific nephron segments has not yet been reported. Affected mice die at 6 months of age. No histological abnormalities were found in any other organ. The *jck* gene was mapped to chromosome 11 and two additional regions, that were associated with disease severity, were identified on chromosomes 1 and 10 (Iakoubova '95). Based on its mapping position, a candidate for one of these modifying loci (on Chr 10) may be the *bpk/jcpk* gene (Guay-Woodford '96). The relatively long lifespan offers a unique opportunity to study the pathophysiology of cyst formation and progressive expansion.

The BPK mouse

We had the opportunity to study the phenotype of this new model of autosomal recessive polycystic kidney disease (Chapter 2.1). In addition to renal abnormalities much like those described for the extensively studied *cpk* model, affected *bpk/bpk mice* also express proliferative abnormalities of the biliary tract. The hyperplastic biliary lesions provide an unique opportunity to culture and study the affected cells in this model (Chapter 2.2). The association of renal and biliary lesions is also a characteristic feature of human ARPKD.

The affected *bpk* gene was localized by Guay-Woodford et al. to a 1.6 cM interval on chromosome 10 (Guay-Woodford '96). Interestingly *bpk* co-localized with *jcpk* and complementation testing indicated that these two loci are allelic. A further detailed discussion is provided in Chapter 2.

The KAT2J mouse

Recently a new C57BL/6J mouse mutant was identified at the Jackson Laboratory (Janaswami '97). Affected mice exhibit autosomal recessive PKD in association with facial dysmorphism, dwarfing, male sterility and anemia. The renal lesions were localized to the proximal tubules and to Bowman's capsule. Approximately half die before weaning and the survivors die between 6 and 12 months of age. The phenotype has not been reported in much detail. The affected gene was localized to a segment on chromosome 8 that is homologous to human chromosome 19p. During the mapping studies it was noted that disease severity was more variable in the intercross progeny than in the parental C57 strain. This variability could be linked to three modifying loci that could be localized to mouse Chr 1, 2 and 19 (Upadhya '99). This model clearly illustrates that genetic factors, other than the primary mutation, may significantly affect the clinical manifestations of PKD.

The Xpl mouse

This X-linked model of limb morphogenesis expresses polydactyly and urogenital malformations including renal cysts (Sweet '80). It was recently found that the mutated gene colocalizes on the X-chromosome with the oral-facial digital syndrome type 1 (OFD1), a cause of human glomerulocystic kidneys disease (Feather '97). The renal lesions of this mouse model have not been described in much detail.

The Chi rat

This recessive model of renal cystic disease arose in Wistar rats, following a spontanous mutation called *chi* (Ohno '89, Inage '91, '93). Unlike human ARPKD this model is characterized by skeletal abnormalities associated with slowly progressive renal disease. Cysts in advanced stages of disease had lectin binding characteristics of collecting ducts. Early cysts have not yet been characterized. Hepatobiliary lesions have not been reported

in this model. Further studies may reveal whether *chi* could be a model for juvenile nephronophthisis, in which renal cysts and more moderate skeletal abnormalities have also been reported (Mainzer '70).

The Cy rat

Autosomal dominant PKD in a Sprague-Dawley rat colony was reported by Kaspareit-Rittinghausen (Kaspareit-Rittinghausen '91) and later in more detail by others (Schäfer '94b, Cowley '93). The model was initially called Han-SPRD and the affected gene was later called *cy*. There is an incomplete dominant mode of inheritance: The disease is more severe in homozygotes than in heterozygotes. Homozygotes develop cysts in all nephron segments throughout the kidney and die in renal failure, at 3-4 weeks of age. Heterozygotes develop cortical and outer-medullary cysts. Approximately 75% of the cysts are derived from proximal tubules. There is a considerable gender effect: cy/+ males develop progressive renal failure and usually die within a year(Gretz '95). Females have milder renal disease. Liver cysts were only noted in 2 year old *cy*/+ females (Kranzlin '97). This gender effect appears to be testosterone related (Cowley '97). Hypertension is present in advanced heterozygous disaese (Kaspareit '91), but is not a consistent finding in earlier stages of disease (Braun '96, Ogborn '95c). In contrast to the phenotypical similarities with ADPKD, is the *cy* mutation not a genetic model of human PKD1 since it does not localize to the homologous region in rats, as discussed in more detail in Chapter 2.3 of this thesis. *Cy* was later mapped to rat chromosome 5 (Bihoreau '97).

The pathophysiology of cyst formation has been studied extensively. Hyperplasia of tubular epithelia and increased expression of oncogenes has been documented in both early and late stages of cyst development (Cowley '93, Ramassubu '98). Changes of the extracellular matrix include thickening of basal lamina and increased expression of collagen type-IV in advanced lesions as well as focal thickening of BM and collagen type-IV overexpression in early cysts (Schäfer '94a, Schaefer '96). Obstruction of the renal tubules has been studied by micropuncture methods and was found to be present in some but not all cysts (Tanner '96). Obstruction appears to be a component, but not the primary event in this model. The role of the renal endothelin system has been studied by Hocher et al., who found increased endothelin-1 (ET-1) expression in cyst-lining epithelia of both *cy* affected rats and human ADPKD (Hocher '98). The system was activated and participated in the regulation of bloodpressure, renal bloodflow and glomerular filtration. Abnormalities of the cell polarity as described for end stage human ADPKD and for *cpk* cysts, was not seen in early *cy*-cysts (Obermuller '97).

The progression of disease can be affected by a number of measures. Accelaration of the lesions and of renal failure was noted after unilateral nefrectomy (Gretz '94), dietary protein overload, potassium depletion, induction of acidosis (Cowley '96). Progression of disease can be ameliorated by dietary protein restriction (Ogborn '95a), soy proteins

instead of casein proteins(Ogborn '98), dietary flaxseed (Ogborn '99), treatment with potassium citrate/citric acid (Tanner '98), taxanes (Martinez '97), methylprednisolone (Gattone '95a) and angiotensine converting enzyme inhibition (Ogborn '95c).

The WPK rat

The *wpk* model is described in more detail in Chapter 2.4. The renal phenotype is much like human ARPKD, exhibiting rapidly progressive cyst formation in homozygotes, proteinuria, hypertension and a collecting tubular cell type in the vast majority of cysts. Localization studies of the affected gene are in progress.

Transgenic mouse models

In addition to these spontaneously developed genetic models of PKD, murine polycystic kidney disease has been associated with a number of artificially induced gene disruptions and overexpression models, as summarized in the following section. The recently acquired ability to study the function of genes by modifying the specific genetic background of mice, has produced a number of interesting PKD-like diseases (Table 2).

Table 2. Transgenic mouse models exhibiting renal cysts

Mouse Model	Genetic defect
PKD1	Knockout of mouse pkd1 gene
PKD2	Knockout of mouse pkd2 gene
ORPK	Disruption of possible cell cycle control gene, TgN737Rpw
JCPK	Disruption (Chlorambucil) of unknown gene
SBM	C-myc oncogene overexpression
AP-2β knockout	Deficiency of transcription factor- apoptosis inhibitor
Bcl-2 'knockout'	Deficiency of apoptosis inhibitor bcl-2
PAX2	Overexpression / deficiency of transcription factor
SV40 early region	Expression of the viral transformation antigen "large-T"
SR2-3 chimeric	v-src proto oncogene, chimeric overexpression
TGF- α	TGF- α overexpression
KGF	Hepatic overexpression of KGF
HGH / GRF / IGF-I	Growth hormone overexpression
HGF/SF	Hepatocyte growth factor / scatter factor overexpression
ET-1	Endothelin 1 overexpression
Tensin	Knockout, Focal adhesion protein

Knockout of human PKD genes

Mutations of either the PKD1 or the PKD2 gene are responsible for most patients with ADPKD. The identification and characterization of these genes has enabled the development of murine knockout models. The mouse *pkd1* gene was disrupted by the

introduction of a truncation mutation. This mutation leads to renal cystic disease with perinatal lethality in homozygotes. Heterozygotes exhibit some renal cysts and hepatic abnormalities, at old age only (Lu '99). Unlike patients with ADPKD these heterozygotes had a normal renal structure until the second half of life and renal failure was never observed (Lu '97). Somatic inactivation of the mouse *pkd2* gene resulted in an ADPKD-like disease including renal and hepatic cystic lesions in heterozygotes (Wu '98). A more detailed characterization of the clinical and cellular abnormalities in this *pkd2* knockout model will further establish its value as a model of human ADPKD.

ORPK

The ORPK model was identified after random insertional mutagenesis (Moyer '94). The disrupted gene was called *TgN737Rpw* and subsequently mapped to chromosome 14 (human Chr.13) (Yoder '95). Sequence analysis revealed motifs common to several cell cycle control genes. The disease expression in mutant animals can be ameliorated by expressing wild type TgN737Rpw as a transgene (Yoder '96, '97). Affected *orpk/orpk* mice manifest renal cysts and hyperplastic biliary abnormalities, similar to human ARPKD. The formation of collecting tubular cysts was associated in this model with increased expression and mislocalization of the EGF receptor to the apical cell membrane, in parallel with similar observations in endstage cysts of human ADPKD and in *cpk/cpk* mice (see earlier sections) (Richards '98, Sweeney '98). Moreover, in a cross with EGFR deficient mice, the double homozygotes had an increased lifespan and less cysts, indicating a direct role for EGFR in collecting tubular cyst formation (Richards '98).

Jcpk

The *jcpk* mutation results from a chlorambucil induced mutation (Flaherty '95). Homozygotes express severe early onset cystic lesions in all nephron segments, associated with pancreatic and biliary abnormalities and die before 10 days of age. Heterozygotes present with glomerulocystic disease. Interestingly, it was found that the *jcpk* mutation co-localizes on chromosome 10 with *bpk*, a phenotypically different PKD mutation as described in Chapter 2.1, and complementation testing indicated that these two mutations are allelic. This implies that distinct phenotypes can result from different mutations within the same gene. It should be noted however, that the chemically induced *jcpk* mutation may well consist of a large deletion involving multiple genes.

SBM, AP-2, Bcl-2

The SBM mouse expresses PKD induced by the overexpression of *c-myc* (Trudel '91). This proto-oncogene is thought to be involved in the regulation of cell proliferation and is transiently expressed during normal nephrogenesis. C-*myc* was found to be overexpressed in cystic epithelia of human ADPKD and of affected *cpk* mice. The development of renal

cysts in SBM is associated with approximately 10 fold increased indices of both proliferation and apoptosis in cystic and noncystic tubules. Interestingly, *c-myc* induced apoptosis in this model appears to be p53 and bcl-2 independent since the expression pattern of these apoptosis associated genes was unaffected and because the *c-myc* induced lesions could also be produced in *p53*(-.-) mutants, as well as in *bcl-2* overexpression mutants (Trudel '97, '98).

AP-2 transcription factors are expressed during embryonic development and may be involved in the regulation of embryonic cell survival since they are capable to suppress c-myc induced apoptosis in vitro. AP-2-β knockout mice express lethal PKD at birth (Moser '97). Following a stage of apparently normal renal development cystic lesions arise in association with downregulation of the bcl-2 inhibitor of apoptosis and massive apoptotic cell death in the distal nephron.

Bcl-2 is another inhibitor of apoptotic cell death and expressed predominantly in adult progenitor cells and more widely in embryonic tissues. Transgenic mice overexpressing bcl-2 exhibit increased cellsurvival and lymphomas. Transgenic mice lacking bcl-2 display cystic kidneys, growth retardation, atrophic lymphoid tissues and accelerated cell death of lymphocytes (Veis '93).

These three observations suggest that PKD may result from a critical imbalance of the regulation of cell proliferation and programmed cell death.

PAX2

PAX2 is member of a transcription factor family involved in embryonic development. Its role during renal development has been elegantly reviewed by Eccles (Eccles '98). This factor is expressed during renal development in association with cell proliferation as well as with the initiation of epithelial differentiation and down regulated postnatally. In contrast, PAX2 is persistently expressed postnatally in the proliferative cystic epithelia of PKD and multicystic dysplasia. Overexpression of PAX2 in transgenic mice leads to microcystic tubular dilatations and glomerular abnormalities similar to congenital nephrotic syndrome. The critical role of PAX2 to renal development is additionally established by the deficiency models. Mutation of PAX2 leads to renal and ocular defects in both human disease states and in mouse models. The renal defects include agenesis, hypoplasia and cystic lesions. Although the renal cystic lesions were frequently observed in a number of mutant mouse models, they are rare in patients with PAX2 mutations.

SV40 early region

The early region of simian virus 40 (SV40) encodes the antigen 'large-T'. In addition to its viral functions, this antigen is capable to transform and immortalize tissue culture cells. Transgenic mice expressing this antigen develop tumours and renal cysts (Mackay '87, Kelley '91). The induction of both tumours and hyperplastic cysts by a single transforming

gene suggests that these may represent different manifestations of the same pathological process, different only in the degree of escape from normal cell cycle control. Interestingly, this combination of multiorgan tumours and renal cysts is a well known feature of the human diseases tuberous sclerosis and von Hippel-Lindau disease.

SR2-3 chimeric mice

Succesfull introduction into the mouse embryo of SR2-3 embryonic stem cells containing the v-src retroviral vector SR2 gave rise to chimeric mice with dominantly inherited cystic kidneys (Boulter '92). Since the v-src oncogene activity in the kidneys of chymeric mice was low, it is not certain whether the hyperplastic abnormalities in these mice are directly caused by the expression of the v-src oncogene as seen in SV40 and c-myc transgenic mice. Alternatively the disease may be caused by a dominant mutation that arose either spontaneously or by insertion of the retroviral vector at a cystic locus.

Overexpression of growth factors

Overexpression of a number of growth factors known to play a role in the physiology of normal renal epithelia, has been associated with cyst formation.

Overexpression of TGF- α has not only been documented as feature of cystic disease, but also causes cysts in transgenic mice (Lowden '94). TGF- α is the fetal ligand of the epidermal growth factor receptor (EGFR) in developing kidneys and involved in the regulation of cell proliferation and differentiation as well as extracellular matrix synthesis. Transient overexpression of TGF- α in transgenic mice during one postnatal month has been associated with increased renal size and the development of renal cysts (Lowden '94). This provides evidence that endogenous stimulation of the EGFR can produce renal cysts. Abnormal regulation of the TGF α /EGF/EGFR axis has been suggested by a number of studies of human and murine PKD (see earlier sections).

Keratinocyte growth factor (KGF) is a potent mitogen of epithelial cells and a renal morphogen. Hepatic overexpression in transgenic mouse embryo's resulted in increased KGF serum levels and cystic dilatations in fetal collecting ducts in association with hyperplasia of the biliary system, much like human ARPKD (Nguyen '96). Additional hyperplastic abnormalities were noted in a number of other organs including lung, intestine, pancreas, skin and hair. KGF overexpression during late gestation resulted in perinatal lethality.

Transgenic overexpression of human growth hormone (hGH) and of hGH releasing factor (GRF) has induced increased body growth as well as a number of organ defects, including cystic tubules, nephron atrophy and glomerulosclerosis (Doi '88, Quaife '89,Wanke '91). Additional lesions included hepatomegaly, hepatocellular carcinoma and myocardial fibrosis. Insulin-like growth factor I (IGF-I) levels were increased in both hGH and GRF transgenic mice. In order to determine whether IGF-I mediates the somatic

effects of hGH, these effects were studied in IGF-I transgenic mice expressing increased IGF-I levels and very low GH levels. Although these animals express some renal changes including glomerular enlargement, no interstitial or cystic lesions were observed in these models. This indicates that the renal cyst formation induced by hGH overexpression is not mediated by IGF-I.

Hepatocyte growth factor/scatter factor (HGF) is a renal morphogen, thought to play a role in normal renal tubulogenesis as well as in renal regeneration after acute renal failure. Its overexpression in transgenic mice induced polycystic kidneys and glomerulosclerosis (Takayama '97).

Endothelin-1

Overexpression of the endothelin-1 gene under its natural promotor in transgenic mice induced renal cysts, interstitial fibrosis and glomerulosclerosis, leading to progressive renal failure (Hocher '97). These abnormalities are also features of human PKD and have been associated with elevated endothelin-1 tissue levels in cystic *cy* rats (Hocher '98). This suggests a potentially mediating role for the endothelin system in the development of these lesions in PKD.

Tensin

Tensin is a focal adhesion protein and widely expressed in many different tissues during embryogenesis. Interestingly, tensin is said to co-localize with Polycystin-1, the primary affected protein in most patients with PKD (Wilson '99b). Transgenic mice lacking tensin initially develop normally. After several months however, they exhibited renal failure and cystic renal lesions (Lo '97). Ultrastructurally the renal lesions are associated with disruption of cell-matrix junctions and abnormal polarity of the tubular cells, whereas no junctional abnormalities were noted in unaffected tubules. These data indicate the cystogenic potential of defective focal adhesion complexes.

Chemically induced animal models

Several chemicals will cause cystic kidney lesions when administered to a number of experimental animal species. These include glucocorticoids (McDonald '90), Tetrahydrocarbazole-3-acetic acid (McGeoch '76) and the antioxidants Diphenylamine (Evan '76), Diphenylthiazole (Carone '74, Ehara '94) and Norhydroguaiaretic acid (Gardner '86, Goodman '70). Although these models have interesting features in common with specific aspects of human renal cysts, none of them represents the complete phenotype of cyst formation in developing kidneys that are typical of one of the genetically determined diseases. They indicate the potential toxicity of the respective drug. These models have limited value to the exploration of cystogenesis in PKD. The pathophysiology

of drug induced cystogenesis is unknown. Most of these drugs affect a large number of different cell systems. It is unknown which of these are involved in the cystogenic process. Moreover and in contrast to the genetic animal models, drug induced cystogenesis is based on postnatal interference only and therefore does not reflect the developmental nature of human PKD.

Summary of animal models

Genetically determined polycystic kidney disease has been identified in a large number of murine models. These models have all contributed to the understanding of human PKD. The various genes primarily disrupted or abnormally expressed are all candidate mediators of the cystogenic process in human PKD. Observations in these models regarding the pathophysiology of cyst formation have contributed considerably to the understanding of human PKD. In addition some were used to explore potential therapeutic interventions of PKD.

The research potential varies from model to model. In general, transgenic models have identified cystogenic properties of a large number of specific genes and gene products. Although renal cysts were present in all, do most transgenic models not present all characteristic features of either human ARPKD or ADPKD. The best exceptions to this might be the pkd2 knockout mouse and the ORPK mouse. In contrast to the transgenic models do spontaneous murine models of PKD often manifest a remarkable phenotypical resemblance to human PKD. However, the genetic defects in these spontaneous models have not yet been characterized in much detail. The characterization of the affected genes and gene products as well as of the genes that affect disease severity in these models may contribute to the future understanding of the cystogenic process.

The ideal animal model for the study of either human ARPKD or ADPKD has yet to be defined. Such a model would obviously be based on a homologous genetic defect and express an identical phenotype, both clinically and histologically. In addition, this model would allow the study of cellular pathophysiology, of physiological parameters like renal funcion and bloodpressure, as well as of therapeutic interventions. Based on genetic and phenotypic characteristics, we arbitrarily suggest as best suitable for such studies, the models listed in Table 3.

Table 3. Selected animal models

Phenotype Clin/histol	Mutation		Characteristics
ADPKD	Mouse	*pcy*	Slow progression allows study of interventions
		pkd2	Gene homologous to human PKD2. Phenotypical similarity; needs further characterization.
	Rat	*cy*	Well characterized and extensively studied Rat allows physiological and interventional studies
ARPKD	Mouse	*cpk*	Well characterized and extensively studied Biliary abnormalities when bred into DBA/2J mice
		orpk	Gene characterized. Similar to CPK, and also biliary lesions.
		bpk	Similar to CPK, and also biliary lesions Enables cell model of hyperplastic biliary cells
	Rat	*wpk*	Rat allows physiological and interventional studies Renal phenotype like ARPKD and mouse models.

In vitro models of cyst formation

The in vitro systems of cyst formation permit highly controled experimental conditions that allow the precise study of abnormalities of cellular metabolism and function involved in cyst development.

Renal organ culture

The culture of metanephric renal explants from fetal mice enables the study of cystogenesis because cysts can be induced, in tissue undergoing advanced differentiation in chemically defined medium in vitro (Avner '88b). The process of cyst formation and cyst enlargements can be experimentally isolated from the effects of glomerular filtration, endothelial-mesangial interactions, phenomena related to intratubular flow and the effects of growth factors and transport substances in serum or urine.

In this system, proximal tubular cysts can be induced by triiodothyronine and glucocorticoids, amid a background of normal organotypic epithelial differentiation (Avner '84, '87b). It was found that this cyst induction was linked to increases in Na-K-ATPase activity and was completely inhibited by specific Na-K-ATPase blockade with ouabaine (Avner '85). This suggested that abnormal transtubular fluid transport under these conditions led to accumulation in discrete nephron segments and ultimately to cyst formation. Additional studies in these models demonstrated that EGF may act as a cystogen and that EGF-receptor inhibition abolished this process (Avner '90, Pugh '95, Sweeney '99). Such studies have delineated mechanisms by which cysts may be produced

in normally developing tubules, including growth factor induced epithelial proliferation and abnormalities of tubular fluid transport.

The culture of fetal mouse kidneys has also extensively been used to study normal nephrogenesis and recently to explore the role during normal nephrogenesis of polycystin-1, the PKD1 gene product. It was found that synthesized fragments of the extracellular domain of polycystin-1inhibited branching tubulogenesis when added to the culture medium of a metanephric organ culture system. As suggested by the author, this effect might result from competitive inhibition of polycystin-1 function, indicating that polycystin-1 may play a role in branching morphogenesis of the ureteric bud (Van Adelsberg '98).

This experimental system is thus particularly suited to study under well standardized conditions, specific aspects of normal nephrogenesis as well as of renal cyst formation.

Cell culture systems

Cells derived from patients with PKD can potentially provide model systems that express critical characteristics of the cystic renal tubule. Cultured cells that retain features of cystic epithelia provide a tool to characterize the ion and solute transport systems, the proliferative properties and the epithelial response of cystic tubules. The creation of cysts in vitro requires cells that proliferate in a supportive matrix to form a confluent epithelium that accumulates intracavitary fluid. Cells used for such systems may either be isolated in primary culture from renal cysts or be derived from immortalized epithelial cell lines. However, the harvesting for primary culture of cyst-lining cells of interest, and in particular the definition and harvesting of tubular segment specific normal control cells, introduces a critical selection to these experiments, given the highly heterogenous cell population of the nephron. It is further noted here, that cystderived cells are usually obtained from end stage kidneys and may not represent the initial disease process.

The immortalized MDCK (Madin-Darby canine kidney) epithelial cell line, expressing characteristics of the distal nephron, has extensively been used to study the in vitro formation of epithelial monolayers, tubules as well as cysts. When grown in three-dimensional collagen gels, MDCK cells form spherical cysts composed of polarized epithelial cells circumscribing a central lumen (McAteer '87). The formation of tubules instead of cysts can be induced by the adddition of HGF to the culture medium (Montesano '91, '97). The use of chemically defined culture media enabled the study of specific factors that affect proliferation and fluid transport (Ye '92, Grantham '89,Yamaguchi '97).

The isolation and culture of ADPKD derived cystwalls yields epithelial cells that proliferate and may form microcysts when cultured in a collagen matrix (Mangoo '89). However, such cystforming properties were also observed in a separate study using cells from normal human kidneys (Neufeld '92). Thus, a genetic defect is not required to

develop a cystic phenotype in vitro. In addition to this three-dimensional system, many investigators have focussed on ADPKD derived monolayers of polarized epithelial cells in culture. Such studies have revealed abberant epithelial cell growth regulation, increased production of extracellular matrix constituents and the secretion of substances that may affect transcellular transport characteristics (Wilson '92, '93, '99a, Du '95). Similarly, PKD-derived murine cell cultures using the *cpk* mouse and the *cy* rat models as a cell source, have also been reported. Primary cultures of *cpk* cells exhibited a normal proliferation rate and a twofold increase above normal of collagen type-IV and laminin synthesis (Taub '90, Rankin '92), while cellcultures derived from cysts of affected *cy* rats were used as a model of in vitro cyst formation (Pey '96).

Alltogether, cultures of cyst-lining cells provide opportunities to study the unique cellular PKD phenotype, in isolation from its normal multicellular environment in cystic tissues. Cells that retain specific features of cyst-lining epithelia may in particular be used to study the proliferative, secretory and matrix abnormalities associated with this cystic phenotype.

Relationship of experimental models to human PKD.

The various experimental models have generated theories that are not mutually exclusive, but rather complementary. It is clear that the pathogenesis of cyst formation is a complex and multifactorial process. It is a relatively common response to mutation or manipulation and it is likely that several different mechanisms can result in this superficially common change. In Figure 1. we provide a theoretical frame work that outlines the relationship of the various components of this common cystogenic pathway.

In this framework a key consideration is the apparent loss of coordinated control of tubular diameter. Under normal conditions this diameter is strictly controled, by yet unknown mechanisms. Cells divide either during development or to replace dead cells and cell divisions can hardly be observed once the tubule has reached its programmed diameter. It is hypothesized that cysts may form when this negative feedback on cell proliferation fails, while at the same time the monolayered organization of the tubular epithelium is maintained and provided that the extracellular matrix gives way and that the intraluminal flow of glomerular filtrate is normally present.

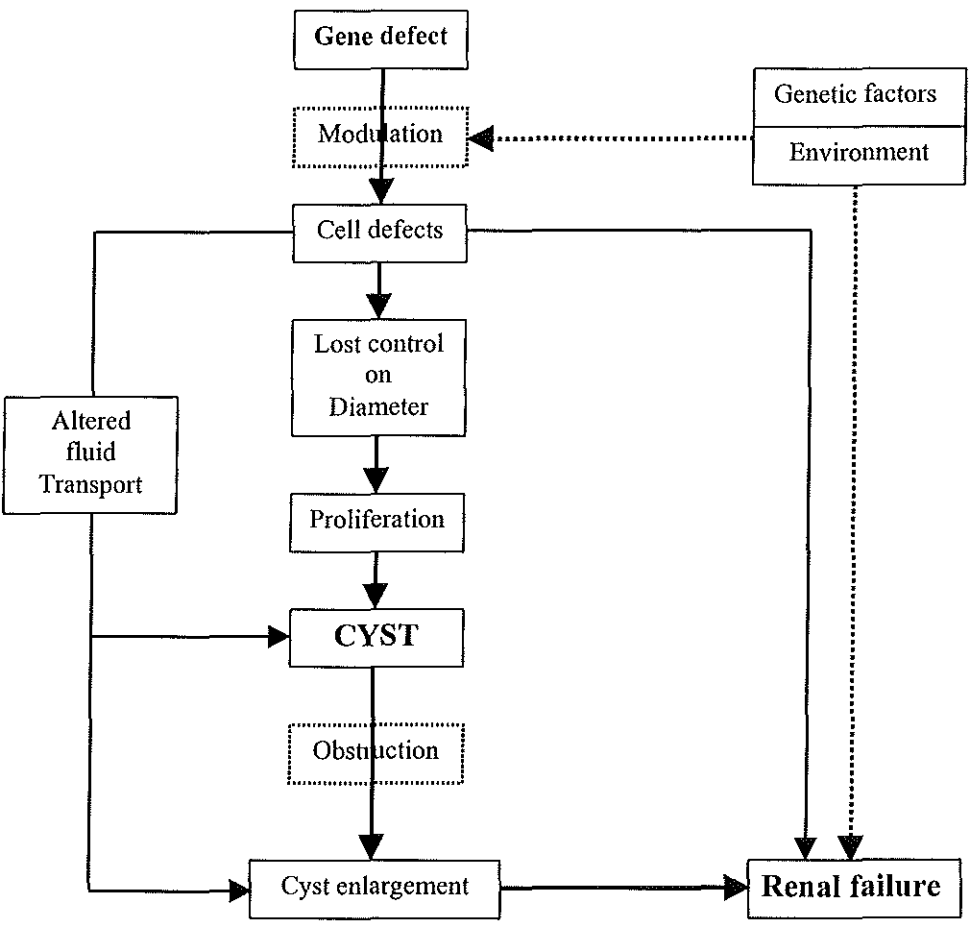
The initial defect in human PKD is genetically determined. However, such a defect is not a prerequisit for the formation of renal cysts since non-genetic, acquired cysts do also occur, in both human disease states and animal models including chemically induced cysts and cysts associated with end stage kidney disease. The extent to which a PKD mutation leads to structural lesions and disease is variable. In ADPKD only about 1% of the renal tubules develop cysts and manifestations of the disease is highly variable, also within affected families. A number of factors have been recognized that may determine disease

severity. First of all, there are indications that cysts in ADPKD result from clonal expansion of cells that underwent a mutation of the unaffected allele (2^{nd} hit) (Qian '96, Brasier '97, Watnick '98, Wu '98, Koptides '99). It can only be speculated that the frequencies of such 2^{nd} hits may vary between individuals and may be determined by both genetic and environmental factors. Gender and genetic background appear to modify the disease course in both murine and human PKD (Kaplan '89, Gretz '96, Schieren '96, Zerres '96, Gabow '92). Several disease modifying genes, have been identified and localized in mice (Iakoubova '97, Wright '97,Upadhya '99, Woo '98). In addition, a number of environmental factors that may modulate disease severity have been recognized, including infections (Werder '84), hypertension (Gabow '92) and testosterone levels (Cowley '97, Fomitcheva '98).

The cellular defects induced by the affected gene may lead directly to the abnormal cell functions that have been associated with renal cystic conditions, such as: 1). Abnormal cell cycle control associated with increased apoptosis and increased expression of oncogenes, growth factors and growth factor receptors (Winyard '96, Veis '93, Trudel '91, Moser '97, Calvet '93, Klingel '92, Nauta '95, Wilson '93, Orellana '95); 2). Abnormal sorting of growth factor receptors (Falkenstein '94, Orellana '95, Sweeney '98), cell adhesion molecules (Du '95) or transport proteins (Wilson '91, Avner '92) 3). Abnormal composition of the lipid membrane (Aukema '92b) 4). Abnormal regulation of cell-matrix receptors and adhesion molecules, possibly including the polycystins (Wilson '99b) and 5). Abnormal extracellular matrix and basement membrane production (Cuppage '80, Wilson '92, Schäfer '94a). These cellular abnormalities may directly disrupt the hypothesized diameter control systems and induce increased epithelial cell proliferation that escapes the normal control of tubular diameter, leading to cystic dilatation and ultimately to cyst formation. This process could further be enhanced by substances in the cyst fluid with mitogenic and/or secretion stimulating properties including EGF, cytokines and lipids , as has been demonstrated in vitro using both human and murine cystfluids (Gardner '91, Grantham '97, Moskowitz '95, Sweeney '96, Yamaguchi '97, Slade '98).

Although early cysts are thought to drain into the tubular lumen, they often obstruct subsequently from cellular debris or epithelial hyperplasia, leading to cysts isolated from the originating nephron. In these obstructed cysts alterations in sodium pump polarity (Wilson '91, Avner '92) and sodium pump-mediated transtubular transport could lead to net intratubular fluid accumulation. Subsequent increases in tubular wall tension may further stimulate epithelial proliferation and cyst enlargement. Although interstitial changes are not a consistent finding in early cystogenesis, they are markedly present during late stages of ADPKD and include the accumulation of a collagen type-IV, laminin, fibronectin, macrophages and fibroblasts. These are thought to play a role in cyst enlargement, and ultimately in the development of interstitial fibrosis and renal failure (Grantham '97).

The subsequent decline in glomerular filtration rate results from the loss of non-cystic parenchyma in association with interstitial fibrosis and mass replacement by fluid filled cysts. This process may be further enhanced by ischaemia, urinary tract infections and uncontroled hypertension.



Figuur 1. Theoretical framework of cystogenesis

Aims of this thesis

Experimental models provide unique opportunities to study the pathophysiology as well as the clinical aspects of Polycystic Kidney Disease (PKD). While genetically determined renal cyst formation has been reported in a number of different animal models, none of these exhibits all genotypic and phenotypic characteristics of either ADPKD or ARPKD in human patients. In **Chapter 1.2** we provide a review of the literature regarding the characteristics of the currently available animal models.

In order to further elaborate mechanisms involved in PKD, we set out to identify and characterize the clinical and histopathological phenotypes of two new murine models of PKD, the '*bpk* mouse'and the '*wpk* rat' (**Chapters 2.1** and **2.4**). In this process we also explored the genetic basis of the *wpk* mutation as well as of the existing '*cy* rat', a model of ADPKD (**Chapters 2.4** and **2.3**). We aim to compare these new models to existing PKD models. Since we noticed that renal and hepatic lesions in *bpk* mice are hyperplastic, as they are in human PKD, we also set out to develop a *bpk* cell culture system that would enable further explorations of the proliferative nature of PKD epithelia (**Chapter 2.2**).

Since PKD can be regarded as an abnormality of normal renal morphogenesis and differentiation and since 'programmed cell death' is thought to play a key role in these developmental processes, we opted to determine whether cyst formation is associated with abnormal regulation of 'programmed cell death' (**Chapter 3.1**).

The recent genetic identification and analysis of the PKD1 gene has revealed the aminoacid sequence of the predicted geneproduct, polycystin-1. To investigate the expression of polycystin-1 in human tissues we set out to develop antibodies to fragments of the predicted polycystin-1 protein and to test these antibodies on tissue sections and homogenates of fetal, adult and cystic human kidneys. We aim to compare the results of these studies with those of others (**Chapter 3.1**).

2

Experimental models of PKD

4 studies

2.1

Renal and biliary abnormalities in a new murine model of ARPKD

Jeroen Nauta[a,b], Yuko Ozawa[b], William E. Sweeney,Jr.[b],
Joe C Rutledge[c] and Ellis D. Avner[b]

Department of Pediatrics[a], Sophia Children's Hospital, Rotterdam, The Netherlands,
Departments of Pediatrics[b] and Laboratory Medicine[c], Children's Hospital Medical Center and the University of Washington School of Medicine, Seattle, Washington, USA

Pediatr Nephrol 7:163-172, 1993

Renal and biliary abnormalities in a new murine model of autosomal recessive polycystic kidney disease

Abstract

Current models of autosomal recessive polycystic kidney disease (ARPKD) fail to demonstrate biliary abnormalities in association with renal cysts. We therefore studied a new murine model of ARPKD in which dual renal tubular and biliary epithelial abnormalities are present. Affected homozygous animals typically die 1 month postnatally in renal failure with progressively enlarged kidneys. Renal cysts shift in site from inner cortical proximal tubules at birth to collecting tubules 20 days later as determined by segment specific lectin binding. Increased numbers of mitosis were demonstrated in proximal and collecting tubular cysts. In addition, epithelial hyperplasia was demonstrated morphometrically in the intra- and extrahepatic biliary tract of affected animals. The number of intrahepatic biliary epithelial cells was increased by 50% at postnatal day 5 and by 100% at postnatal day25 ($p<0.01$). Despite an increased frequency of "chaotic" portal areas in mice with renal cysts, no intrahepatic cysts or shape abnormalities of the biliary lumen were detected using biliary casts and morphometry. Additionally there was nonobstructive hyperplastic dilatation of the extrahepatic biliary tract which was linked in all animals to the presence of renal cysts. The hyperplastic abnormalities in both renal and biliary epithelium make this new mouse strain a good model for the study of the dual organ cellular pathophysiology of ARPKD.

Introduction

Autosomal recessive polycystic kidney disease (ARPKD) is a congenital disorder generally leading to end stage renal failure within the perinatal period or early childhood. The disease is highly associated with hepatic abnormalities which can lead to cholangitis and portal hypertension (1-5). The expression of disease in both kidney and liver is variable and has led to a number of different disease classifications (6-8). On the basis of extensive family and clinical studies, it now appears that ARPKD is a single autosomal recessive disease complex with a wide clinical spectrum in which renal cystic disease is linked to biliary tract abnormalities and congenital hepatic fibrosis (CHF) (9,10). This disease complex includes the clinical entities previously classified as infantile polycystic disease, juvenile polycystic kidney disease, autosomal recessive polycystic kidney disease, autosomal recessive polycystic liver disease, biliary duct ectasia, Caroli's disease and CHF. In the different clinical entities of this disease complex, liver lesions are variable. They consist of proliferation and dilatation of intrahepatic bile ducts in conjunction with

progressive fibrosis of portal connective tissue (9,11). Epithelial hyperplasia seems to be a hallmark for both hepatic and renal abnormalities (12,13). In both organs hyperplasia may be related to the expression of abnormal developmental programs (11,14,15).

Studies into the mechanisms of the formation and progressive enlargement of renal cysts have focussed largely on animal models (16). The C57BL/6J cpk/cpk (CPK) mouse has attracted considerable interest as a genetic model for ARPKD (12,17,18). However the absence of hepatic lesions in affected animals is an important shortcoming of this model. Hepatic lesions have thus far only been identified in aged CPK animals which are heterozygous for the cystic trait (19,20). In the current study we describe a new mutant strain of Balb/c mice in which ARPKD is expressed in conjunction with abnormalities of the intra- and extrahepatic biliary system.

Materials and methods

Animals

In 1985 polycystic kidney disease was recognized in an inbred strain of otherwise healthy Balb/c mice at the National Institutes of Health. These animals were subsequently maintained as an inbred strain for over 25 generations at the University of Minnesota and expressed renal cystic disease as a stable autosomal recessive trait. In 1990, heterozygous breeding pairs from the Minnesota colony were obtained from Dr R Vernier and an inbred colony was established at the University of Washington. In the current study affected homozygous BPK (Balb/c Polycystic Kidneys) mice were obtained from the controlled breeding of animals known to be heterozygous for the cystic disease. Wild type Balb/c mice (Simonsen Laboratories Inc. Gilroy, Cal.) served as controls. All animals were fed standard mouse chow containing 24% protein.

Light microscopy

For histologic analysis, kidneys were obtained from cystic and control animals at postnatal days 0, 5, 10, 15, 20 and 25 and livers were obtained at postnatal days 5, 15 and 25. No livers were included at day 0 because identification of bile ducts proved to be inaccurate at this age due to the immaturity and small size of the ducts and the relatively heterogenous background caused by extensive hematopoetic activity. Histological analysis of the liver sections was performed blind by an experienced pediatric pathologist (J.R.). Other structures and organs were studied at only one postnatal age. Heart, sternum, spleen, brain, intestine and salivary gland were obtained at day 25, which is the age of terminal disease in BPK mice. Gallbladders, common bile ducts, choledochoduodenal junctions and pancreases were studied at day 10 when the moderately enlarged kidneys do not cause mechanical obstruction of the biliary tract.

The tissue was fixed in 3.5% paraformaldehyde for 30 minutes at 4°C, washed, dehydrated in graded acetone, infiltrated and embedded in Immunobed® plastic embedding medium (Polysciences Inc, Warrington, Pa, USA). Sections of 3 microns were mounted on glass and stained with hematoxylin. Additionally, staining for bilirubin was performed, using Fouchet's reagent, on paraffin embedded liver sections from 10 day old mice.

Immunolocalization

Cyst localization was studied by segment specific lectin binding using Dolichos Biflorus Agglutinin (DBA) as a marker for collecting tubules and Lotus Tetragonolobus Agglutinin (LTA) as a marker for proximal tubules (21,22). Renal cysts from 6 BPK mice per age group on days 0, 5, 10, 15 and 20 postnatally were lectin-profiled at 3 different depths of the tissue following standardized embedding and serial sectioning. Depths were chosen as 10, 30 and 50 % of the thickness of each embedded kidney. In each section all cysts were classified as either DBA positive, LTA positive, or negative for both LTA and DBA. No cysts were positive for both lectins.

Biliary tract epithelium was identified utilizing a rabbit polyclonal antibody against human cytokeratin (Accurate Chemical & Scientific Corp), which has reactivity with developing rodent biliary epithelium as previously demonstrated (23).

The immunostaining procedure used was our previously described postembedding technique specifically developed for immunolocalization of antigens and lectins in plastic sections of developing murine tissue (24,25). Etched and trypsinized Immunobed® sections were incubated overnight at 4°C followed by 30 minutes at room temperature with either biotinylated lectins (6.25µg/ml for LTA and DBA; Sigma) or , after blocking with normal goat serum (1:50 for 30 minutes) with the cytokeratin antibody (1:250). This was followed for lectins by aviden-peroxidase (1:500) for 90 minutes and for antibody staining by sequential incubations in bridging goat anti rabbit IgG (1:40) for 90 minutes and rabbit peroxidase-antiperoxidase complex (1:100) for 45 minutes. Sections were then stained with 0.05% diaminobenzidine, 0.01% hydrogen peroxide and counterstained with hematoxylin.

Mitotic indices

Mitotic figures in both cystic and normal tubular walls were counted in lectin stained kidney sections from 0, 5, 10 and 20 day old BPK and control mice. The tissue was fixed, embedded in plastic and stained with segment specific lectins as described in the section on immunolocalization. We analyzed 6 mice per age group. Data were obtained at 3 different depths at 10, 30, and 50% of the thickness of the tissue and pooled for each kidney. Data are expressed as mitotic indices, being defined as the mean number of mitotic figures per 1000 cells in each group.

Counting of portal structures and bile duct morphometry

Due to the patterned arborization of the biliary tract, it was essential to standardize depth and angle of liver sections for histologic and morphometric analysis. In each case the major left lobe was embedded with the convexity to the surface of the plastic. Sections were cut parallel to the surface of the liver at a depth of 30% of the thickness of the liver. In this manner a standardized population of peripheral and more central bile ducts was consistently obtained. Sections from control and BPK livers were blinded to the observer (J.N.) prior to analysis. Structures were counted in one liver section from each of 5-7 animals from both control and BPK groups at postnatal days 5,15 and 25. In each section all veins, portal areas, bile ducts and biliary epithelial cells were counted. A portal area was identified when a vein was accompanied by at least one bile duct. A biliary epithelial cell was defined as an epithelial cell lining a bile duct. In addition computer assisted morphometric analysis (Sigmascan®) was performed at day 15 and 25 on the same coded sections as used for the counting of structures. Surface area of the section and surface area and circumference of each bile duct lumen were analyzed, as well as a size independent measure of circularity of bile duct profiles. This "shapefactor" was defined as the square root of the surface area divided by the circumference (26).

Electronmicroscopy

For electron microscopy, representative samples of kidney, liver and common bile duct obtained on postnatal days 5, 15 and 25 were fixed in a mixture of 2% paraformaldehyde / 2.5% glutaraldehyde for 2 hours at 4^{o} C and postfixed in 0.5% osmium tetroxide for 1 h at 4^{o} C. The tissue was then dehydraded through a series of graded acetone and infiltrated and embedded in Spurr® (Polysciences). Ultrathin sections were mounted on copper mesh grids and stained with uranyl acetate and lead citrate.

Microfil casts of the biliary tract

Casts of the biliary tract of 3 control and 3 BPK animals of 20 days of age were made using yellow silicone rubber (Microfil®, Canton Bio-Medical Products, Inc. Boulder, Colorado, USA). This age was chosen in order to permit the study of potential liver lesions as late as possible during their development prior to the stage of terminal renal disease. The common bile duct was punctured with a 30 G needle and silicone rubber infused into the biliary tract under a pressure of 30 cm H2O. The duct was ligated after the rubber was visualized in the distal duct radicals using a stereomicroscope. The liver was fixed in graded alcohol, digested in 1% potassium hydroxide, further cleared in a 1:1 mixture of glycerin and 70% ethyl alcohol and examined for cystic dilatations by stereomicroscopy.

Pressure-flow study on choledochoduodenal junctions

In addition to the study of cholestatic features by serum biochemistry, light microscopy and electron microscopy, pressure-flow studies of the choledochoduodenal junction were performed in a limited number of 10-day-old animals. At this age, bile duct cannulation was technically feasable. Unlike older mice, interference of the progressively enlarging kidneys with the bile flow could be excluded at this stage. In 4 control and 4 BPK mice the outflow resistance of the common bile duct was calculated from a pressure-flow study using constant pressure (27). The BPK mice presented with massive dilatation of the extrahepatic biliary tract typically present in all affected animals. The flow of 0.9% saline at a constant pressure of 30cm H2O through a 30 G needle was measured and the resistance calculated and reduced by the baseline resistance of the needle. This baseline resistance was 25 +/- 2 cm H2O/µL/sec and constant in the pressure range from 10 to 50 cm H2O. During these experiments the duodenum was opened in order to permit free outflow. Backflow into the pancreatic duct was never observed.

Blood chemistry

Plasma urea nitrogen values were obtained from 9 control animals and from 11 BPK animals at 3 distinct postnatal developmental stages. Plasma conjugated and unconjugated bilirubin were obtained at day 10 in 6 control and 5 BPK animals. Samples were analyzed by Kodak Ektachem 700 spectrophotometry.

Statistics

Data are expressed as means and standard deviations. Differences between control and BPK animals studied at a single time point were analyzed by a paired two tailed t-test. Strain effects studied at multiple time points were analyzed by ANOVA.. When significant overall strain effects were present the Bonferroni method of adjustment for multiple comparisons was used to determine the specific ages at which this effect was significant. An effect was considered to be significant when $p<0.05$.

Results

Clinical

In a series of 219 mice born from proven breeding pairs, 21% were found to be affected by the cystic trait. This percentage was relatively stable (20%-25%) over a 5–year period of inbreeding at the University of Minnesota and the University of Washington. Males and females were equally affected. At birth affected BPK mice were clinically normal and subsequently developed progressive renal enlargement with massive abdominal distension, which was detectable in all affected mice by physical examination at the age of 2 weeks. Renal failure and death followed at the age of 4 weeks. At the ages of 2 and 4 weeks

plasma urea values of affected animals deviated significantly and progressively from control values (At day 15: 31 +/- 9 VS. 18 +/- 1 mg/dL, $p<0.01$ and at day 25: 88 +/- 14 vs. 24 +/- 4 mg/dL, $p<0.01$). Plasma bilirubin was not different in the 10 day BPK and control animals (0.08+/-0.11 vs 0.18+/-0.13 mg/dL for conjugated bilirubin and 0.02+/-0.02 vs 0.03+/-0.05 mg/dL for unconjugated bilirubin).

Kidneys

Morphologic alterations in BPK kidneys were present at all postnatal stages (fig1). At birth they consisted of tubular dilatations and early cyst formation in the inner cortical zone. During successive postnatal stages, tubules underwent progressive cystic enlargement, with consequent nephron destruction. During postnatal life there was a gradual shift in site of cystic nephron involvement from proximal tubules to collecting tubules as defined by lectin binding (table 1). A small proportion of the cysts did not bind LTA or DBA.. Binding of both LTA and DBA never occurred in the same cysts.

Table 1. Localization of renal tubular cysts during cystogenesis as a percentage of total cysts [a,b]

Age (days)	Proximal	Collecting	Unclassified
0	97 (3)	0	3 (3)
5	73 (5)	13 (3)	14 (5)
10	58 (6)	23 (5)	19 (6)
15	38 (5)	46 (4)	16 (4)
20	11 (4)	78 (7)	11 (3)

[a] According to segment-specific lectin binding
[b] Expressed as mean with SD in parenthesis

Ultrastructurally, cyst lining epithelial cells from 5 day old affected animals were unremarkable and normally differentiated as compared to age-matched controls (fig 2A). Most cysts were lined by cells with the morphology of normal proximal tubules. Microvilli were well formed and cell junctions, basal lamina and the distribution of cell organelles were normal. In 15 day old animals the cyst lining cells were slightly flattened and had fewer and shorter microvilli in cysts of both proximal and collecting tubules. The apical cell junctions and the interdigitating pattern of cell-cell contacts were normal. Compared with controls, the mitochondria were decreased in number and slightly swollen as were the apical microvesicles. By day 25, cyst lining cells were even more flattened and microvesicles rare (fig 2B,C). Proximal tubular characteristics were rarely seen in direct correlation with the lectin profiling studies. Most cyst-lining epithelium had collecting

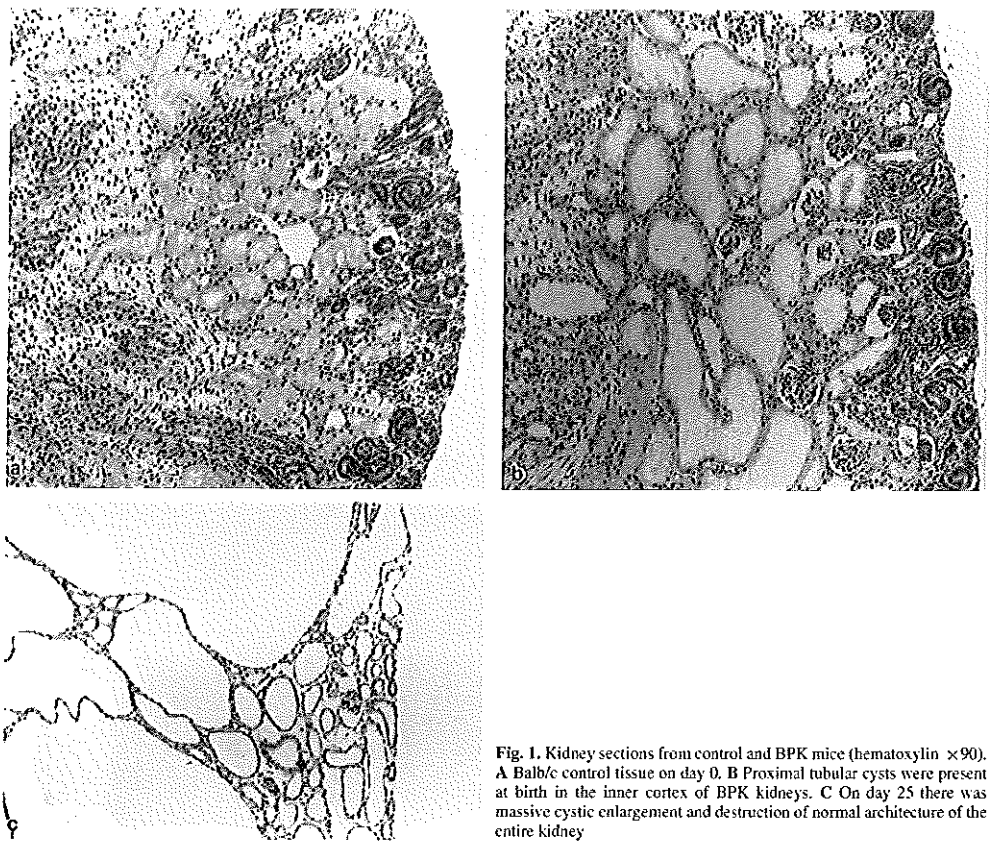


Fig. 1. Kidney sections from control and BPK mice (hematoxylin ×90). **A** Balb/c control tissue on day 0. **B** Proximal tubular cysts were present at birth in the inner cortex of BPK kidneys. **C** On day 25 there was massive cystic enlargement and destruction of normal architecture of the entire kidney

tubular features. The opposing lateral surfaces of cyst-lining cells were decreased and the interdigitation simplified both laterally and basally with replacement by small membrane bound spaces at the basal and basolateral surfaces. Intratubular polyps, piling of cells, wall thickening, intercellular space expansion or luminal projections were not observed. Despite gross disruption of overall renal architecture in the latter stages of disease progression, the basal laminae were intact, unsplit and of normal thickness.

There was a significant increased number of mitosis in cyst lining epithelial cells from both early proximal cysts at day 0 and 5 and early collecting tubular cysts at day 5 and 10 (table2). Undilated tubules in cystic kidneys demonstrated mitotic indices similar to age and segment matched control tubules. The numbers of cystic collecting tubules at day 0 and of undilated collecting tubules in cystic kidneys at day 20 were insufficient to allow appropriate statistical analysis.

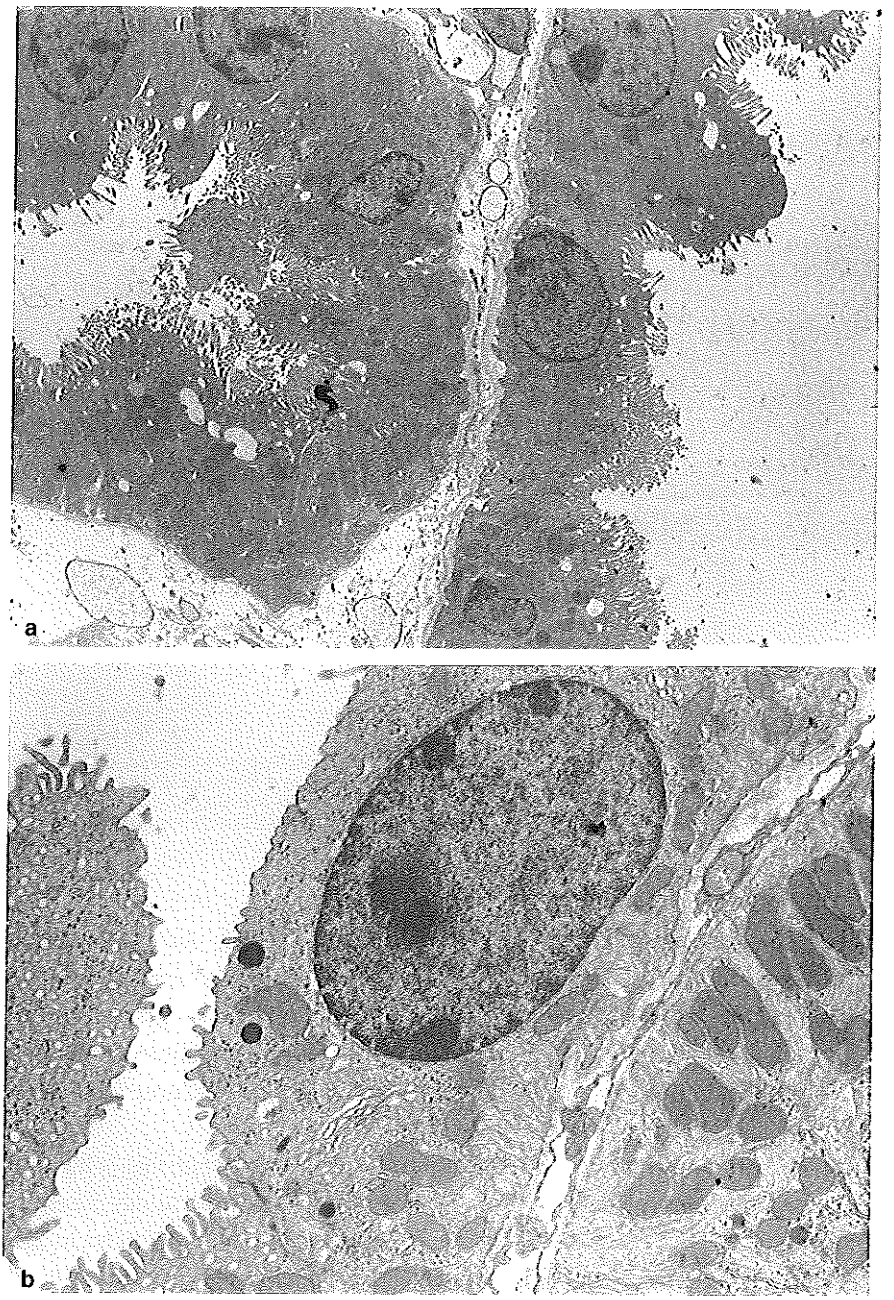
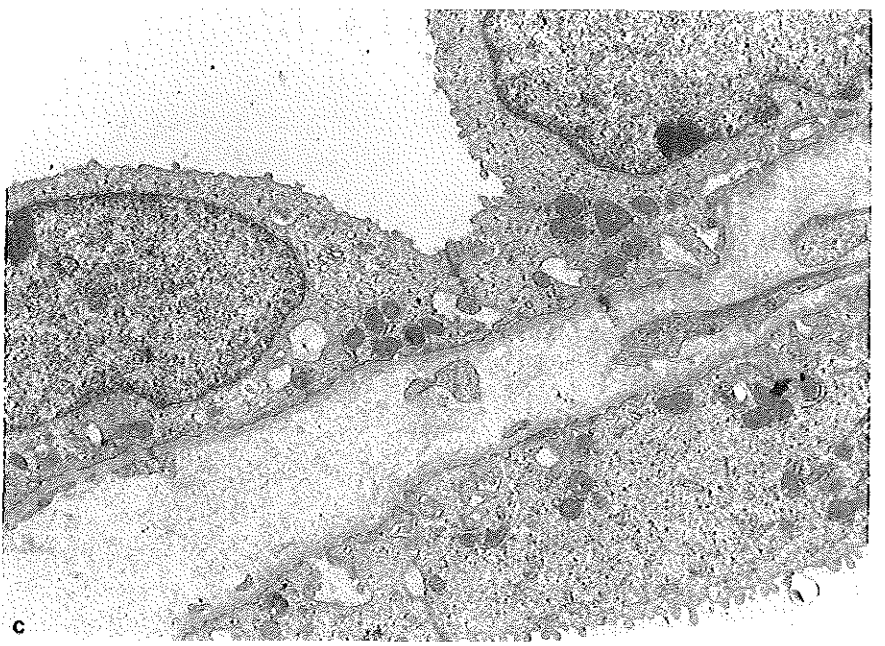


Fig. 2A, B

Fig. 2. Transmission electron microscopy of renal lesions on day 5 and 25. **A** Proximal tubules of a 5-day-old BPK mouse. One appears normal and the other is dilated forming a cyst. The lining cells of the dilated tubule maintain proximal tubule characteristics similar to those of the normal tubule which include microvilli, an apical-vacuolar network, normal intracellular organelles, basal nuclei and a regular basal lamina (×5,300). **B** Collecting tubule of Balb/c control on day 25 demonstrates a principal cell (*center*), including a fairly smooth apical membrane, extensive infoldings of the basal region, a lack of lateral cell processes and interdigitations, scarce mitochondria which are scattered randomly in the cytoplasm and, in contrast to the neighboring intercalated cell (*right*), a lack of apical tubulovesicular membrane structures (×13,300). **C** Collecting tubular cyst of BPK on day 25. Cyst-lining cells with morphological characteristics of principal cells. Organelle numbers and basal organization are decreased compared with controls (×5,300)



c

Fig. 2 C

Table 2. Mitotic indices in renal tubules and cysts [a,b]

	Proximal Tubules			Collecting Tubules		
Age (days)	Control tubules	BPK noncystic tubules	BPK cystic tubules	Control tubules	BPK noncystic tubules	BPK cystic tubules
day 0	3.9 (0.6)	3.7 (0.5)	9.3 (1.3)*	4.1 (0.5)	3.6 (0.8)	
day 5	4.0 (1.1)	5.5 (2.7)	7.5 (1.5)*	2.5 (1.0)	4.0 (1.4)	7.2 (1.6)*
day 10	4.8 (1.2)	5.3 (1.6)	5.3 (1.9)	2.5 (1.2)	3.2 (1.3)	7.3 (1.7)*
day 20	3.2 (1.2)	3.7 (1.3)	4.3 (1.3)	2.2 (1.5)		3.8 (1.1)

* $p < 0.05$ compared with control tubules

[a] Mitotic indices = mitotic figures per 1.000 cells

[b] Mean with SD in parenthesis of mitotic indices in lectin-defined proximal and collecting tubular segments of control and homozygous BPK mice

Liver

The liver size of 10 day old BPK mice was not different from controls as determined by the ratio of wet liver weight to body weight (BPK:2.9 +/- 0.12%,Controls: 2.8 +/- 0.04%). Liver weight was not affected by the animal's sex at this age.

Light microscopy revealed an increased frequency of "chaotic" portal areas in BPK mice. These consisted of portal areas where the portal vein was surrounded by multiple, irregularly shaped bile ducts and epithelial cell proliferation (fig3). In a blinded study on 1

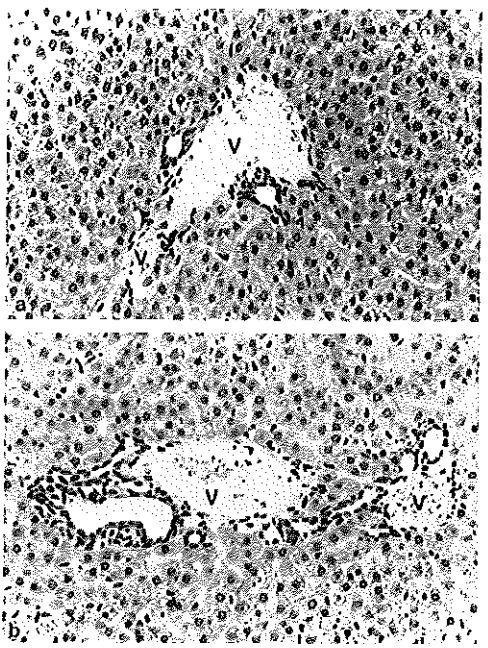


Fig. 3. Liver sections of 15-day-old mice (hematoxylin ×170). A A representative portal area in Balb/c control tissue with erythrocytes in the portal vein (v), accompanied by two bile ducts. B A representative "chaotic" portal area in BPK tissue. Two portal veins (v) are accompanied by multiple, irregularly shaped bile ducts

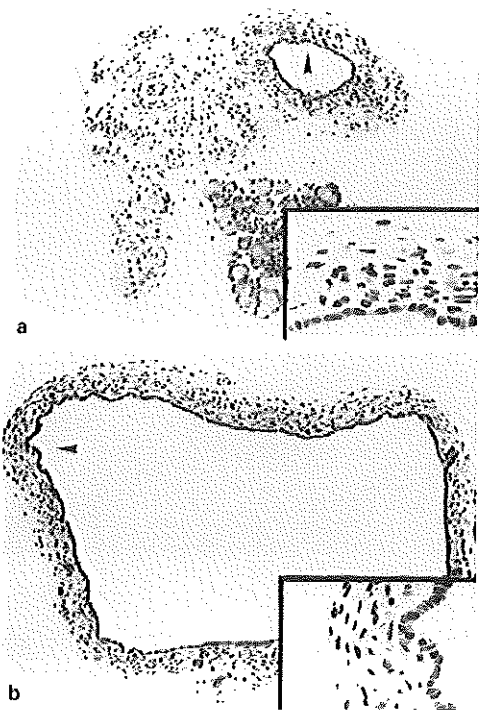


Fig. 4. Common bile ducts of 15-day-old mice (hematoxylin ×120). The area indicated by an *arrowhead* is shown at higher power (×250) in the insert. A Tissue from a control Balb/c mouse with some attached connective and pancreatic tissue. B The common bile duct of an affected BPK mouse. Note the normal cellular composition of the duct wall compared with control tissue (*insert*)

section of each of 6 BPK and 6 control animals of 15 days old, a trained pediatric pathologist (J.R.) identified 8.3 +/- 2.6 such portal areas in BPK and 2.3 +/-1.2 in control mice ($p<0.01$). No other abnormalities were seen. There were no cystic abnormalities or signs indicative of portal or periportal fibrosis. Staining for bile was negative in all sections.

Ultrastructurally, the intrahepatic ducts in the BPK and age matched control mice were lined by similar, morphologically normal epithelium. Cell organelles, junctions, appositions and basement membranes were unremarkable. The hepatocytes exhibited no evidence of bile inspissation or blunting of canalicular microvilli and had a normal distribution of hepatocellular organelles.

Counting of portal structures

Standardized liver sections from age-matched control and BPK animals obtained for the counting of portal structures, were comparable with regard to section size and number of vein profiles (table 3). During postnatal development considerable liver growth was accompanied in both strains by an increase in recognized portal areas (veins accompanied by bile ducts), a decrease of bile ducts per portal area and a decrease of epithelial cells per bile duct ($p<0.05$ for all three age effects).

In BPK tissue there was marked epithelial hyperplasia. Biliary epithelial cells were increased by 50% at day 5, by 60% at day 15 and by 100% at day 25 when expressed per square mm of the section and compared with control values. This was associated with an increased number of bile ducts per square mm. Proliferation of bile ducts in BPK animals occurred in different patterns in the different postnatal age groups. In 5 day old mice it was associated with an increase of bile ducts per portal area, while the number of portal areas was normal. In older animals bile duct proliferation was associated with an increased number of recognized portal areas.

Quantitation of bile duct shapes

Despite the earlier noted increase in "chaotic" portal areas in BPK mice, computerized morphometric analysis did not reveal any consistent abnormalities in bile duct surface area, circumference or "shape factor". Subgroups of bile duct profiles categorized according to their shape factor were represented equally in control and BPK livers.

Casts of the intrahepatic biliary tree of 20-day-old BPK mice were not different from controls. No cysts, dilatations or von Meyenburg complexes were identified.

Extrahepatic biliary tract

Dilatation of the cystic, the hepatic and the common bile ducts was clearly visible after laparotomy in all postnatal homozygous BPK animals. This finding was linked to the presence of renal cysts in all instances. The outer diameter of the common bile duct was <0.25 mm in all control mice and between 0.4 and 1.5 mm in BPK mice ($p<0.01$). No gender difference was noted. The duct walls of dilated bile ducts demonstrated cellular profiles which were not different morphologically from normal bile ducts other than an increased number of epithelial cells (Fig.4). Ultrastructurally the dilated ducts were lined by intact cuboidal epithelium with an intact microvillar surface. Apical tight junctions gave way to complex interdigitations of the lateral cell membranes. Cytoplasmic organelles were dispersed throughout the cell. The underlying basement membrane was unfrayed and of normal caliber and underlaid by collagen and fibroblasts with an active endoplasmic reticulum. The ultrastructural morphology did not differ from controls (fig 5).

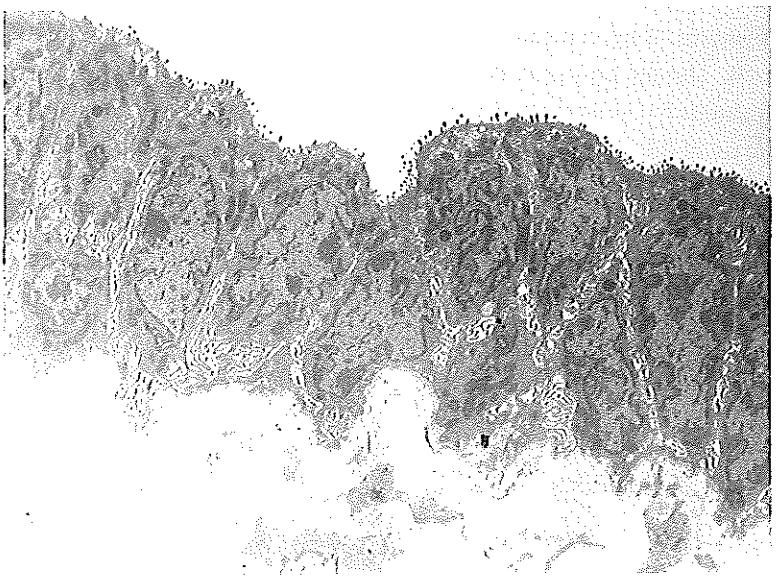


Fig. 5. Epithelial lining of a dilated common bile duct from a 30-day-old BPK mouse. The cell features are not distinguished from control ductal epithelium including superficial microvilli, apical organelles and extensive basolateral infoldings (×5,300)

The gallbladder was not dilated and had a normal histology. The weights of the sutured undrained gallbladder in 10 day old BPK and control mice were 0.9 +/- 0.2 mg and 1.2 +/- 0.6 mg respectively.

Choledocho-duodenal junction

Light microscopy of the choledochoduodenal junction did not reveal any abnormalities. The intraduodenal segment of the common bile duct was serially sectioned and found to be open and free of stenosis, fibrosis and inflammation.There was no restriction of the bile flow into the duodenum as measured by pressure flow studies. The calculated resistances at 10 days of age were 4, 4, 6 and 8 cm/μL/sec in BPK mice and 6, 7, 9 and 14 cm/μL/sec in control mice.

Light microscopy of organs other than kidney or liver was unremarkable. No cystic or other abnormalities were seen and there were no signs of inflammation or fibrosis.

Discussion

The BPK mouse demonstrates autosomal recessive progressive tubular cyst formation associated with biliary abnormalities. This association is a well recognized feature of the human ARPKD /CHF disease complex (9). At one end of the clinical spectrum are patients

Table 3. Portal structures in standardized liver sections at different postnatal developmental stages [a]

		Day 5	Day 15	Day 25
Surface area (mm^2)	Contr	19 (6)	39 (2)	55 (9)
	BPK	28 (3)	40 (8)	51 (12)
Veins/mm^2	Contr	8.7 (1.8)	7.7 (1.2)	6.4 (0.9)
	BPK	6.8 (1.2)	8.0 (0.7)	7.0 (1.1)
Cells/mm^2	Contr	27 (8)	29 (8)	21 (5)
	BPK	40 (19)	45 (6) *	43 (10) **
Cells/Bileduct	Contr	16 (4)	14 (1)	11 (2)
	BPK	15 (5)	12 (1)	11 (2)
Bile ducts/mm^2	Contr	1.7 (0.3)	2.1 (0.5)	2.0 (0.3)
	BPK	2.6 (0.7)	3.7 (0.5) **	3.8 (1.0) **
Bile ducts/Portal area	Contr	1.5 (0.2)	1.4 (0.1)	1.3 (0.1)
	BPK	2.5 (0.5) **	1.6 (0.2)	1.7 (0.3)
Portal area/mm^2	Contr	1.1 (0.2)	1.4 (0.4)	1.5 (0.2)
	BPK	1.0. (0.1)	2.3 (0.2)**	2.3 (0.6) **
Portal area/100 Veins	Contr	13 (2)	18 (3)	23 (3)
	BPK	15 (2)	28 (2) **	32 (6) **

* $p<0.05$ vs. controls
** $p<0.01$ vs. controls
[a] mean with SD in parentheses

with "classic" ARPKD who present neonatally with renal failure, only microscopic hepatic abnormalities and no clinical symptoms of liver disease. At the other end, are patients with CHF, presenting in early childhood with symptoms of portal hypertension and cholangitis but with normal renal function and only microscopic renal lesions. The clinico-pathological classification of ARPKD introduced by Blyth and Ockenden was based on the variable degree of renal and hepatic involvement as well as the age at presentation (6). It is now accepted that the different clinical entities introduced by Blyth and Ockenden are different manifestations of the same genetic defect (5,9). The increased survival of even very young patients undergoing treatment for end-stage renal disease, in concert with the progressive nature of the hepatic abnormalities in ARPKD, leads to the expectation that many of these patients may eventually develop portal hypertension and progressive liver fibrosis. Therefore hepatic lesions are not only inherent to human ARPKD but also of increasing clinical importance.

Studies of the pathophysiology of human ARPKD are hampered by its low incidence, variable clinical expression and advanced pathology at presentation. As a consequence, research has largely focused on animal models. The animal model studied most

extensively to date is the C57BL/6J cpk/cpk mouse strain (CPK) which expresses a lethal ARPKD much like the human disease (12,17,18,28). However, the validity of this model has been questioned since unlike its human counterpart, biliary abnormalities could not be demonstrated in affected animals. Extensive studies identified hepatic cysts only in aged heterozygous breeders of the CPK strain but not in homozygous animals with renal cysts (19,20). The invariably present biliary abnormalities demonstrated in cystic BPK mice not only emphasize the value of this strain as a new model of dual organ epithelial pathophysiology in ARPKD but also suggests it may have value as a model for the study of biliary dysgenesis.

The pattern of renal cyst formation and the progression of renal failure in the BPK strain are similar to those described for the CPK strain and, in latter stages, to those of human ARPKD. The two murine models demonstrate similar ultrastructural features of cyst-lining epithelium, although dilatation of intercellular spaces, previously noted in proximal tubular lesions of CPK animals (12), was not a feature of BPK mice. The localization of cysts to lectin-defined segments has yielded a number of interesting observations. The lesions in early stages of disease in BPK mice consist of dilatations exclusively localized to proximal tubules. These proximal tubular cysts most likely develop in functioning, non-obstructed nephrons as indicated by the normal cellular ultrastructure. This is consistent with microdissection data from the CPK model (12). The contribution of proximal tubular cysts to the total population of cysts decreases during disease progression. This is most likely due to restriction of proximal tubular cystogenesis to a specific developmental stage in the context of ongoing nephrogenesis and progressive formation of collecting tubular cysts (12). Regression of proximal tubular cysts has never directly been demonstrated in vivo but has been demonstrated in vitro (29,30). Although proximal tubular cyst formation has not yet been demonstrated as an early feature of human ARPKD it should be emphasized that little tissue from early disease stages has been available for detailed microdissection or lectin profiling analysis. Of particular interest in this regard is a recent histopathological study of ARPKD kidneys in which two of five specimens demonstrated slight positive staining for lectin markers specific for proximal tubules (31).

The histological picture of latter BPK disease stages is dominated by collecting tubular cysts. This is similar to data from patients with end stage ARPKD in which systematic immunohistologic and microdissection studies have localized most cysts to collecting tubular segments (12, 32). In addition to proximal tubular cysts and collecting tubular cysts, there was a small population of BPK cysts which could not be localized by lectin-profiling to either one of these segments (Table 1). Whether these cysts originate from a different nephron segment or represent phenotypic changes of preexisting proximal or collecting tubular cysts can only be determined by further studies. Involvement of nephron segments other than the proximal tubule or the collecting duct has not been suggested by

ultrastructural analysis in the BPK strain and does not play a role in the cystogenesis of the CPK strain or ARPKD.

The intrahepatic abnormalities in standardized liver sections of BPK mice consist of hyperplasia of the biliary tract and an increased number of "chaotic" portal areas. In human CHF and related liver disorders, similar lesions have been described and attributed to a defective remodelling of the ductal plate (11). According to current views this plate is formed during normal liver development by transformation of hepatocytes into a double epithelial sheet surrounding the intrahepatic portal veins. During further development, this plate is then remodelled into multiple and later single bile ducts. The increased number of bile ducts and the abnormal shapes of bile duct profiles in liver sections of patients with ARPKD or CHF have been attributed to insufficient breakdown of the ductal plate and an arrest of the normal remodelling process (11). The demonstrated increased number of bile ducts per portal area in early BPK disease stages is consistent with a remodelling defect, such that remodelling of the ductal plate has started but not been completed to the stage of single bile ducts. Further, the accelerated postnatal development of the biliary tract along the portal venous tract in BPK mice, indicated in our data by the increasing numbers of portal areas, relative to controls, suggests additional abnormalities of the ductal plate remodeling process. Whether the multiple, irregular shaped bile ducts observed in a limited number of portal areas from affected mice represents the curving biliary plates described for the 'ductal plate malformation' in human disease by Jorgensen (13) cannot be concluded from this study. A separate study utilizing stereological reconstruction from serial sections is required to resolve this issue.

The observation in this study of an increased number of "chaotic" portal areas consisting of abnormal numbers and shapes of bile duct profiles was made by an experienced observer in a blinded study. It was therefore surprising and in contrast to data from human studies that no consistent abnormalities in bile duct shape or size could be demonstrated by quantitative morphometry (33). It appears that the number of "chaotic" portal areas relative to the number of normal portal areas was insufficient to affect the quantitative morphometric analysis. The absence of intrahepatic fibrosis and cystic biliary dilatations in BPK mice contrasts with data from most patients with advanced CHF and related disorders (3,9,34). It should be emphasized however that the early lethality of the renal disorder in the murine model necessarily restricted this study to early stages of liver disease.

Dilatations of the entire extrahepatic biliary tract with the exception of the gallbladder were strikingly present in all animals with cystic kidneys at all postnatal ages and never seen in unaffected animals. Biliary outflow restriction as the primary cause of this dilatation and of the intrahepatic biliary hyperplasia was excluded by the absence of gallbladder dilatation, normal serum bilirubin, the normal ultrastructure of the liver and common bile duct, and the normal resistance of the choledochoduodenal junction.

Moreover the massive lumenal dilatation in conjunction with the apparently normally structured wall, in which increased numbers of biliary cells were paralleled by hyperplasia of the surrounding stroma, suggests a primary hyperplastic abnormality. Although the common bile duct dilatation invariably present in affected BPK animals has not been specifically associated with human ARPKD, it is a well recognized finding in patients with Caroli's disease (35). It thus appears that extrahepatic biliary dilatation is part of the variable hepatobiliary phenotypic expression of the ARPKD/CHF disease complex.

The renal and hepatic abnormalities demonstrated in this model suggest a common pathogenetic pathway. In both organs, epithelial hyperplasia is an early feature of the lesions although the specific patterns of renal and biliary hyperplasia are somewhat different. The hyperplasia of tubular cystogenesis and the hyperplasia of the extrahepatic bile duct both are associated with luminal dilatation. In contrast intrahepatic biliary epithelial hyperplasia in affected BPK mice is expressed as an increased number of bile ducts and thus elongation, rather than dilatation, of the biliary tract.

Differences in the three-dimensional structural consequences of hyperplasia in kidney and liver may reflect the fact that these organs are in different developmental stages at the time that lesions develop. Since the renal cystic lesions develop in a segment specific pattern in preexisting, structurally normal nephrons (12), elongation of hyperplastic segments is anatomically restricted by the attachment at both ends to fixed normal tubular segments. Uncontrolled growth of cells in such spacially restricted tubular segments would then lead to expansion perpendicular to the long axis of the tubules, i.e. dilatation. Similarly, elongation of the extrahepatic bile duct is restricted by the attachment to liver and duodenum, and thus localized cellular proliferation would lead to dilatation. In contrast the proliferative elongation of the intrahepatic biliary tract is part of the pattern of normal murine postnatal development, as illustrated by our data from control mice (table 3). The intrahepatic biliary tract is not physically restricted by attachment to other organ structures until the normal developmental process is complete. It would be of interest to know whether biliary hyperplasia continuing beyond the end point of biliary tract development would lead to increased numbers of bile ducts per portal area and luminal dilatations, and whether such abnormalities would be accompanied by fibrotic changes in the portal areas as seen in human disease. At present, such a course of events can only be speculated since affected BPK animals die of renal failure prior to this developmental stage.

We conclude that the presence of intra and extrahepatic biliary hyperplasia in conjunction with progressive, hyperplastic renal tubular cyst formation make the BPK mouse strain an excellent model for the study of the ARPKD/CHF disease complex.

Acknowledgments

A preliminary report of these findings was presented at the 1991 Annual Meeting of the American Society of Nephrology, and has appeared in abstract form (36). These studies were supported by the Sophia Foundation for Medical Research in Rotterdam, the Dutch Kidney Foundation and Grants no. DK34891 and DK44875 from the National Institutes of Health. We gratefully acknowledge Dr. R. Vernier who initially provided us with breeder stock of the BPK mouse strain.

References

1. Lieberman E (1971) Infantile polycystic disease of the kidneys and liver: clinical, pathological and radiological correlations and comparison with congenital hepatic fibrosis. Medicine 50:277-318
2. Murray-Lyon IM, Shilkin KB, Laws JW, Illing RC, Williams R (1972) Non-obstructive dilatation of the intrahepatic biliary tract with cholangitis. Quart J Med 164: 477-489
3. Alvarez F, Bernard O, Brunelle F, Hadchouel M, Leblanc A, Odièvre M, Alagille D (1981) Congenital hepatic fibrosis in children. J Pediatr 99:370-375
4. Kaplan BS, Fay J, Shah V, Dillon MJ, Barratt TM (1989) Autosomal recessive polycystic kidney disease. Pediatr Nephrol 3:43-49
5. Mc Donald R, Avner ED (1991) Cystic Disease in Children. Seminars in Nephrology 11 :632-42
6. Blyth H, Ockenden BG (1971) Polycystic disease of kidneys and liver presenting in childhood. J Med Gen 8:257-284
7. Resnick J, Vernier RL (1987) Renal cystic disease and renal dysplasia. Chapt 24 in Holliday MA, Barratt TM, Vernier RL (eds) Pediatric Nephrology, 2nd edn. Williams and Wilkins, London, p 371-383
8. Summerfield JA, Nagafuchi Y, Sherlock S, Cadafalch J, Scheuer PJ (1986) Hepatobiliary fibropolycystic diseases, a clinical and histological review of 51 patients. J Hepatology 2:141-156
9. Picolli DA, Witzleben CL (1991) Disorders of intrahepatic bile ducts. Chapt 28, part 26 in Walker WA, Durie PR, Hamilton JR, Walker-Smith, Watkins JB (eds) Pediatric Gastrointestinal Disease, Decker Inc., Philadelphia, Vol 2, p 1124-1140
10. Kaplan BS, Kaplan P (1990) Autosomal recessive polycystic kidney disease. Chapt 13 in Spitzer A and Avner ED (eds) Inheritance of Kidney and Urinary Tract Diseases, Kluwer, The Netherlands, p 265-276
11. Desmet VJ (1985) Intrahepatic bileducts under the lens. J Hepatology 1:545-549
12. Avner ED, Studnicki FE, Young MC, Sweeney Jr WE, Piesco NP, Ellis D, Fetterman GH (1987) Congenital murine polycystic disease. I. The ontogeny of tubular cyst formation. Pediatr Nephrol 1: 587-596
13. Jørgensen MJ (1977) The ductal plate malformation. Acta Pathol Microbiol Scand A Suppl 257: 1-88
14. Avner ED, Sweeney WE, Nelson WJ (1992) Abnormal sodium pump distribution during renal tubulogenesis in congenital murine polycystic kidney disease. Proc Nat Acad Sci 89:7447-7451
15. Harding MA, Chadwick LJ, Gattone VH, Calvet JP (1991) The SGP-2 gene is developmentally regulated in the mouse kidney and abnormally expressed in collecting duct cysts in polycystic kidney disease. Dev Biol 146: 483-490
16. Avner ED, McAteer JA, Evan AP (1990) Models of cysts and cystic kidneys. Chapt 4 in Gardner KD, Bernstein J (eds) The cystic kidney, The Netherlands, p 55-98

17. Avner ED, Sweeney Jr WE, Young MC, Ellis D (1988) Congenital murine polycystic kidney disease. Pediatr Nephrol 2:210-218
18. Fry JL, Koch WE, Jennette JC, Mc Farland MA, Mandel J (1985) A genetically determined murine model of infantile polycystic kidney disease. J Urol 134: 828-833
19. Crocker JFS, Blecher SR, Givner ML, McCarthy SC (1987) Polycystic kidney and liver disease and corticosterone changes in the cpk mouse. Kidney Int 31: 1088-1091
20. Grimm PC, Crocker JFS, Malatjalian DA,Ogborn MR (1990) The microanatomy of the intrahepatic bile duct in polycystic disease: comparison of the cpk mouse and human. J Exp Path 71: 119-120
21. Laitinen L,Virtanen I, Saxén L (1987) Changes in the glycosylation pattern during embryonic development of mouse kidney as revealed with lectin conjugates. J Histochem Cytochem 35: 55-65
22. Avner ED, Sweeney Jr WE (1990) Polypeptide growth factors in metanephric growth and segmental nephron differentiation. Pediatr Nephrol 4: 372-377
23. Van Eyken P, Sciot R,Desmet VJ (1988) Intrahepatic bile duct development in the rat: A cytokeratin-immunohistochemical study. Lab Invest 59: 52-59
24. Sweeney Jr WE, Avner ED (1991) Intact organ culture of murine metanephros. J Tissue Cult Meth: 13: 163-168
25. Morris SM, Sweeney WE, Kepka DM, O'Brien WE, Avner ED (1991) Localization of arginine biosynthetic enzymes in renal proximal Sausolito, California. Jandel Scientific 1987, pp 3.28-3.34
26. Acker CJ, Mitchel D (1987) Sigmascan manual. Jandel Scientific, Sausolito, California, pp 3.28-3.34
27. Woodbury PW, Mitchell ME, Scheidler DM, Adams MC, Rink RC, McNulty A (1989) Constant pressure perfusion: a method to determine obstruction in the upper urinary tract. J Urol 142: 632-635
28. Preminger GM, Koch WE, Fried FA, Mc Farland E, Murphy ED, Mandell J 1982) Murine congenital polycystic kidney disease: a model for studying development of cystic disease. J Urol 127: 556-560
29. Avner ED, Sweeney Jr WE, Piesco NP, Ellis D (1986) Regression of genetically determined polycystic kidney disease in murine organ culture. Experiencia 42: 77-80
30. Avner ED, Sweeney Jr WE, Ellis D (1989) In vitro modulation of tubular cyst regression in murine polycystic kidney disease. Kidney Int 36: 960-968
31. Verani R, Walker P, Silva FG (1989) Renal cystic disease of infancy: results of histochemical studies. Pediatr Nephrol 3: 37-42
32. Holthofer H, Kumpulainen T, Rapola J (1990) Polycystic disease of the kidney. Evaluation and classification based on nephron segment and cell-type specific markers. Lab Invest 62:363-369
33. Jørgensen MJ (1973) A stereological study of intrahepatic bile ducts. Acta Pathol Microbiol Scand A 81: 670-675
34. Kerr DNS, Harrison CV, Sherlock S, Milnes Walker R (1961) Congenital hepatic fibrosis. Quart J Med 30:91-117
35. Caroli J, Corcos V (1964) Maladies des voies biliaires intrahépatiques segmentaires. Masjon and Cie, Paris
36. Nauta J, Sweeney WE, Avner ED (1991) Epithelial hyperplasia and cell polarity abnormalities in murine autosomal recessive polycystic kidney disease. (abstract) JASN 2: 257

2.2

Biliary epithelial cells from mice with congenital polycystic kidney disease are hyperresponsive to epidermal growth factor.

J.Nauta, W.E. Sweeney Jr., J.C. Rutledge, E.D. Avner.

Department of Pediatrics, Sophia Children's Hospital, Rotterdam, The Netherlands.
Departments of Pediatrics and Laboratory Medicine, Children's Hospital Medical Center and the University of Washington School of Medicine, Seattle, Washington U.S.A.

Pediatric Research 37:755-763, 1995

Biliairy epithelial cells from mice with congenital polycystic kidney disease are hyperresponsive to epidermal growth factor

Abstract

Epithelial hyperplasia is an early feature of the renal and biliary lesions in autosomal recessive polycystic kidney disease (ARPKD). To explore the cellular basis of this hyperplasia we isolated, cultured and characterized biliary tract epithelium from common bile duct explants of mice with ARPKD (the BPK mouse) and controls. Primary cultures resulted in dense colonies of contact inhibited epithelial cells with a homogenous growth pattern. Colony growth in serum free basal medium (BM) of BPK derived cells was not different from controls. Supplementation of BM with epidermal growth factor (EGF) induced a proliferative response in BPK derived cells that was significantly increased over controls as assessed by ^{3}H-Thymidine uptake and expressed as percent change over growth in BM (BPK 239% and controls 131% of BM growth). In contrast no differences between BPK and Control derived cells were found with regard to the effects of BM supplementation with IGF I, IGF II, aFGF, KGF, HGF or TGFß.
Primary culture of biliary epithelium may provide a useful in vitro model for the study of the cellular pathophysiology of ARPKD. Our data demonstrate that increased epithelial sensitivity to EGF-like proteins may play a role in biliary epithelial proliferative changes which parallel renal tubular epithelial proliferation in ARPKD.

Introduction

Genetically determined polycystic kidney disease (PKD) affects about one person in thousand and is responsible for significant morbidity and mortality (1). Usually it is the hallmark of a unique autosomal dominant (ADPKD) or autosomal recessive (ARPKD) disorder. The cystic renal tissue is characterized by fluid accumulation in progressively enlarging dilatations of preexisting renal tubules which lead to distortion of renal architecture and ultimately to renal failure. Renal failure develops in about half of patients with ADPKD and in virtually all those patients with ARPDK that survive the perinatal period (2). ARPKD typically presents perinatally or during the first years of life. Many patients die at birth from pulmonary hypoplasia which is thought to result from oligohydramnis and inadequate prenatal renal function. In contrast ADPKD typically is a disease of adults with disease manifestations generally developing during the second half of life. However a small proportion of patients may present during childhood. PKD is

highly associated with liver disease. Hepatic lesions develop in 50-90% of patients with ADPKD and in all patients with ARPKD (1). In ADPKD proliferative intrahepatic biliary lesions resulting in discrete cyst formation are characteristic. In ARPKD the lesions are variable and consist of proliferation and dilatation of intra- and extrahepatic bile ducts ("ductal ectasia"), and periportal fibrosis as well. The clinical course of the associated liver disease, is generally mild in ADPKD and much more severe in patients with ARPKD and may include cholangitis, portal hypertension and ultimately liver failure (3-5). Genes responsible for human ARPKD and ADPKD have been localized (6,7). In addition the ADPKD gene has been identified in part (7).

Studies into the pathophysiology of PKD have largely focused on the renal lesions using a variety of in vivo and in vitro experimental models (8). Such studies demonstrate that two basic criteria are necessary for renal tubular cyst formation: 1) increased tubular epithelial cell proliferation and 2) altered transtubular fluid transport (9-11). The mechanisms responsible for aberrant renal tubular epithelial cell growth are not known. Recent reports suggest a role for abnormal regulation and/or activation of the TGF-α/EGF/EGFR axis (8, 12-17). It has been theorized that the presence of mitogenic quantities of TGFα and/or EGF-like peptides in cyst fluid coupled with aberrant apical tubular epithelial expression of EGF-R may drive an autocrine/paracrine cycle of cellular proliferation.

In contrast to the focus on renal pathophysiology in PKD, little is known about the pathophysiology of the hepatic lesions. Studies at the cellular level have been hampered by the low availability of human hepatic tissue and the lack of an animal model. Recently we reported a new spontaneous mutation in Balb/c mice which is a new model of human ARPKD, the BPK strain (18). In BPK mice renal cystic lesions are associated with intra- and extrahepatic hyperplastic lesions of the biliary tract. The disease is transmitted in an autosomal recessive pattern and leads to terminal renal failure and death in affected animals at the age of 1 month. The demonstrated hyperplastic abnormalities in both renal and biliary epithelium makes this new strain a good model to study the dual organ cellular pathophysiology of human ARPKD. Studies were therefore undertaken in order to establish a cellular model of the abnormal growth regulation of biliary epithelia in murine ARPKD.

Methods

Animals

All experiments were approved by the Institution Animal Use and Care Committee. Throughout the experiments we have maintained stable colonies of wild type Balb/c control mice (Jackson Laboratories) and Balb/c BPK mice. The BPK strain expresses PKD

in an autosomal recessive pattern and arose from a spontaneous mutation in 1985 in an otherwise healthy Balb/c colony at the National Institutes of Health. The BPK strain has been maintained as an inbred strain at the University of Washington since 1990. Recently we reported the presence of hepatic as well as renal lesions in these mice (18). In the current experiments 15-25 day old offsprings from wild-type matings served as controls, while affected BPK animals of the same age were obtained from matings of known heterozygotes for the BPK trait. The age group was chosen such that affected mice can be readily identified by physical examination while terminal renal failure is not yet present. Affected mice demonstrate enlarged kidneys as well as macroscopically dilated extrahepatic bile ducts.

Culture medium

Two types of media were initially used in culture of biliary epithelium. Eagle's minimal essential medium (EMEM) was supplemented with 10% fetal calf serum, 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 microgr/ml streptomycin sulfate and 100 IU/ml penicillin (19). The second medium consisted of equal volumes of Dulbecco's modified Eagle's medium (DMEM) and Ham F12 supplemented with HEPES (10 mM), sodium bicarbonate 13.4 mM (15 ml 7,5% per L), glutamine (2 mM), transferrin (6.2 x 10^{-8} M), insulin (2,7 x 10^{-8}M), triiodothyronine (2 x 10^{-9} M), Hydrocortisone (2,9 x 10^{-8}M), selenic acid (6.8 x 10^{-9} M), prostaglandin E1 (7.1 x 10^{-8} M), penicillin (50 U/l), streptomycin sulfate (50 microgr/mL) and nystatin (50 U/ml). Since the two media gave equal results and since serum would interfere with the intended studies we used the serum free medium as basal medium (BM) throughout the current study.

Preparation of culture dishes

Explants were grown on either coated or uncoated 35 mm plastic culture dishes (Falcon, Oxnard, CA). Coating was performed with either Vitrogen-100 (Celltrix, Palo Alto, CA), or with Matrigel (Collaborative Research, Lexington, MA) to determine the most reproducible culture conditions. Vitrogen was used after dilution at 4°C with equal volumes of culture medium. Matrigel was used according to the instructions of the manufacturer. Dishes were coated with approximately 100 microL, placed in the incubator at 37° C for 30 minutes, than filled with 2 ml culture medium and kept at 37°, 5% CO_2 for use the same day. In preliminary studies Vitrogen coating gave the best results in terms of colony growth, viability and cellular morphology and was therefore utilized for all of the subsequent studies. For electron microscopy explants were grown on Vitrogen coated 22 x 22 mm coverslips placed in 35 mm culture dishes. Growth and ^{3}H-Thymidine incorporation studies were performed on explants grown on Vitrogen coated 10 x 10 mm coverslips placed in 35 mm culture dishes.

Common bileduct explantation and primary cultures

Mice were killed by decapitation and laparotomy was performed under sterile conditions. The common bile duct was carefully dissected from extraneous tissue and from the pancreatic duct. The common bile duct was transsected proximally at the origin of the cystic duct and distally near the choledocho-duodenal junction, immediately placed into ice cold culture medium and transferred into a laminar flow hood.

Under the stereomicroscope the duct was then cut into 0.2 mm^2 explants. Each control duct thus provided approximately 6 explants, while markedly enlarged BPK ducts provided approximately 30 explants for culture. Explants were transferred to fresh medium free culture dishes and incubated overnight at 37° C, 5% CO_2 to permit attachment. The next morning 2 ml of culture medium was gently added to each dish and subsequently changed every 2 days.

Immunohistological characterization

The presence of specific epithelial cell markers was tested on both intact tissue and cultured cells which grew from cultured explants using: 1) a rabbit polyclonal antibody against human cytokeratin which previously was shown to bind specifically to murine biliary epithelial cells (Accurate Chemical and Scientific Corp., Westbury, NY, no. ax 1698) (18) and 2) Lectin Dolichos Biflorus Agglutinin (DBA) which also showed specific binding to biliary epithelium in preliminary studies. Monoclonal anti Vimentin antibody (Bio Genex, San Ramon, CA) staining was used as a marker for fibroblasts. Immunohistology was performed using our previously described methods (20).

Bile ducts from control mice were rapidly fixed in 3.5% parafomaldehyde (pH 7.4) for 30 minutes at 4° C. Fixed tissue was then washed, dehydrated through a graded acetone series and infiltrated and embedded in Immunobed plastic embedding medium (Polysciences, Warrington, PA). Immunobed sections (3 micrometer each) were etched with alcoholic NaOH, trypsinized and incubated overnight at 4° C with biotinylated DBA (6.25 microgr/ml) or after blocking with normal goat serum (1 : 50) with anticytokeratin antibody (1 : 250) or with anti-vimentin. This was followed for DBA by aviden peroxidase (1 : 500) for 90 minutes, for the cytokeratin antibody by sequential incubations with bridging goat anti rabbit IgG (1 : 40) for 90 minutes and rabbit peroxidase-anti peroxidase complex (1 : 100) for 45 minutes, and for the anti Vimentin monoclonal by biotinylated anti mouse (1 : 200) for 90 minutes and avidin peroxidase (1 : 500) for 90 minutes. Sections were then stained with diaminobenzidine 0.05%, hydrogen peroxide 0.01%.

Cells which were identified as outgrowth from cultured explants were stained after first passage. Multiple primary cultures grown for 10 days were incubated in 0.25% trypsin, 0.1% ethylenediamine tetra acetic acid (EDTA) for 30 seconds and after aspiration of the supernatant for another 10 minutes at 37° C, 5% CO_2. The cells were then harvested,

washed, seeded on multiwell microscope glass slides and cultured for 48 hours. The slides were then fixed in methanol (-20° C) for 10 minutes and dried. Staining was performed with the same antibodies as given for intact tissue. Primary antibody was applied for 1 hour, and secondary antibody and peroxidase for 30 minutes each. This was followed by diaminobenzidine staining as described for intact tissue.

Electron microscopy

Transmission electron microscopy was performed on both intact bile ducts and cultured biliary epithelial cells from BPK and control animals. Common bile ducts were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde for 2 hours at 4° C and postfixed in 0.5% O_sO_4 for 1 hour at 4° C, then dehydrated through a series of graded acetone and infiltrated and embedded in Medcast (Ted Pella Incorp., Redding, CA.). Primary cell cultures grown on Vitrogen coated glass cover slips were fixed in 1% paraformaldehyde, 1.25% glutaraldehyde for 2 hours at 4° C and postfixed in 1% O_sO_4 for 30 minutes at 4° C, then dehydrated through graded ethanols and infiltrated and embedded in Medcast. Ultrathin sections were mounted on copper mesh grids and stained with uranylacetate and lead citrate.

Assessment of mitogenic activity

The mitogenic activity of control and BPK derived cell cultures was quantitavely assessed using serial colony size measurements and by ^{3}H-Thymidine incorporation assay. Colony drawings made using a CK_2-TRC-2 Olympus inverted microscope with a BH_2-DA drawing attachment were analyzed using computer assisted morphometry (Sigmascan^R). Colony size was measured with 99% reproducability. Colony growth curves were prepared using daily surface area measurements of control (n = 36) and BPK-derived (n=19) primary cell cultures. In addition the mitogenic effect of various polypeptide growth factors was assessed by serial surface area measurements and by a ^{3}H-Thymidine incorporation assay as follows. Control and BPK derived primary cell cultures were grown on Vitrogen coated glass coverslips in basal culture medium (BM). After 6 days of culture the media were replaced by BM, with or without supplementation with one of the following polypeptide growth factors: human Insulin-like Growth Factor I and II (IGF I and II, 25 ng/ml, U.S. Biochemical Corp., Cleveland, Ohio), mouse Epidermal Growth Factor (EGF, 20 ng/ml, Sigma), bovine acidic-Fibroblast Growth Factor (aFGF 12 ng/ml, Sigma), human Transforming Growth Factor ß (TGF ß, 2.5 ng/ml, Collaborative Research Corp., Bedford, Massachusetts), human Hepatocyte Growth Factor (HGF, 20 ng/ml, Coll. Research Corp.) and human recombinant Keratinocyte Growth Factor (KGF, 12 ng/ml, a generous gift from Dr. Stuart. A. Aaronson, Bethesda) (21). Each experimental group consisted of 28 primary cultures.

The effect of serum and growth factor supplementation over basal growth was assessed by both surface area increase during the first 24 hours and by ^{3}H Thymidine incorporation following this 24 hour incubation. ^{3}H Thymidine (5 microCi/ml) was added to freshly prepared basal or growth factor-supplemented medium. After 6 hours incubation the cover slips were transfered to scintillation vials and washed with 10% trichloracetic acid. The cells were then dissolved in Protosol (Dupont) and mixed with Aquassure scintillation cocktail (Dupont). Counts were expressed as desintegrations $x10^{-2}$ per minute ($dpm10^{-2}$) and related to the surface area of the colony at the start of the ^{3}H-Thymidine incubation ($dpm.10^{-2}$). In order to visualize light microscopically the distribution of mitogenic activity in individual colonies we used a 9-hour Nocodazole (0.2 microgr/mL) induced arrest of mitosis prior to fixation.

Statistics

Data are expressed as means and standard deviations. Differences between growth factor supplemented growth and basal growth were analyzed by unpaired two tailed t-test. Interactions of growth factor effects and strain effects were tested by analysis of variance.

Results

Explantation and primary culture conditions.

In all BPK mice fusiform dilatation of the entire extrahepatic biliary tract was seen in association with renal cystic disease (18). The outer diameter of the common bile duct was increased in all cases as compared to control values (250-1500 micrometer vs. < 200 micrometer).

The technique of stereomicroscopic fragmentation of the explants did not allow standardized sizing of cultured fragments. The optimal tissue fragment size was approximately 0.2 mm^2. Smaller pieces yielded inconsistent culture results. For adequate attachment of the explants to the culture dishes a collagen coating was indispensable. The attachment efficiency was 90% for Vitrogen 100 and Matrigel-coated dishes alike and less than 10% for uncoated dishes. Explants cultured on coated dishes formed an expanding cellular outgrowth in 90% of cases, which was first noticable after 2 days of culture. This plating efficiency was not different for Vitrogen 100 and Matrigel coated dishes. With continuing growth better results were obtained with Vitrogen 100 where cellular outgrowth was consistently homogenous during 10 days of culture.

In contrast, cultures grown on Matrigel for more than 5 days occasionally formed cell free patches in initially homogenous cell colonies. This was interpreted to be due to the fragility of the substratum and the early breakdown of the coating. Vitrogen-100 coating

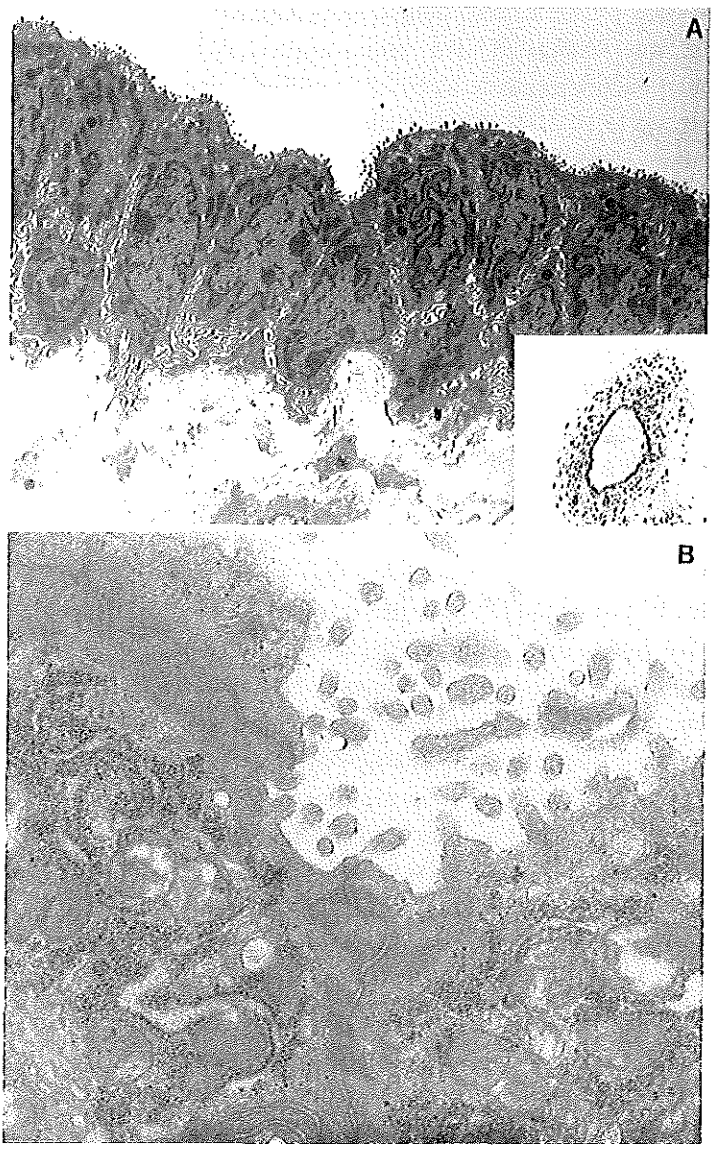


Fig.1. Common bile duct epithelium prior to culture. (A) Inset: Overview of an intact undilated common bileduct (hematoxylin x 200). Transmission electron microscopy: Epithelial lining of a dilated common bile duct from a 30 day old BPK mouse. The cell features are undistinguishable from control ductal epithelium including superficial microvilli, apical secretion vesicles, extensive basolateral infoldings and an intact basement membrane (x 6000). (B) At higher magnification rough endoplasmatic reticulum with ribosomes as well as free ribosomes can be identified. Apically tight intercellular junctions including desmosomes can be identified (x 32.000).

was therefore used for the current studies. In preliminary studies two different types of culture media were compared. The serum-supplemented and the serum-free media produced similar culture results in terms of attachment, plating efficiency, colony growth and cell density up to 10 days of culture. Based on these findings and on the requirement of defined culture conditions for the intended experiments on cell growth, we used the serum free medium as basal medium (BM) throughout the current experiments.

Characterization of the cells

Cells in and on the primary explants.

Before microsurgical dissection, the explants consisted of ducts lined by an epithelial monolayer and surrounded by connective tissue (fig.1). In some cases explanted bile ducts were attached to pancreatic and/or to fatty tissue. This could clearly be identified and removed using the stereomicroscope. Only "clean" ducts were used for the culture experiments. The duct walls of dilated bile ducts demonstrated cellular profiles that were not different from normal bile ducts other than an increased number (not density) of epithelial cells. Ultrastructurally the dilated ducts were lined by intact cuboidal epitelium with an intact microvillar surface. Apical tight junctions gave way to complex interdigitations of the lateral cell membranes. Cytoplasmic organelles were dispersed throughout the cell. The underlying basement membrane was intact and of normal caliber and underlayed by collagen and fibroblasts with an active endoplasmatic reticulum. The ultrastructural morphology did not differ from controls.

During culture the explants were covered entirely by a single layer of cuboidal epithelial cells (fig.2). These cells had ultrastructural characteristics much like those seen in vivo (fig.1). Unlike the epithelium in vivo no basement membrane could be identified at this stage. The underlying tissue consisted of a loose stroma with ultrastructurally viable stromal cells. During culture multiple ductular structures could be observed in most explants. The cells lining these ducts invariably had the ultrastructural appearance of epithelial cells not different from the explant covering cells in figure 2.

Cellular outgrowth from the primary explants.

Cellular outgrowth was first seen after 3 days of culture at the edges of most explants (fig.3). This outgrowth expanded progressively and developed into a dense confluent cell colony in which the cell density was homogenous up to 10 days of culture (fig.4). Fibroblast-like cells were never seen. Two different growth patterns were recognized in these cell colonies. The outer rim of the colony with a width of approximately 20 cells exhibited a relatively low cell density and a high mitogenic activity as illustrated by the nocodazole-induced arrest of mitosis (fig.5). The central area of the cell colonies was made up of densily packed cells with low mitogenic activity.

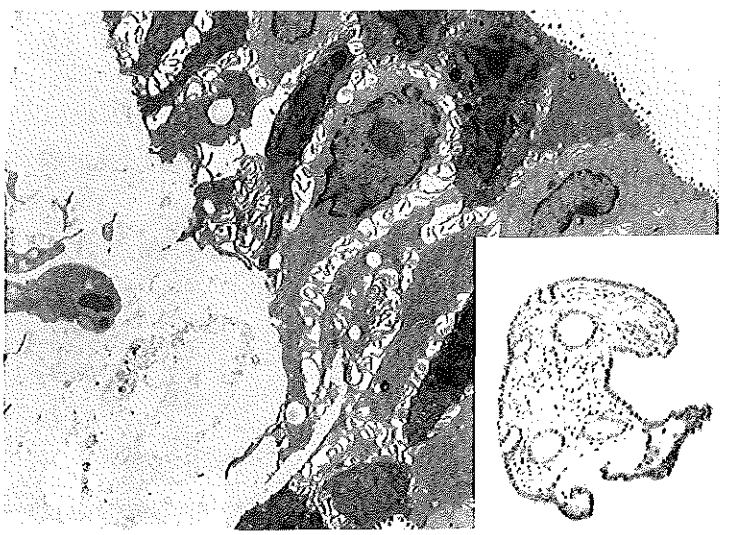


Fig.2 Common bile duct explant cultured for 7 days. Inset: Overview demonstrating the complete coverage of the explant by a dense epithelium as well as the presence of multiple ductular structures within a loose and apparently viable stroma (hematoxylin x 100). Transmission electron microscopy: Epithelial characteristics include apical microvilli, tight junctions and extensive intercellular digitations. Note the reduced number of secretary granules and the absent basement membrane as compared to the in vivo epithelium shown in Fig 1. (x 8000)

Ultrastructurally a uniform sheet of cells could be identified. Cultured cells formed tight monolayers with the characteristics of polarised epithelia including numerous apical microvilli, tight junctions, extensive intercellular digitations, and the apical distribution of secretory granules and mitochondria (fig.6). Compared to the intact biliary epithelium, the cultured cells were more spread and exhibited no basement membrane at this stage.

Immunocytochemical staining revealed specific binding of anti cytokeratin antibody to the apical cell membrane of biliary epithelial cells in intact tissue (fig.7) as well as to the cultured cells in a delicate filamentous pattern (fig.8). Similarly DBA staining revealed specific apical binding to biliary epithelial cells in vivo and positive staining of the cultured cells. Biliary epithelial cells of intact tissue as well as cultured cells did not express vimentin.

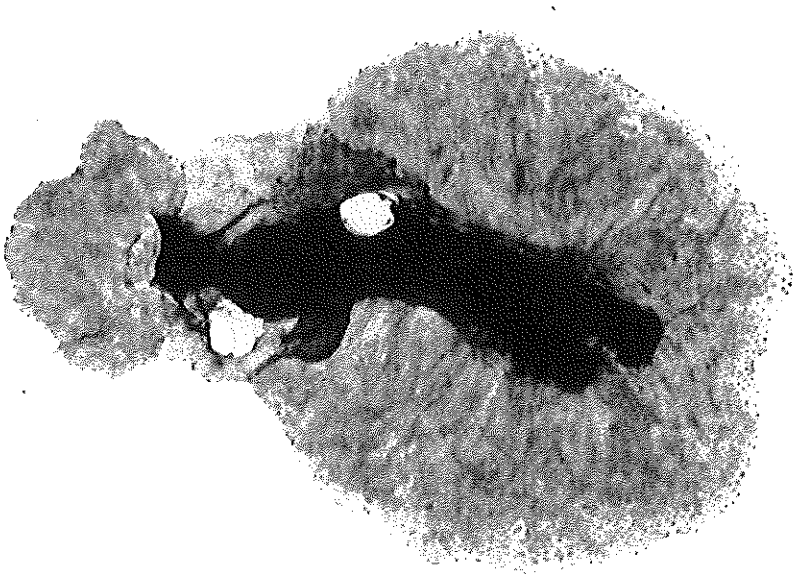


Fig.3 Representative example of a tightly confluent cellular outgrowth from a common bile duct explant (dark central area) cultured for 3 days in basal medium (hematoxylin x 50).

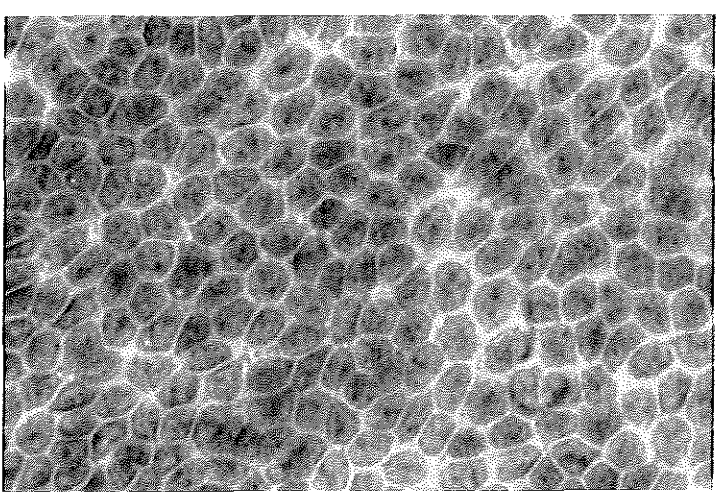


Fig.4 Primary epithelial cell culture from a common bile duct explant cultured for 10 days in basal medium. Note the homogeneity and high density of the epithelial monolayer (hematoxylin x 300)

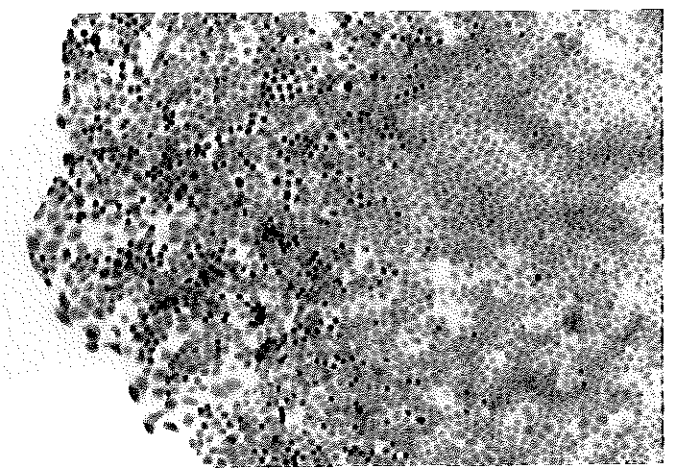


Fig.5 Homogeneous epithelial monolayer cultured for 7 days. Mitogenic activity, (visible as dark nuclear chromatin aggregates) is confined to the periphery of the colony. For better visualisation these cells were arrested in mitosis for 9 hours using Nocodazole (0.2 µg/ml) incubation (hematoxylin x 50).

Cellular growth studies

Linearity of colony growth up to 10 days of culture under basal conditions was demonstrated in both BPK and control derived cell colonies (fig.9). Basal growth of BPK derived cells was not different from controls.

Cellular growth was assessed by measurement of: 1) 6 hour ^{3}H-Thymidine uptake and 2) 24 hour colony size increments. Data are expressed relative to colony size. These two parameters correlate well when measured simultanously in individual cell colonies grown in basal medium ($r = 0.75$). Because the size of individual colonies varied considerably we wondered whether size is a determinant of colony growth. Under basal medium conditions no such correlation could be demonstrated between colony size and percent cellular growth as assessed by either thymidine uptake or colony size increments.

The effects of individual growth factors on ^{3}H-Thymidine uptake are presented in Table 1. The results of two sets of experiments are presented separately. As compared to uptake under basal medium conditions we found a stimulatory effect of over 130% for EGF, KGF and aFGF in cells derived from both BPK and control mice. TGFß significantly inhibited the thymidine uptake of both cell populations (61% of basal uptake). HGF and IGF-I inhibited the thymidine uptake to a lesser extent and in control cells only.The proliferative response to exogenous EGF was significantly different for BPK and control cells. This observation was later confirmed by the second set of experiments. BPK derived cells demonstrated a higher proliferative response to EGF than control cells (mean increase over BM-growth for BPK 239% and for control 131%, $p < 0.001$). A similar pattern of growth factor induced proliferative responses was noted when measuring colony size increments (Table 2). This parameter appeared to be less sensitive than the thymidine uptake assay.

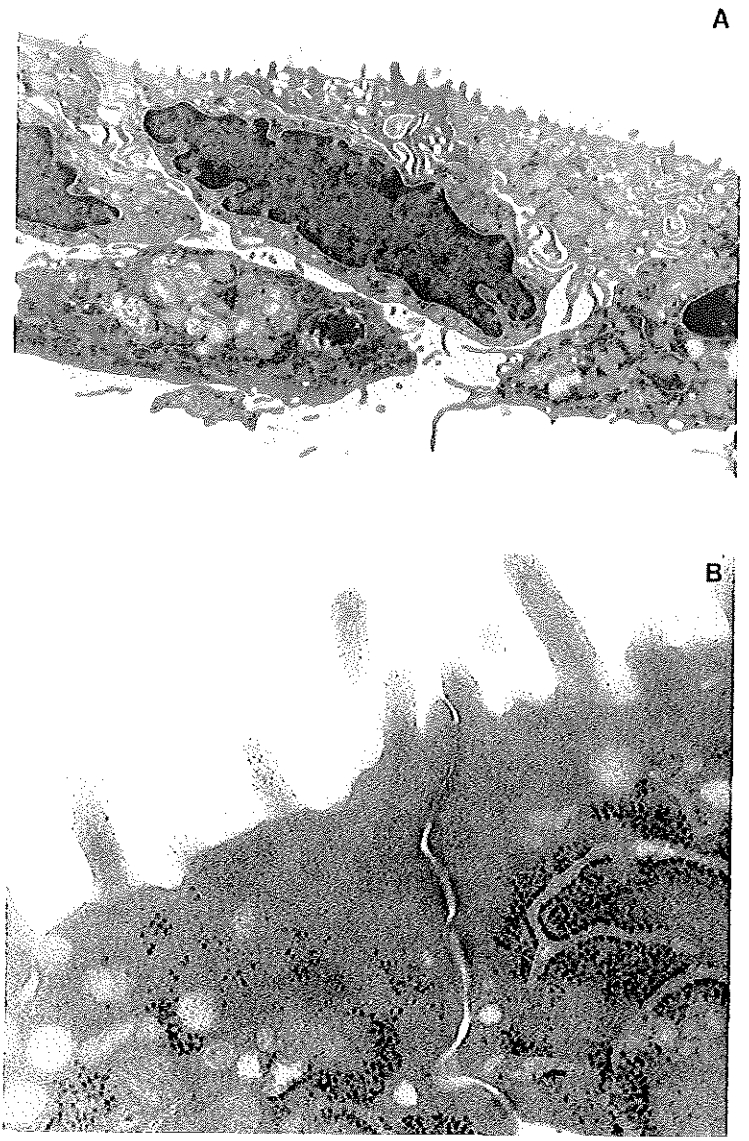


Fig.6 Ultrastructural characteristics of a cellular monolayer cultured from a common bile duct explant derived from a 20 day old Balb/C control mouse and cultured for 1 week. A:. Overview, featuring elongated cells with a rough apical surface, microvilli, extensive intercellular digitations, apical secretory vesicles, lysosomes, mitochondria and large nuclei with indentations and a regular chromatine pattern (x 18.750). B: Rough endoplasmatic reticulum with ribosomes as well as free ribosomes can be identified at higher magnification. Tight apical cell-cell contact includes desmosomes (x 82.500).

Fig.7 Longtudinal section of a normal common bile duct stained with a polyclonal anticytokeratin antibody (diaminobenzidine x 200).

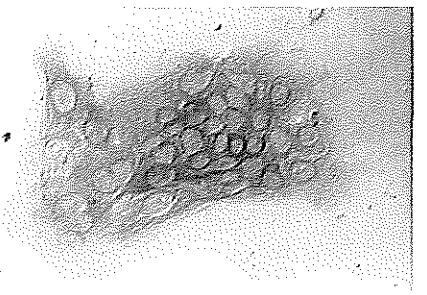


Fig. 8 First passage biliary epithelial cells stained with polyclonal anti human cytokeratin antibody (diaminobenzidine x 200).

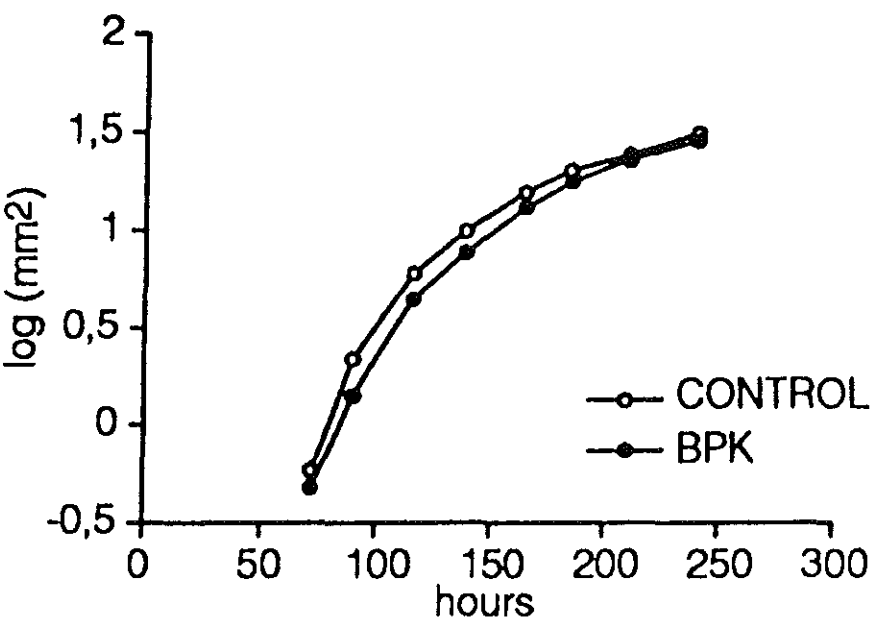


Fig.9 Surface area growth pattern during 10 days of culture of primary cell colonies derived from common bile duct explants form Balb/C BPK and control baby mice of 20-25 days of age.

Table 1. The effect of growth factor supplementation on the uptake of ^{3}H-Thymidine by control and BPK cell cultures.

		Control		BPK	
Exper.	Factor	dpm/mm^2	Percent of BM	dpm/mm^2	Percent of BM
1	BM	39 (17)		29 (16)	
	EGF	46 (17)	118	76 (31) *	262 ##
	IGF-I	29 (12) *	74	29 (15)	101
	HGF	29 (8) *	75	29 (19)	100
2	BM	39 (19)		30 (24)	
	EGF	56 (30) *	143	65 (42) **	217 #
	aFGF	54 (17) *	139	40 (29)	133
	KGF	61 (32) *	156	49 (30) **	169
	IGF-II	50 (44)	109	30 (16)	100
	TGFß	24 (18) *	61	18 (19) *	64

The uptake of ^{3}H-Thymidine by primary biliary cell cultures derived from BPK and control animals in the presence or absence (BM) of various growth factors. Data from 2 sets of experiments are presented separately and expressed as mean (sd) desintegrations per minute per surface area of the cell colonies (10^{-2}dpm/mm^2) (n=25-30 for each data point). The growth factor data are additionally presented relative to the thymidine uptake in defined basal medium.
* p<.05, growth factor vs BM; ** p<.001, growth factor vs BM
# p<.05, BPK vs control; ## p<.001, BPK vs control

Table 2. The effect of growth factor supplementation on 24-h colony growth of control and BPK cell cultures.

	Control		BPK	
Factor	Growth	Percent of BM	Growth	Percent of BM
BM	32 (17)		26 (11)	
EGF	42 (14) ##	131	42 (17) ##	157
aFGF	36 (9)	110	30 (12)	113
KGF	30 (14)	93	30 (18)	114
HGF	29 (19)	90	26 (14)	99
IGF-I	29 (9)	80	23 (10)	85
IGF-II	33 (22)	102	30 (14)	112
TGFß	28 (15)	87	18 (8) ##	67

Colony growth of primary biliary cell cultures derived from BPK and control animals in the presence or absence of various growth factors. Data are expressed as mean (sd) percentual increase of surface area during 24-h observations (n=25-30 for each data point). Growth factor data are additionally presented relative to growth in basal medium (% over BM). ## p<.001, BPK vs control

Discussion

We have recently reported a new murine model of autosomal recessive polycystic kidney disease expressed in the Balb/c BPK/BPK mouse strain (18). In affected animals both renal tubular and biliary intra- and extrahepatic epithelial hyperplasia were early features of disease as in human ARPKD. We noted that this dual organ epithelial hyperplasia preceded the overall disruption of normal organ architecture that is characteristic of more advanced disease stages. Our current data on in vitro growth characteristics of biliary epithelial cells from BPK mice may therefore reflect early mechanisms involved in the development of hepatic lesions in PKD.

Our system of primary cultures of extrahepatic biliary cells carries several potential advantages over culture systems based on cell isolation from hepatic tissue. The relative simple structure of the common bileduct permits isolation by microsurgery only. The excellent homogeneity of the obtained epithelial cell population makes further isolation steps unnecessary. This prevents the potential hazard of unwanted selection of epithelial cell subsets by stringent isolation procedures. Moreover surgical isolation allows straightforward matching of control cells. The confounding effect of isolation steps mandatory for whole organ derived cell suspensions can be omitted. Since such steps are based on physical and biochemical cell properties they may act differently on control and affected cells, consequently complicating the matching process.

Biliary epithelial cells have been isolated and cultured by others from either the intrahepatic biliary tract or the gallbladder from various animal species (19, 22-24). None of these methods could be used in the current study. The reported methods necessary for isolation and consequent propagation of biliary cells from liver homogenates potentially affect the characteristics of the obtained cell population through less than perfect purification and through selection. Methods using the gallbladder as an epithelial cell source could not be used in the current study because of its small size in mice and foremost since the gallbladder was the only apparantly unaffected part of the biliary tract in the murine disease model.

The linear growth pattern which was maintained up to ten days of culture allows the comparison of the growth characteristics of BPK and control derived cell colonies. The two parameters used in this study to assess colony growth, surface area increment and thymidine uptake, correlate well when measured simultaneously in individual colonies under basal medium conditions. However the effect of growth factor supplementation on these 2 parameters (Table 1) was disparate in some cases. The fact that growth factor effects on colony size were assessed over the first 24 hours of factor supplementation while the thymidine uptake was measured during the following 6 hours may explain this apparent disparity.

Because the colony growth curves of BPK derived cell colonies in serum free basal medium were not different from controls, we had the opportunity to test the proliferative response of the cells to specific growth factors. Several significant growth factor effects were noted in both BPK and controls. Only EGF repeatedly induced a significantly higher effect in BPK derived cell colonies when compared to controls. The potential biological significance of the increased sensitivity of BPK derived biliary epithelial cells to EGF is highlighted by the reported secretion of EGF from the circulation into the bile in an immunoreactive and biologically active form. Moreover the appearance of intact EGF in bile of suckling rats is several-fold higher than in adult rats (25). In addition cultured bile duct epithelial cells are known to be capable of receptor mediated binding and endocytosis of EGF (26).

Such findings parallel data from renal cystogenesis in PKD. Overexpression of the EGF receptor and of it's messengerRNA has been demonstrated using whole kidney homogenates from both human (12) and murine (13) cystic kidneys. In additon abnormal localization of the EGFR to the lumenal membrane of renal tubular epithelia in contrast to the normal basolateral localization has been reported for human as well as murine PKD (14). The biological significance of these findings is demonstrated by the report of mitogenic concentrations of EGF in cyst fluid (15), the increased EGF induced mitogenic response of human cyst derived cells (16) and the cystogenic properties of EGF in metanephric organ culture (27) and in cultures of normal kidney cells in collagen gel (28).

In conclusion we report a reproducable serum free cell culture system in which minimal selection by isolation procedures and excellent homogeneity of the cell colonies provide optimal conditions for the study of normal and abnormal biliary epithelial cell growth in murine PKD. The demonstration of increased sensitivity of BPK-derived cells specifically to the proliferative effect of EGF contributes to the hypothesis that epithelial hyperproliferation is an underlying pathogenic mechanism of the renal and biliary lesions in PKD and that abnormal regulation of the EGF/EGF receptor is involved in this process.

Acknowlegdments

A preliminary report of these findings was presented at the 1992 Annual Meeting of the American Society of Nephrology, and has appeared in abstract form (29). These studies were supported by the Sophia Foundation for Medical Research in Rotterdam, The Netherlands, the Dutch Kidney Foundation and grants no. DK 34891 and DK 44875 from the National Institutes of Health. We gratefully acknowledge the technical advise of Dr Sum P. Lee and Dr Dolphine Oda (Departments of Oral Biology and Medicine, University of Washington, Seattle, Washington) and the technical assistence of Rob Willemsen (Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands).

References

1. Welling LW, Grantham JJ 1991 Cystic and developmental diseases of the kidney. In: Brenner B, Rector F (eds) The Kidney W.B. Saunders, pp 1657-1694.
2. Churchill DN, Bear JC, Morgan J, Payne RH, McManamon PH, Gault MH 1984 Prognosis of adult onset polycystic kidney disease re-evaluated. Kidney Int 26:190-193.
3. Picolli DA, Witzleben CL 1991 Disorders of intrahepatic bile ducts. In: Walker WA, Durie PR, Hamilton JR, Walker-Smith JA, Watkins JB (eds) Pediatric gastrointestinal disease Decker Philadelphia, pp 1124-1140.
4. Kaplan BS, Kaplan P 1990 Autosomal recessive polycystic kidney disease. In:Spitzer A, Avner ED (eds) Inheritance of kidney and urinary tract diseases. Kluwer, Dordrecht pp 265-276.
5. Desmet VJ. 1985 Intrahepatic bileducts under the lens. J Hepatol 1:545-549.
6. Zerres K, Mücher G, Bachner L, Deschennes G, Eggermann T, Kääriäinen H, Knapp M, Lennert T, Misselwitz J, van Mühlendahl KE, Neumann HPH, Pyrson Y, Rudnik-Schöneborn, Steinbicker V, Wirth B and Schärer K 1994 mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. Nature Genetics 7:429-32.
7. European Polycystic Kidney Disease Consortium 1994 The Polycystic Kidney Disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. Cell 77:881-894.
8. Avner ED, McAteer JA, Evan AP 1990 Models of cysts and cystic kidneys. In Gardner KD, Bernstein J (eds) The Cystic Kidney. Kluwer Publishers, Dordrecht pp 55-98.
9. Grantham JJ, Geiser JL, Evan AP 1987 Cyst formation and growth in autosomal dominant polycystic kidney disease. Kidney Int 31:1145-1152.
10. Avner ED 1988 Renal cystic disease. Nephron 48: 89-93.
11. Wilson PD, Sherwood AC 1991 Tubulocystic epithelium. Kidney Int 39:450-463.
12. Klingel R, Dippold W, Störkel S, Meyer zum Büschenfelde KH, Köhler H 1992. Expression of differentiation antigens and growth-related genes in normal kidney, autosomal dominant polycystic kidney disease, and renal cell carcinoma. Am J Kidney Dis 19:22-30.
13. Orellana S, Sweeney WE, Neff C, Avner ED 1995. Epidermal growth factor receptor expression is abnormal in murine polycystic kidney. Kidney Int (In press).
14. Avner ED, Sweeney WE 1992. Epidermal growth factor (EGF) and altered membrane localization of epidermal growth factor receptor (EGFR) modulate in vitro renal collecting tubule cystogenesis. Ped Res 328A (abstr.).
15. Horikoshi S, Kubota S, Martin RS, Yoshihiko Y, Klotman EP 1991. Epidermal growth factor expression (EGF) in the congenital polycystic mouse kidney. Kidney Int 39:57-62.
16. Wilson PD 1991. Aberrant epithelial cell growth in autosomal dominant polycystic kidney disease. Am J Kidney Dis 17:634-637.
17. Wilson PD, Du J, Norman JT 1993. Autocrine, endocrine and paracrine regulation of growth abnormalities in autosomal dominant polycystic kidney disease. Eur J Cell Biology 61:131-138
18. Nauta J, Ozawa Y, Sweeney Jr WE, Rutledge JC, Avner ED 1993. Renal and biliary abnormalities in a new murine model of autosomal recessive polycystic kidney disease. Ped Nephrology 7:163-172.
19. Oda D, Lee SP, Hayashi A 1991. Long term culture and partial characterization of dog gallbladder epithelial cells. Lab Invest 64:682-692.
20. Sweeney WE, Avner ED 1991. Intact organ culture of murine metanephros. J Tiss Cult Meth 13:163-168.
21. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. PNAS 86:802-806.
22. Ishii M, Vroman B, LaRusso NF 1989. Isolation and morphologic characterization of bile duct epithelial cells from normal rat livers. Gastroenterology 97:1236-1247.

23. LaRusso NF, Hoerl BJ, Vroman BT, Scott RE 1989. Biological characteristics of cultured human gallbladder epithelial cells (abstr). Hepatology 10:636.
24. Vierling JM 1989. Biliary victories: studies of isolated biliary epithelial cells. J Lab Clinical Med 113:663-664.
25. Kong W, Koldovsky O, Rao RK 1992. Appearance of exogenous epidermal growth factor in liver, bile and intestinal lumen of suckling rats. Gastroenterology 102:661-667.
26. Ishii M, Vroman B, Larusso NF 1990. Morphologic demonstration of receptor mediated endocytosis of epidermal growth factor by isolated bile duct epithelial cells. Gastroenterology 98:1284-1291.
27. Avner ED, Sweeney WE 1990. Polypeptide growth factors in metanephric growth and segmental neprhon differentiation. Ped Nephrol 4:372-377.
28. Neufeld TK, Douglas D, Grant M, Ye M, Silva F, Nadasdy T, Grantham JJ 1992. In vitro formation and expansion of cysts derived from human renal cortex epithelial cells. Kidney Int 41:1222-1236.
29. Nauta J, Sweeney WE, Avner ED 1993. In vitro model of epithelium hyperplasia in murine autosomal recessive polycystic kidney disease (ARPKD). Kidney International 43:466.

2.3

The Han:SPRD rat is not a genetic model of human ADPKD-type 1.

J. Nauta[1], M.A. Goedbloed[1], T.M. Luider,
A.Th. Hoogeveen[2], A.M.W. van den Ouweland[2] & D.J.J. Halley[2]

[1]Department of Pediatrics, Sophia Childrens Hospital, Molewaterplein 60, 3015GJ Rotterdam, The Netherlands and [2]Department of Clinical Genetics, Erasmus University and University Hospital Dijkzigt, PO Box 1738, 3000DR Rotterdam, The Netherlands

Laboratory Animals 31:241-247, 1997

The Han:SPRD rat is not a genetic model of human autosomal dominant polycystic kidney disease type 1.

Abstract

Human autosomal dominant polycystic kidney disease (ADPKD) is a high incidence disorder, leading to renal failure in many patients. The majority of cases results from a mutation in the PKD1 gene. The only well documented animal model of ADPKD is the Han:SPRD-*Pkd* strain. Its genetic basis is unknown as yet. In the current study we determined whether the disease in these rats is genetically linked to the rat homologue of the PKD1 gene. We used the protamine gene as a polymorphic marker (*Prm1*) of the PKD1 region. Matings of Han:SPRD-*Pkd* with BB rats and backcross of the offspring with BB yielded animals informative for linkage analysis. This analysis revealed random segregation of the defect and the *Prm1* marker, indicating that the model is not caused by a mutation in the PKD1 gene. We conclude that the Han:SPRD-*Pkd* rat strain is not a genetic model of PKD1.

Introduction

Human polycystic kidney disease (PKD) is one of the most common inherited disorders (Welling 1996). Two subtypes can be recognised: Autosomal dominant PKD (ADPKD) typically presenting during adult life and autosomal recessive PKD (ARPKD) leading to renal failure, either prenatally or during early childhood. These subtypes are distinct in terms of clinical presentation, histopathology and pattern of inheritance. ADPKD has an incidence of 1 in 1000 and gives rise to considerable morbidity. The major feature of the disease is cystic enlargement of the renal tubules leading to destruction of the normal renal architecture, typically resulting in end stage renal failure during adult life (Gabow et al. 1992).

Recent studies in affected families explored the genetic basis of ADPKD. Locus heterogeneity has been established with at least two genes that independently can cause the disease. The locus involved in 85% of the families with ADPKD (now called PKD1) has been linked to the tip of the short arm of chromosome 16 (Reeders et al. 1985). Most of the remaining cases of ADPKD have been linked to a locus on chromosome 4 (PKD2) (Kimberling et al. 1993, Peters et al. 1993). In addition, families have been reported in which the mutation must have occurred in a third gene which has not been localised as yet (Daoust et al. 1995). The PKD1 gene has been identified by the European Polycystic Kidney Disease Consortium (1994). This group analysed a chromosomal translocation associated with ADPKD that disrupts a gene encoding a 14 kb transcript in the PKD1

candidate region. Mutation analysis in PKD1 patients confirmed that the gene involved is the PKD1 gene. Recently full length genomic and cDNA sequences of this gene have been published (International Polycystic Kidney Disease Consortium 1995, Hughes et al. 1995). The gene product, Polycystin, is predicted to be an integral membrane protein with several extracellular domains. These domains are suggested to be involved in protein-protein interactions or protein-carbohydrate interactions. Polycystin may therefore play a role in cell-cell and cell-matrix communications (American PKD1 Consortium 1995).

The study of genetically transmitted PKD in animal models has the potential of identifying specific cystogenic gene products as well as biological and environmental factors that modulate the formation and progressive enlargement of the cysts. Genetically-transmitted renal cystic diseases have been described in mouse, rat, rabbit, cat, dog, antelope and pig (Avner et al. 1990, Nauta et al. 1993, Aziz 1995). The Han:SPRD-*Pkd* rat strain described by Kaspareit-Rittinghausen and subsequently in more detail by others is the best documented animal model of inherited PKD with an autosomal dominant inheritance pattern, resembling several features of the human disease (Kaspareit-Rittinghausen et al. 1991, Gretz 1992, Cowley et al. 1993). This model has been used to study the cystogenic process as well as the progressive nature of renal cystic diseases (Gretz et al. 1994, Schäfer et al. 1994, Torres et al. 1994, Zeier et al. 1994, Cowley et al. 1996).

In contrast to the detailed documentation of the Han: SPRD-*Pkd* phenotype no information is available as yet on the genotype of this rat model of PKD. We wondered whether the disease in these rats results from a mutation in the PKD1 gene. This gene is involved in most human cases of ADPKD and is conserved in rodents (European PKD Consortium 1994). Here we report a linkage analysis of the disease with the PKD1 region using the protamine gene *Prm1* as a genetic marker. According to recent reports this polymorphic gene is located near the PKD1 gene in humans as well as in rats (Yeung et al.1993, 1994, Kobayashi et al.1995) .

Materials and methods

Animals and breeding program

Han: SPRD-*Pkd* rats heterozygous for the PKD mutation were kindly provided by Dr. F. Deerberg in Hannover. A breeding colony was established and maintained for 5 generations using brother-sister matings. Animals homozygous for the disease die at 3 weeks of age and can be identified by physical examination 1 to 2 weeks after birth. Heterozygotes are detected either retrospectively when transmitting PKD or at postmortem by renal histology.

The polymorphic *Prm1* locus was selected as a genetic marker linked to the PKD1 gene. This *Prm1* locus exhibits a diallelic "restriction fragment length polymorphism"

(RFLP). Southern blots of EcoR1 digested genomic rat DNA were hybridised with a *Prm1* cDNA probe. This revealed 2 different DNA fragments, sized approximately 9 and 12 kb. All rats of our Han:SPRD-*Pkd* colony are homozygous for the 12kb allele (see results). In order to obtain information with respect to the *Prm1* RFLP, a crossbreeding program was set up using a Han:SPRD-*Pkd* male known to be heterozygous for PKD and 2 female BB rats and backcross of the offspring with BB (Yeung et al. 1993). BB rats are maintained at our institution as an inbred colony and were kindly provided by Dr H.A. Drexhage, Dept of Immunology, Erasmus University. This strain was selected for the crossbreeding experiments because it proved to be homozygous for the 9kb allele (see results). Backcross of 9 females with BB males ((BBxSPRD-*Pkd*)F1xBB) yielded informative N2 offspring being either unaffected or heterozygously affected by the disease causing mutation. The offspring was killed at 3 weeks of age for DNA isolation and histological determination of heterozygosity.

DNA isolation

Genomic DNA was isolated from spleen using the simplified mammalian DNA isolation procedure according to Laird *et al.* (1991). DNA was digested overnight with the restriction enzyme EcoR1 at 37^{0}C, separated on a 0.7% agarose gel and blotted overnight to Hybond-N+ (Amersham) in 10 x SSC. Southern blots were hybridized with a ^{32}P-labeled mouse cDNA *Prm1* probe (kindly provided by Dr. R. Yeung, Fox Chase Cancer Center, Philadelphia), overnight at 55^{0}C in 10 x Denhardts, 10% dextran sulfate, 3 x SSC, 0.1% SDS, 5microgr/mL salmon sperm DNA.. Blots were briefly washed twice in 3 x SSC, 0.1% SDS at room temperature, followed by 20 minutes at 60^{0}C in 0.3 x SSC, 0.1% SDS. The signal was detected using a phosphor imager.

Statistics

Linkage was tested using both chi-squared and lod score analysis using the MLINK program of the LINKAGE package (Lathrop et al. 1984).

Results

Southern blot analysis of EcoR1 digested genomic DNA samples of Han:SPRD-*Pkd* rats, using *Prm1* as a probe revealed homozygosity for the 12 kb allele in all tested animals. To obtain an informative situation for the *Prm1* RFLP, other rat strains were analysed. The inbred strain of BB rats showed homozygosity for the 9kb fragment. Therefore this strain was selected to set up a cross with Han:SPRD-*Pkd*. Nine female crosses of BB x Han:SPRD-*Pkd* (F1) were backcrossed to BB resulting in an N2 in which six out of 9 litters included offspring with cystic kidneys (heterozygotes) as detected by histology at 3 weeks of age (fig. 1). The remaining 3 unaffected litters consisted of at least 10 animals

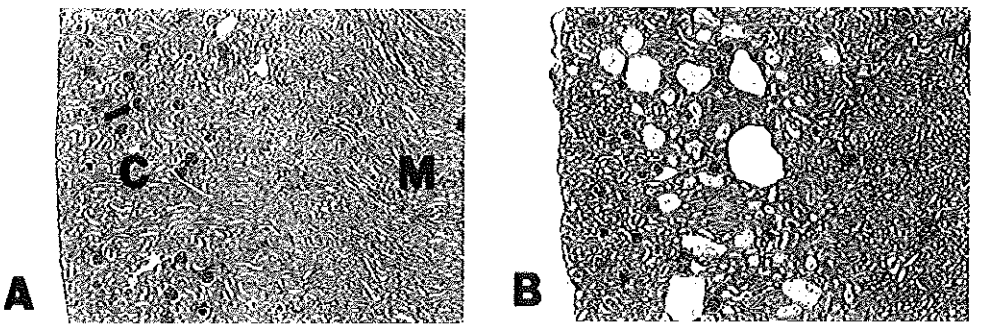


Figure 1. Representative kidney sections of Han:SPRD-Pkd x BB backcross progeny at the age of three weeks. Panel A: Unaffected animal. Panel B: Affected animal with early cysts in the subcortical region. Arrow = glomerulus, C = renal cortex, M = renal medulla. (Hematoxylin x 100)

each. Four of the couples that produced affected litters had a second N2 litter. Within the total of 10 affected litters PKD could be demonstrated in 22 of 60 female and 29 of 67 male animals. The cysts were histologically similar to the lesions in Han:SPRD-*Pkd* rats. Samples from 42 cystic animals were used for DNA analysis. Given the previously established tight linkage (3.9% recombination) between *Prm1* and the Eker mutation, located adjacent to the rat homologue for PKD1, it was to be expected that of the cystic animals (heterozygous for the pkd mutation) a majority of approximately 96.1% would be heterozygous at the *Prm1* locus and that only 3.9% would be homozygous for the 9 kb *Prm1* allele as a consequence of a recombination between the disease locus and the *Prm1* polymorphism (Yeung et al. 1993). In our experiments we observed however that 23 out of 42 affected rats were homozygous for the 9kb allele (Figure 2, Table 1).

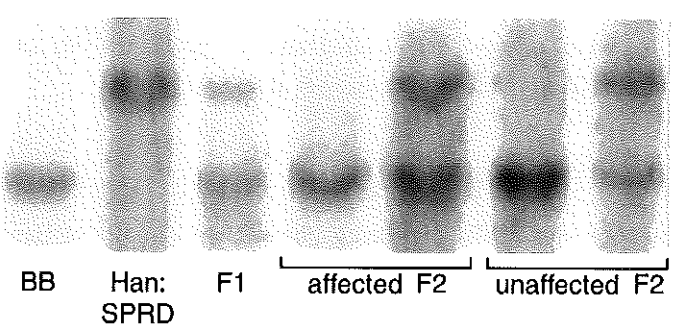


Figure 2. Absence of linkage between the PKD phenotype and the protamine gene, *Prm1*. The probe detected a 12-kb DNA fragment in the Han:SPRD-*Pkd* strain and a 9-kb DNA fragment in the BB strain. The 12-kb fragment did not cosegregate with the presence ('affected') or absence ('unaffected') of renal cysts in the Han:SPRD-*Pkd* x BB backcross progeny (N2).

A chi-squared test against the expected proportion of recombinants yielded significant evidence against such tight linkage (chi-square = 298.4, $p < 10^{-6}$). Lod score analysis also provided strong evidence against linkage, a region of 36 cM on either side of the *Prm1* locus can be excluded as location for the PKD locus in the Han:SPRD-*Pkd* rat.

Discussion

This study indicates that the Han:SPRD-*Pkd* strain is not a genetic model of PKD1. This is concluded from the absence of genetic linkage of the SPRD-*Pkd* mutation with the *Prm1* gene. Consistent with previous reports *Prm1* was found to be a diallelic genetic marker expressing DNA fragments with an estimated size of 9 and 12 kb respectively (Yeung et al. 1993). The apparent difference in size when compared to fragments reported by Yeung et al. (9.5 and 15 kb) is most likely based on inaccuracy of sizing in this size range. The validation of *Prm1* as a marker of the PKD1 locus is based on the following combination of reports. The human *Prm1* gene was localised near the PKD1 gene (Reeders et al. 1990). In rats the *Prm1* gene has been located on chromosome 10 and linked to a gene involved in the Eker rat model of Renal Carcinoma (lodscore = 18) (Yeung et al. 1993). The genetic distance between the *Prm1* gene and the Eker mutation was estimated to be 3,9 cM. The Eker mutation has recently been located within the rat homoloque (Tsc2) of the human tuberous sclerosis 2 (TSC2) gene (Yeung et al. 1994). As in humans (European PKD Consortium 1994), the rat Tsc2 gene has been localised adjacent to the PKD1 gene in a tail-to-tail orientation (Kobayashi et al. 1995). The intergenic distance between these two genes is very small (approximately 60bp) and highly conserved between human and rodents (Ollson et al.). This indicates that the genetic distance between *Prm1* and the rat homologue of PKD1 equals the 3,9 cM distance between *Prm1* and Eker.

Our results suggest incomplete penetrance of PKD in young pups of our crosses. In 10 affected litters we observed renal cystic disease in only 51 of 127 pups. This is less than the 50% predicted by a Mendelian pattern of inheritence, suggesting that not all mutants express the cystic phenotype at this age. Incomplete penetrance indicates heterogeneity of disease expression and suggests the involvement of genetic or environmental factors modifying the expression of disease in addition to the primary genetic defect. Whether this pattern of incomplete penetrance matches a similar pattern of disease expression in the original Han:SPRD-*Pkd* mutants can not be established since no systematic studies of heterozygous baby Han:SPRD-*Pkd* rats have been reported. We note that a variable expression of disease would not affect the outcome of the current linkage analysis because this analysis was restricted to phenotypically proven heterozygotes only.

In contrast to reports on Han:SPRD-*Pkd* rats, our study of the BB x Han:SPRD-Pkd cross did not reveal a gender effect. Young male and female animals from the crossbreed

were equally affected. In adult Han:SPRD-*Pkd* rats , however, cystic males develop renal failure, while female animals with renal cysts maintain a normal renal function. Studies of a gender effect in baby Han:SPRD-*Pkd* rats have not yet been reported. The lack of a sex effect in our rats may reflect the prepubertal stage of the animals studied. At this age sexual endocrine differentiation has not yet been completed. In addition, any possible phenotypical difference between our crossbreed and Han:SPRD-*Pkd* may be explained by differences in genetic background. It is likely, that disease modifying genetic factors are different between the inbred BB strain used for this study and the SPRD strain in which the mutation originally arose. Such strain associated genetic modfying factors have also been reported for other animal models of PKD (Guay-Woodford et al. 1996).

Our observation questions the value of the Han:SPRD-*Pkd* strain as a model of human disease. The affected gene in these rats is different from the gene affected in most human patients with ADPKD: the PKD1 gene. Nevertheless we still consider the Han:SPRD-*Pkd* rat a valuable model of inherited renal cyst formation, because this model may still serve the study of genetical, pathophysiological as well as clinical aspects of human cystic disease states.

Han:SPRD-*Pkd* may still be considered a candidate for the genetic homologue of one of the non-PKD1 types of human renal cystic diseases (Welling et al. 1996). These include ADPKD type 2 localised on chromosome 4 (Kimberling et al. 1993, Peters et al. 1993), autosomal recessive polycystic kidney disease localised on chromosome 6p21 (Zerres et al. 1994), medullary cystic disease and nephronophthisis localised on chromosome 2p (Antignac et al. 1993). Linkage analysis of the rat model with loci thought to be involved in these human diseases awaits the definition of appropriate genetic markers in the rat.

In addition Han:SPRD-*Pkd* rat is still considered one of the best animal models available for physiological, nutritional and biomedical research of PKD. Its phenotypical similarities with human disease states made it the subject of a number of clinical, experimental and pathophysiological studies. Such studies suggest that different forms of human and experimental renal cysts share common pathways regarding both pathophysiology and clinical expression. Pathophysiological phenomena observed in both human PKD and Han:SPRD-*Pkd* include the involvement of all nephronsegments, the proliferation of cyst lining epithelial cells (Grantham et al. 1987, Kaspareit-Rittinghausen et al. 1991) - associated with increased proto-oncogen expression as was shown in the rat model (Cowley et al. 1993), thickened and multilaminated basement membrane abnormalities in areas of cellular immaturity (Cowley et al. 1993,Wilson et al. 1991) and sex-dependent differences in clinical and histological severity of the disease indicating that other factors can alter the expression of the primary genetic defect in polycystic kidney disease (Cowley et al. 1993). Clinical similarities of human ADPKD and Han:SPRD-*Pkd* include (Kaspareit-Rittinghausen et al. 1991) the mode of inheritance, the slow progression to end stage renal failure in heterozygotes, the presence of proteinuria and hypertension

during the progression of disease, as well as the phenomena associated with renal failure including anaemia, renal osteodystrophy and hypercholesterolaemia.

We conclude that the Han:SPRD-*Pkd* rat is not a genetic model of ADPKD type 1. Nevertheless this rat strain is still considered to be a valuable animal model of human PKD and chronic renal failure.

Acknowledgments

This work was approved by the Erasmus University Animal Care and Ethics Committee. The research was supported by the Dutch Kidney Foundation (Grant 94.1400). The authors thank Professor H Galjaard for continuous support, S van der Flier and E Muris for help in preliminary studies, Dr LAJ Janssen and Dr LA Sandkuijl for statistical advice, Dr F Deerberg (Zentralinstitut für Versuchstierzucht, Hannover) for providing mutant Han:SPRD-*Pkd* rats, Dr Drexhage (Erasmus University, Rotterdam) for providing BB rats and Dr RS Yeung (Fox Chase Cancer Center, Philadelphia) for providing the *Prm1* probe.

References

American PKD1 Consortium (1995) Analysis of the genomic sequence for the autosomal dominant polycystic kidney disease (PKD1) gene predicts the presence of a leucine-rich repeat. Human Molecular Genetics 4, 575-82

Antignac C, Arduy CH, Beckman JS, Benessy F, Gros F, Medhioub M, Hildebrandt F, Dufier JL, Kleinknecht C, **Broyer** M (1993) A gene for familial juvenile nephronophthisis (recessive medullary cystic kidney disease) maps to chromosome 2p. Nature Genetics 3, 342

Avner ED, McAteer JA, Evan AP (1990) Models of cysts and cystic kidneys. In: The cystic kidney (Gardner KD, Bernstein J, eds). Dordrecht: Kluwer, pp55-98

Aziz N (1995) Animal models of polycystic kidney disease. Bioessays 17, 703-12

Cowley BD, Gudapaty S, Kraybill AL, Barash BD, Harding MA, Calvet JP, Gattone VH (1993) Autosomal dominant polycystic kidney disease in the rat. Kidney International 43, 522-34

Cowley BD, Rupp JC (1996) Abnormal expression of epidermal growth factor and sulfated glycoprotein sgp-2 messenger RNA in a rat model of autosomal dominant polycystic kidney disease. Journal of the American Society of Nephrology 6, 1679-81

Daoust MC, Reynolds DM, Bichet DG, Somlo S (1995) Evidence for a third genetic locus for autosomal polycystic kidney disease. Genomics 25, 733-6

European Polycystic Kidney Disease Consortium (1994) The Polycystic Kidney Disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. Cell 77, 881-94

Gabow PA, Johnson AM Kaehny WD, Kimberling WJ, Lezotte DC, Duley IT, Jones RH (1992) Factors affecting the progression of renal disease in autosomal dominant polycystic kidney disease. Kidney International 41, 1311-9

Grantham JJ, Geiser JL, Evan AP (1987) Cystformation and growth in autosomal dominant polycystic kidney disease. Kidney International 31, 1145-52

Gretz N, Hocker A Baur S, Lasserre JJ, Bachmann S, Waldherr R, Strauch M (1992) Rat models of polycystic kidney disease. In: Contributions in nephrology 97. Polycystic kidney disease (Breuning MH, Devoto M, Romeo G, eds). Basel: Karger, pp 35-46

Gretz N (1994) Accelerated renal death after unilateral nephrectomy in a rat strain suffering from autosomal dominant polycystic kidney disease. Journal of the American Society of Nephrology 4, 1925-26

Guay-Woodford LM, Bryda EC, Brian C, Lindsey JR, Collier WR, Avner ED, D'Eustachio P, Flaherty L (1996) Evidence that two phenotypically distinct mouse PKD mutations, *bpk* and *jcpk*, are allelic. Kidney International 50, 1158-1165

Hughes J, Ward CJ, Peral B, Aspinwall R, Clark K, San Millán JL, Gamble V, Harris PC (1995) The polycystic kidney disease1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. Nature Genetics 10, 151-60

International Polycystic Kidney Disease Consortium (1995) Polycystic Kidney Disease: The complete structure of the PKD1 gene and its protein. Cell 81, 289-98

Kaspareit-Rittinghausen J, Deerberg F, Wcislo A (1991) Hereditary polycystic kidney disease: Adult polycystic kidney disease associated with renal hypertension, renal osteodystrophy, and uremic enteritis in SPRD rats. American Journal of Pathology 139, 693-6

Kimberling JW, Kumar S, Gabow PA, Kenyon JB, Connolly CJ, Somlo S (1993) Autosomal dominant polycystic kidney disease: localization of the second gene to chromosome 4q 13-q23. Genomics 18, 467-72

Kobayashi T, Hirayama Y Kobayashi E, Kubo Y, Hino O (1995) A germline insertion in the tuberous sclerosis (Tsc2) gene gives rise to the Eker rat model of dominantly inherited cancer. Nature Genetics 9, 70-74

Laird PW, Zijderveld A, Linders K, Rudnicky MA, Jaenisch R, Berns A (1991) Simplified mammalian DNA isolation procedure. Nucleic Acids Research 19, 4293

Lathrop GM, Lalouel JM, Julier C, Ott J (1984) Strategies for multilocus linkage analysis in humans. Proceedings of National Academy of Science USA 81, 3443-3446

Nauta J, Ozawa Y, Sweeney Jr WE, Rutledge JC, Avner ED (1993) Renal and biliary abnormalities in a new murine model of autosomal recessive polycystic kidney disease. Pediatric Nephrology 7, 163-72

Olsson PG, Löhning C, Horsley S, Kearney L, Harris PC, Frischauf AM (1996) The mouse homologue of the polycystic kidney disease gene (pkd1) is a single-copy gene. Genomics 34, 233-5

Peters DJM, Spruit L, Saris JJ, Ravine D, Sandkuijl LA, Fossdal, R, Boerma J, van Eijk R, Norby S, Constantinou-Deltas CD, Pierides A, Brissenden, JE, Frants RR, van Ommen, JB, Breuning MH (1993) Chromosome 4 localization of a second gene for autosomal dominant polycystic kidney disease. Nature Genetics 5, 359-62

Reeders ST, Breuning MH, Davies,K.E., Nicholls,R.D., Jarman,A.P., Higgs,D.R., Pearson,P.L., and Weatherall,D.J. (1985) A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. Nature 317, 542-4

Reeders ST, Hildebrand CE, Sutherland GR (1990) Report of the committee on the genetic constitution of chromosome 16. Cytogenet Cell Genet 55, 194-7

Schäfer K, Gretz N, Bader M, Oberbäumer I, Echardt K, Kriz W, Bachmann S (1994) Characterisation of the Han:SPRD rat model for heriditary polycystic kidney disease. Kidney International 46, 134-52

Torres VE, Mujwid DK, Wilson DM, Holley KH (1994) Renal cystic disease and ammoniagenesis in Han:SPRD rats. Journal of the American Society of Nephrology 5, 1193-1200

Welling LW, Grantham JJ (1996) Cystic and developmental diseases of the kidney. In: The Kidney 5th ed. (Brenner B, ed) Philadelphia: WB Saunders, pp1828-63

Wilson PD, Sherwood AC (1991) Tubulocystic epithelium. Kidney International 39, 450-63

Yeung RS, Buetow KH, Testa JR, Knudson Jr AG (1993) Susceptibility to renal carcinoma in the Eker rat involves a tumor suppressor gene on chromosome 10. Proceedings of the National Academy of Science, USA 90, 8038-42

Yeung RS, Xiao GH, Jin F, Lee W-C, Testa JR, Knudson AG (1994) Predisposition to renal carcinoma in the Eker rat is determined by a germline mutation of the tuberous sclerosis (Tsc2) gene. Proceedings of the National Academy of Science, USA 91, 11413-16

Zeier M, Pohlmeyer G, Deerberg F, Schönherr R, Ritz E (1994) Progression of renal failure in the Han: SPRD polycystic kidney rat. Nephrology Dialysis and Transplantation 9, 1734-39
Zerres K, Mücher G, Bachner L, Deschennes G, Eggerman T, Kaariainen H, Knapp M, Lennart T, Misselwitz J, van Muhlendahl KE, Neumann HPH, Pirson Y, Rudnik-Sconeborn S, Steinbicker V, Wirth B, Scharer K (1994) Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. Nature Genetics 7, 429-32

2.4

A new rat model that phenotypically resembles ARPKD

Jeroen Nauta, MD[1], Miriam A Goedbloed[1], Harry van Herck, PhD[4], Dennis A Hesselink, MD[1], Pim Visser[3], Richard PE van Dokkum, PhD[2], Christopher J Wright[5], Lisa M Guay-Woodford, MD[5,6,7].

Departments of [1]Pediatrics, [2]Pediatric Surgery and [3]Cell Biology, Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands, [4]Central Laboratory Animal Institute (GDL), Utrecht University, Utrecht, The Netherlands and Departments of [5]Cell Biology, [6]Pediatrics and [7]Medicine, University of Alabama at Birmingham, Birmingham, AL, USA.

A new rat model that phenotypically resembles autosomal recessive polycystic kidney disease.

Abstract

Numerous murine models of polycystic kidney disease (PKD) have been described. While mouse models are particularly well-suited for investigating the molecular pathogenesis of PKD, the rat is well-established as an experimental model of renal physiology. The Han:SPRD *cy* rat has been proposed as a model for human autosomal dominant PKD. We have now identified a new spontaneous rat mutation, designated *wpk*. The mutant phenotype resembles human autosomal recessive PKD (ARPKD). The current study was designed to characterize the clinical and histopathologic features of *wpk/wpk* mutants and to map the *wpk* locus. Homozygous mutants developed nephromegaly, hypertension, proteinuria, impaired urinary concentrating capacity, and uremia, resulting in death at 4 weeks of age. Early cysts were present in the nephrogenic zone, at embryonic day 19. These were localized by specific staining and electron microscopy to differentiated proximal tubules, thick limbs, distal tubules and collecting ducts. In later stages the cysts were largely confined to collecting ducts. While the renal histopathology is strikingly similar to human ARPKD, *wpk/wpk* mutants had no evidence of biliary tract abnormalities. The *wpk* locus maps just proximal to the *cy* locus on rat Chromosome 5, and complementation studies have demonstrated these loci are not allelic.
We conclude that the clinical and histopathologic phenotype of this new rat model strongly resembles human ARPKD. While homology mapping indicates that rat *wpk* and human ARPKD involve distinct genes, this new rat mutation provides an excellent experimental model to study the molecular pathogenesis as well as the renal pathophysiology of recessive PKD.

Introduction

Renal cystogenesis occurs with a number of inherited, developmental, and acquired diseases (1). A common feature among this otherwise heterogeneous set of disorders is the development of epithelial-lined cysts arising from various nephron segments and the collecting ducts.

Of these conditions, the inherited renal cystic diseases have been most extensively investigated. These disorders, which are transmitted as single Mendelian traits, include: 1). autosomal dominant polycystic kidney disease (ADPKD), 2). autosomal recessive polycystic kidney disease (ARPKD), 3). juvenile nephronophthisis-medullary cystic

disease complex (JN-MCD), 4). tuberous sclerosis complex (TSC), 5). von Hippel-Lindau (VHL) disease, and 6). several multiple malformation syndromes (reviewed in (2,3)). These disorders cause significant morbidity and mortality in both adults and children. For example, ADPKD causes 6-8% of adult end stage renal disease (ESRD) (2). In ARPKD, 30-50% of affected neonates die in the perinatal period. Surviving ARPKD patients, coupled with children who have JN, comprise 6-14% of all pediatric ESRD patients (4,5).

Recent genetic studies have identified the principal genes involved in ADPKD (*PKD1* and *PKD2*), JN (*NPHP1*), TSC (*TSC1* and *TSC2*) and VHL (*VHL*) (6-15). In addition, linkage studies have defined the predominant, if not exclusive locus for ARPKD (*PKHD1*), as well as distinct loci for several of the malformation syndromes (16-18). While many of these disorders are inherited as dominant traits, multiple lines of evidence suggest that a second, somatic mutation in these disorders is necessary for disease expression (19-27). Thus, like ARPKD and JN, ADPKD, TSC, and VHL are thought to involve recessive mechanisms at the cellular level. In addition, recent evidence indicates that some of these genes interact, either directly at the protein level or in the pathogenesis of cystic disease (28-30). Together, these data suggest that inherited human cystic disease is initiated by cellular recessive mechanisms and common molecular pathways may be involved in different cystic diseases. However, the molecular pathogenic events involved in the initiation and progression of renal cystic diseases remain largely unknown.

In addition to these human renal cystic disease genes, numerous murine (mouse and rat) models of polycystic kidney disease (PKD) have been described. Most models involve disruption of a single gene and the mutant phenotypes closely resemble human PKD with regard to morphology, cyst localization and disease progression. Several models are the result of spontaneous mutations (31-42), while others were engineered either through chemical mutagenesis (43) or transgenic technologies (44-59). In addition, experimental models of PKD have been induced by chemical cystogens, primarily in rats (reviewed in (60)).

Investigations of the murine model systems have identified several common pathogenic features, that are common to both murine PKD models and human disease. These include: (1) dysregulated epithelial cell proliferation and differentiation; (2) alterations of tubular basement membrane constituents and the associated extracellular matrix; (3) abnormalities of epithelial cell polarity with apical mislocalization of key receptors and enzymes; and (4) abnormalities in transepithelial fluid transport.

In addition, gender and genetic background also appear to modify the disease course in both murine and human PKD (2,61-63). Recent investigations suggest that a modifying gene, *Jckm2*, on distal chromosome 1 may influence the disease phenotype of the mouse *jck*, *bpk*, and *kat* models (64-66) while a locus on proximal chromosome 4 appears to modify the disease severity in the *cpk*, *pcy*, and *bpk* models (65,67,68).

Therefore, a multigenic pathway appears to be involved in renal cystogenesis in both human PKD and murine models. In both human disease and these animal models, the disease-susceptibility genes appear to act by cellular recessive mechanisms. While the numerous mouse PKD mutations provide powerful models to characterize the genetic factors that regulate renal cyst initiation and disease progression, these models have limited utility with regard to renal physiologic investigations. In contrast, the rat is a well-established model system for investigating renal physiologic parameters such as renal blood flow, glomerular filtration rate, renal tubular transport and blood pressure regulation. Among the genetically-determined and chemically-induced rat PKD models, only the Han SPRD: *cy* rat has been well characterized (62). This rat mutant has been proposed as a model of human ADPKD.

In this report, we describe a new rat PKD model. Affected homozygotes develop rapidly progressive PKD that clinically and histologically resembles human ARPKD. This mutation occurred spontaneously in an outbred Wistar strain. We therefore have designated the mutant locus, *wpk* (*w*istar *p*olycystic *k*idneys). We have localized *wpk* to Chromosome 5. This locus is distinct from the rat Han SPRD: *cy* locus, and its mouse and human orthologs are not allelic with any previously described mouse PKD model or human PKD gene. However, based on its phenotypic similarities with human ARPKD, the *wpk* rat provides a new experimental model for investigating the pathogenesis of recessive PKD. Given that renal physiology has been well-studied in the rat, the *wpk* rat should provide the first model system to evaluate the abnormalities in renal tubular transport and systemic blood pressure regulation associated with recessive PKD.

Materials and Methods

Rats

The *wpk* mutation was first recognized in 1994 in a colony of outbred Wistar rats, U:WU (Cpb), at Utrecht University, The Netherlands. This breeding colony was initiated in 1989 from 10 ancestral breeding pairs obtained from the 'National Institute of Public Health and the environment (RIVM)", Bilthoven, The Netherlands. No new rats were introduced to the colony from 1989 to the present. In 1996, a breeding pair of test-proven heterozygotes were transferred to the University of Rotterdam, The Netherlands, and a new subcolony was initiated. This colony has been maintained by brother x sister matings for over 5 generations. Individuals heterozygous for the mutant allele were identified in each generation by test-crossing phenotypically normal offspring from known heterozygotes.

The Han:SPRD-*cy* rat model of PKD (40) was used for complementation experiments and as a phenotypic reference for our evaluation of the homozygous *wpk/wpk* rats. In this model, *+/cy* heterozygotes develop slowly progressive PKD that leads to renal failure in

males only, at ≥ six months of age, whereas *cy/cy* homozygotes develop a severe form of PKD within the first few weeks of life. Heterozygous Han:SPRD *+/cy* breeding pairs were kindly provided in 1994 by F. Deerberg (Central Institute for Laboratory Animal Breeding, Hannover, Germany) and a breeding colony was established at the University of Rotterdam. BB-DP rats, an inbred strain (F30) derived from the biobreeding-diabetic prone rat were kindly provided by H.A. Drexhage (Dept of Immunology, Erasmus University).

To map the *wpk* locus, Wistar-*+/wpk* heterozygotes of both sexes were bred to BB-DP rats, and F1 progeny heterozygous for the *wpk* mutation were intercrossed to generate F2 rats. Affected homozygotes could be identified by abdominal palpation at postnatal day 12.

All animals were fed standard rat chow containing 24% protein and had free access to acidified tap water. All experiments were conducted in accordance with the Dutch guidelines for the care and use of laboratory animals.

Clinical parameters

Kidney weight, body weight, blood pressure, and urine and blood chemistries were assessed in groups of 6-8 rats at the age of 3-4 weeks. Affected *wpk/wpk* rats were compared to phenotypically unaffected littermates. Affected *cy/cy* rats were compared to +/+ littermates. In contrast to *+/wpk* heterozygotes, *+/cy* rats could be identified histologically and these pups were excluded from further analysis.

The weights of the left and right kidneys of *wpk/wpk* homozygotes did not differ. The kidney weights were thus combined and expressed as percentage of body weight. Data from male and female pups were pooled, since gender effects were never observed in prepubertal animals.

Bloodpressure was measured in 6 pairs of 3-4 weeks old rats anesthetized with ketamine and thiopental, using an indwelling catheter in the femoral artery. Blood was obtained by cardiac puncture in six pairs of *wpk/wpk* homozygotes and unaffected littermates. Plasma urea nitrogen, creatinine and total bilirubin were measured in the plasma using Kodak Ektachem 700 spectrophotometry. Urine was collected after 2 hours of fasting in 4 weeks old affected and unaffected wpk rats. Urinary creatinine was measured by the Jaffé method and urinary protein by absorption spectroscopy.

In older animals, urinary protein excretion, blood pressure and blood chemistries were compared between test proven heterozygous *+/wpk* rats and wild-type controls at the age of 1.5 years for females and 1 year for males. Urine specimens were collected over 24 hours using a metabolic cage. Blood pressure was determined by tail plethysmography. The animals were trained for this procedure prior to the measurements (69). Blood was obtained by aortic puncture under ether anesthesia.

Tissue preparation for histology and electronmicroscopy

For histological analysis, kidneys were obtained from *wpk/wpk* and control animals on embryonic day 19 and on postnatal days 0, 7, 14 and 21. The cystic phenotype was established by histology. A multiple-organ autopsy, including heart, lung, liver, pancreas, spleen, brain and intestine, was performed on 21 day old homozygous mutants and on adult heterozygotes. Heterozygous females were studied at the age of 1.5 years and males at the age of 1 year.

For light microscopy and immunohistochemistry, the kidneys and livers were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4 and embedded in paraffin. For liver histology, the left ventral lobe was dissected, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin with the convexity to the surface of the block.

For electron microscopy (EM) representative samples of the kidneys were fixed in 2.5% glutaraldehyde, postfixed in 1% osmiumtetraoxide, and dehydrated in graded alcohol series. For scanning EM (SEM) the samples were then critical point dried, mounted on stubs, coated with gold/paladium and examined in a JEOL JSM 25 scanning electron microscope. For transmission EM (TEM) the fixed and dehydrated samples were embedded in epon. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips C100 electron microscope.

Human kidney from a neonate with ARPKD and end stage renal failure was used as a comparative reference for the SEM analysis of *wpk/wpk* kidneys.

Evaluation of the biliary ductal plates

In addition to the renal cystic lesion, the intrahepatic morphology was evaluated in six affected *wpk/wpk* homozygotes and six phenotypically unaffected animals, aged 21 days. The depth and angle of liver sections was standardized for histological and morphometric analysis as previously described (35). Bile ductules and recognizable portal areas were systematically examined in each section by one blinded observer. A recognizable portal area was defined as a venule accompanied by one or more bile ductules.

Immunohistochemistry

The localization of the renal cysts was evaluated by immunodetection of nephron segment-specific proteins and lectin binding sites at postnatal days 0, 7, 14 and 21. Based on preliminary studies, the following reagents were selected. As a rat proximal tubular marker, we used polyclonal goat anti-rat dipeptidyl peptidase 4 (dpp4) (kindly provided by E. de Heer, Leiden) (70) at dilutions of 1:500. As a marker of the thin limb of Henle's loop (medulla) and proximal tubules (cortex) we used polyclonal rabbit anti-rat aquaporin 1 (Aqp 1) at 1:100 (generous gift of P. Deen, University of Nijmegen, The Netherlands). As a marker of the thick ascending limb we used polyclonal rabbit anti-human Tamm Horsfall

glycoprotein (TH) at 1:200 (Biomedical Technologies, Stoughton, MA, USA). As a marker of the cortical and medullary collecting tubules we used polyclonal rabbit anti rat aquaporin 2 (Aqp2) at 1:100 (generous gift of P. Deen, University of Nijmegen, The Netherlands). All antibodies were diluted in PBS, 0.5% dried milk, 0.15% glycine.

Schäfer et al. have reported increased expression of collagen type IV and laminin in the renal cysts of 2 month old Han:SPRD *+/cy* rats (40). Therefore, for comparative purposes, kidney sections from 21 days old Wistar *wpk/wpk* rats, 21 day old Han:SPRD *cy/cy* rats and 2 month old *+/cy* rats, were analyzed with Jones' silver stain as well as with a polyclonal rabbit anti-mouse collagen IV antibody at 1:200 (Collaborative Medical Products; (71)) and polyclonal rabbit anti-mouse laminin antibody at 1:50 (Eurodiagnostica, Arnhem, The Netherlands; (72)).

The tissue sections were pretreated with pronase 0.1% in PBS for 5-10 minutes prior to the incubations with anti-TH, anti-dpp4 and anti-Col IV and with 0.2% SDS in PBS for 5 minutes prior to the incubations with anti-Aqp2. Anti laminin and anti aqp1 were used without such pretreatment of the tissue sections. Primary rabbit antibodies were detected using swine anti-rabbit peroxidase conjugate at 1:100 (DAKO). Goat anti-dpp4 was detected using rabbit anti-goat peroxidase conjugate at 1:100 (DAKO). The sections were stained with 0.05% diaminobenzidine, 0.01% hydrogen peroxide and counterstained with hematoxylin.

Statistics

Clinical data are expressed as means and standard deviations (SD). Differences between *wpk* and control animals were analysed by *t*-test.

PCR-based genotyping

To type progeny for inheritance of alleles of anonymous DNA microsatellite markers, spleen genomic DNA was prepared according to standard protocols. Initial mapping was performed using interval haplotype analysis (73). For these studies, we selected microsatellite markers whose Wistar and BB-DP alleles differed in size by at least 6 base pairs (bp) and which mapped within ~10 centimorgan (cM) of proximal and distal ends of each chromosome. Once a chromosomal localization was determined, further mapping was performed using markers spaced at 20-cM intervals spaced along Chr 5 (low-resolution linkage mapping study) and then within 4 cM of *D5Rat73* (high-resolution linkage mapping study). All markers were chosen from the on-line Whitehead/MIT database (accessible at http://www-genome.wi.mit.edu/; described by Szpirer et al. (74)). PCR primer pairs for these markers were purchased from Research Genetics, Inc. (Huntsville, AL).

Forward primers were end-labeled with 32P-adenosine triphosphate, and PCR amplification was performed as described by (75). Amplified fragments were analyzed on denaturing 6% polyacrylamide gels.

Analysis of genetic data

Genotype data obtained by analyzing 35 affected F2 progeny for microsatellite markers known to map to the ends of each autosome were subjected to interval haplotype analysis exactly as described by Neuhaus and Beier (73)). To construct low- and high-resolution linkage maps, individual chromosomal haplotypes were inferred from F2 genotypic data as described previously (75) and markers were ordered so as to minimize the numbers of crossover events needed to account for the inferred haplotypes.

Results

Wistar-wpk/wpk phenotype

Our Wistar-+/*wpk* colony breeding has been maintained for over 5 generations using a scheme of brother-sister matings. Heterozygotes had a normal phenotype and bred as productively as wild-type Wistar rats. A typical litter contained 9 to 14 pups. As expected for an autosomal trait, approximately 1:4 offspring of test-proven heterozygotes expressed polycystic kidney disease (PKD) and males and females were affected in equal numbers (23.5% and 24.2%, respectively). Of note, approximately 10% of the F1 pups died within the first 2 weeks of postnatal life of as yet undetermined causes.

Table. 1 Comparative clinical features at 3-4 weeks of age.

	Wistar		Han SPRD: cy	
	wpk/wpk	Unaffected	*cy/cy*	+/+
Body weight (BW)	43.3 ± 6.8	48.2 ± 7.2	34.3 ± 3.5	38.1 ± 5.8
Kidney weight/BWx100	10.9 ± 0.6 *	1.1 ± 0.1	21.8 ±1.4 *	1.0 ± 0.5
Blood pressure (mean)	139 ± 26 *	90 ± 10	ND	ND
Plasma urea	29.3 ± 2.8 *	8.9 ± 0.7	51.2 ± 8.8 *	13.2 ± 1.7
Urinary protein excretion	580 ± 307 *	33 ± 26	ND	ND
Urinary osmol.	394 ± 87 *	961 ± 584	ND	ND

Clinical features of homozygous mutants and unaffected littermates. Body weight (grams), kidney weight (% of body weight (% of BW) and plasma urea (mmol/L) were studied in 3 week old rats; Proteinuria (mg/mmol creatinin), urine osmolality (mosm/kg) and mean blood pressure (mmHg) in 3-4 week old rats. Numbers are given as means ± SD. ND-not done. * $p < 0.01$ vs. controls.

The phenotype in homozygous mutants was characterized by progressive nephromegaly and abdominal distension. Nephromegaly was first palpable at postnatal day 12. By 4 weeks of age, homozygous mutants were runted and had large, palpable kidneys. Affected rats died at 4-6 weeks of age. The total kidney weight to body weight (KW/BW)

ratio at 4 weeks was greater than 10% (Table 1). In comparison, the KW/BW was 1% in unaffected littermates and 20% in Han:SPRD *cy/cy* mutants at the same age. At three weeks of age, *wpk/wpk* homozygotes had elevated plasma urea levels, proteinuria and a low urine osmolality after 2 hours fasting, relative to unaffected littermates. The mean arterial blood pressure assessed by direct measurement in the femoral artery was markedly elevated (Table 1). The weight of the liver relative to kidney free-body weight and the plasma bilirubin levels were not significantly different in homozygous mutants versus unaffected littermates.

Test-proven heterozygotes were clinically unaffected. There was no significant difference between 18-month old female *+/wpk* heterozygotes and age-matched, wild-type controls with respect to body weight (345 ± 24 vs. 344 ± 29 grams); systolic blood pressure (135 ± 9 vs. 128 ± 7 mm Hg); plasma creatinine (41 ± 4 vs. 46 ± 5 μmol/L) and (20 ± 13 vs. 27 ± 18 mg/day/100 grams bodyweight). Similarly, one-year old males *+/wpk* heterozygotes did not differ from age-matched controls with respect to BW (547 ± 31 vs. 517 ± 25 grams); systolic blood pressure (130 ± 7 vs. 127 ± 8 mm Hg); plasma creatinine (47 ± 5 vs. 49 ± 7 μmol/L) and urinary protein excretion rate (40 ± 26 vs. 38 ± 21 mg/day/100 grams bodyweight).

Pathology of wpk/wpk kidneys

At a gross level, neonatal *wpk/wpk* kidneys had a normal architecture including normal lobulation, medullary rays, and a well defined corticomedullary demarcation. In affected pups, the kidneys progressively enlarged but maintained a reniform shape, despite the progressive cystic changes in the parenchyma. The capsular surface was smooth. The renal pelvis and calices were not enlarged and maintained a normal relation to the parenchyma (fig.1). The ureters were present and non-dilated. This pattern is very similar to that observed in the kidneys of human ARPKD neonates.

The histopathology was characterized by progressive cystic dilatation of renal tubules. Early cysts were noted at 19 days of embryonic development (Fig. $2^{A,B}$). At this stage, as well as in neonatal kidneys, the lesions were predominantly localized to the renal cortex. The cysts were round or oval-shaped and lined by either a single layer of cuboidal cells or flattened epithelia (Fig. 2^{C}). Some of the cysts were lined by a brush-border bearing cell type, suggestive of proximal tubular epithelia. The cell density of the epithelial lining is high in most, but not in all cysts. Glomerular cysts were not observed.

With subsequent disease progression, cysts developed throughout the entire kidney (fig. 2^{D}) with cortical cysts arrayed in a radial orientation, while medullary cysts were generally round in shape and of variable size. In kidneys with advanced cystic disease, there was an apparent reduction in the number of glomeruli and non-dilated tubules and those remaining appeared to be compressed between innumerable tubular cysts. Interstitial

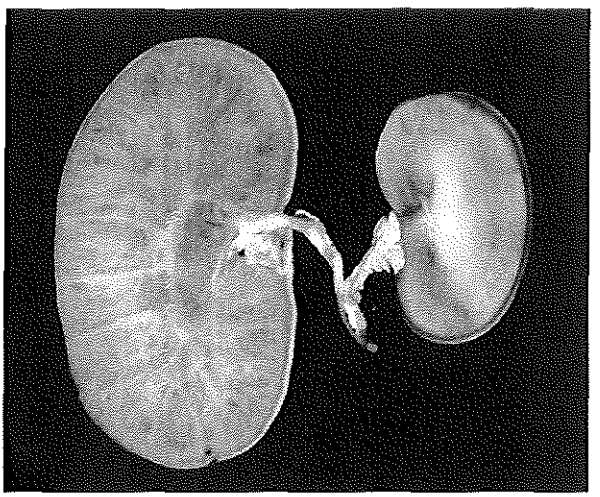


Fig. 1. Normal and cystic kidneys at 3 weeks of age (x 2.3). The affected kidney has a well maintained reniform shape, a smooth capsular surface and radially oriented cysts. The renal pelvis and ureters are undilated.

fibrosis was not observed and the renal vasculature was unremarkable. In contrast to the early cystic kidneys, the cysts in more advanced disease were lined with a relatively homogenous cell type. Within individual cysts the cell density varied considerably. Interestingly, areas of high cell density were often juxtaposed to similar areas in neighbouring cysts (Fig. 2^{E}). This observation suggests that focal epithelial cell proliferation within cysts may be determined by the local biological environment. However, true epithelial hyperplasia with associated polyps or microadenomas, as noted in human ADPKD (76), in *cy/cy* rats (40), and in the c-myc overexpression model of PKD (77), was never observed.

Immunohistochemistry

Cysts in neonatal kidneys stained either anti-dpp4, anti Tamm Horsfall protein, or (from day 1 onwards) anti-Aqp2 antibody, indicating cellular characteristics typical for proximal tubules, thick ascending limb or collecting tubules, respectively. Staining of medullary cysts with anti-Aqp1 indicating a thin loop-derived cell type was rarely seen. Cortical cysts occasionally stained with anti-Aqp1 in a weak pattern. These cysts were lined by a brushborder baring cell type and also stained with anti-dpp4, indicative of proximal tubules. Each individual cyst was typically lined with only one cell type and overlapping staining patterns, other then Aqp1 in proximal tubules, were not observed. By two weeks of age, the vast majority of cysts appeared to derive from collecting ducts, as the majority

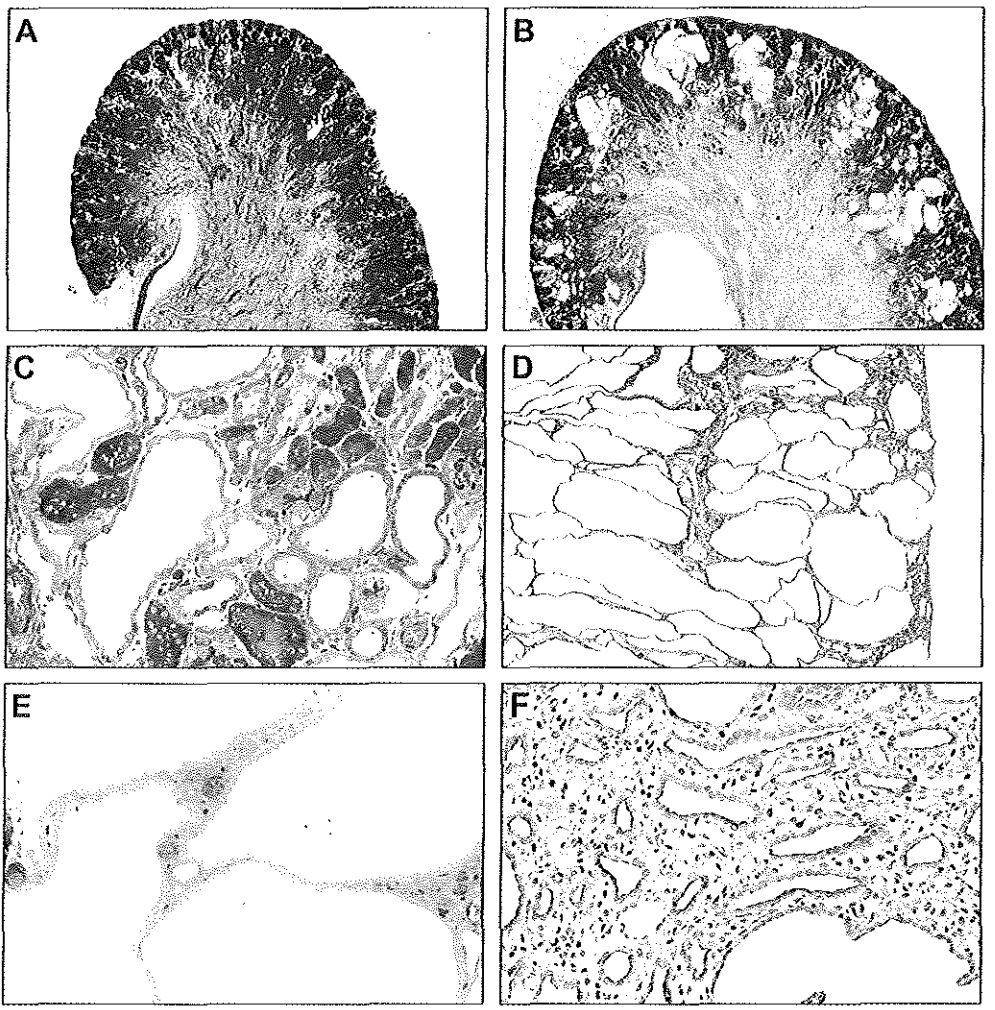


Fig. 2. Lightmicroscopy of kidneys at different age. (A,B): Normal and cystic kidney at embryonic day 19. Clusters of cysts in the cortex suggest that adjacent cysts are dilations of tubular loops of the same nephron. Medullary cysts are markedly smaller (Hematoxilin x 30). (C): Neonatal kidney with cortical cysts lined by various types of epithelium (Methylene blue x 120 (D): Cortical area of an end stage cystic kidney exhibiting large epithelial cysts. Glomerular cysts were not observed. The few glomeruli and undilated tubules appear to be compressed between innumerable cysts (Hematoxilin x 30) (E) . The cell density varied considerably within individual cysts. Areas of high cell density were often juxtaposed to similar areas in neighbouring cysts (methylene blue x 80). (F). Aquaporin-2 staining of the vast majority of cysts in a 3 weeks old cystic kidney (Diaminobenzidin, Hematoxilin x 100).

of cysts stained with anti-Aqp2. At three weeks of age, over 90% of the cysts expressed aquaporin 2 (Fig. 2^{F}). In comparison, renal cysts in 3-week old *cy/cy* homozygotes appeared to arise from all nephron segments (data not shown).

In *wpk/wpk* homozygotes, the renal tubular basement membrane (TBM) morphology, as assessed by Jones' silver staining, was not significantly different between cystic tubules and normal tubules in either early or advanced disease. Moreover, there was no difference in the expression of the TBM constituents, collagen type IV and laminin, when cystic and non-cystic tubules were compared. In contrast, collagen type IV and laminin were overexpressed in the TBM associated with some, but not all renal tubular cysts in SRPD-*+/cy* and SRPD-*cy/cy* kidneys (not shown; (40)).

Electron microscopy

Scanning electron microscopy was consistent with the light microscopic observation that early and late cysts have different epithelial linings (Fig. 3). The cell surface characteristics in early cystic tubules varied considerably between individual cysts. Some had a homogeneous brush border, charactistic of proximal tubules (fig. 3^{A}). Others had more distal tubule or collecting duct characteristics, including less microvilli and a more smooth apical surface. Some had the typical characterisics of collecting ducts expressing a prominent single cilium, as illustrated for 3 weeks old tissue (fig. 3^{E}).

Cysts in kidneys with advanced disease were radially oriented. In contrast to early cysts, these cysts were uniformly lined by a homogenous cobblestone cell-type, indicative of collecting duct origin (fig. 3^{B-D}). These cells had the typical phenotype of principal cells, including a relatively smooth apical surface and a well-differentiated, conspicious single cilium (fig. 3^{E}). These cilia were prominently evident in the vast majority of cortical cysts and less prominent or absent in medullary cysts. In addition, the epithelial lining of most cysts contained a small number of rough-surfaced cells with characteristics previously described for rat type B intercalated cells (fig. 3^{F}) (78). This mixed pattern of principal and intercalated cells is characteristic of cortical and outer medullary collecting ducts in the rat and is very similar to the pattern observed in the vast majority of the cysts in our human ARPKD reference sample (fig. $3^{G,H}$) (78).

Transmission electron microscopy was consistent with both the light microscopic and SEM findings. Early renal cysts in *wpk/wpk* rats were lined by well-differentiated cells with the phenotypic characteristics of either proximal tubules, thick ascending limbs, distal tubules, or collecting ducts (Fig. 4). Microvilli were well-formed, cell junctions and basal lamina were intact, and the cell organelles were normally distributed. At 3 weeks of age, the vast majority of cortical as well as medullary cysts were lined by a homogeneous layer of cells that had the characteristic phenotype of collecting duct cells (79). Abnormalities evident in human ADPKD and in the Han:SPRD *cy/cy* model, e.g. basement membrane thickening, intercellular vacuoles, or intratubular micropolyps were not observed (76). The basal laminae were intact with normal architecture and thickness.

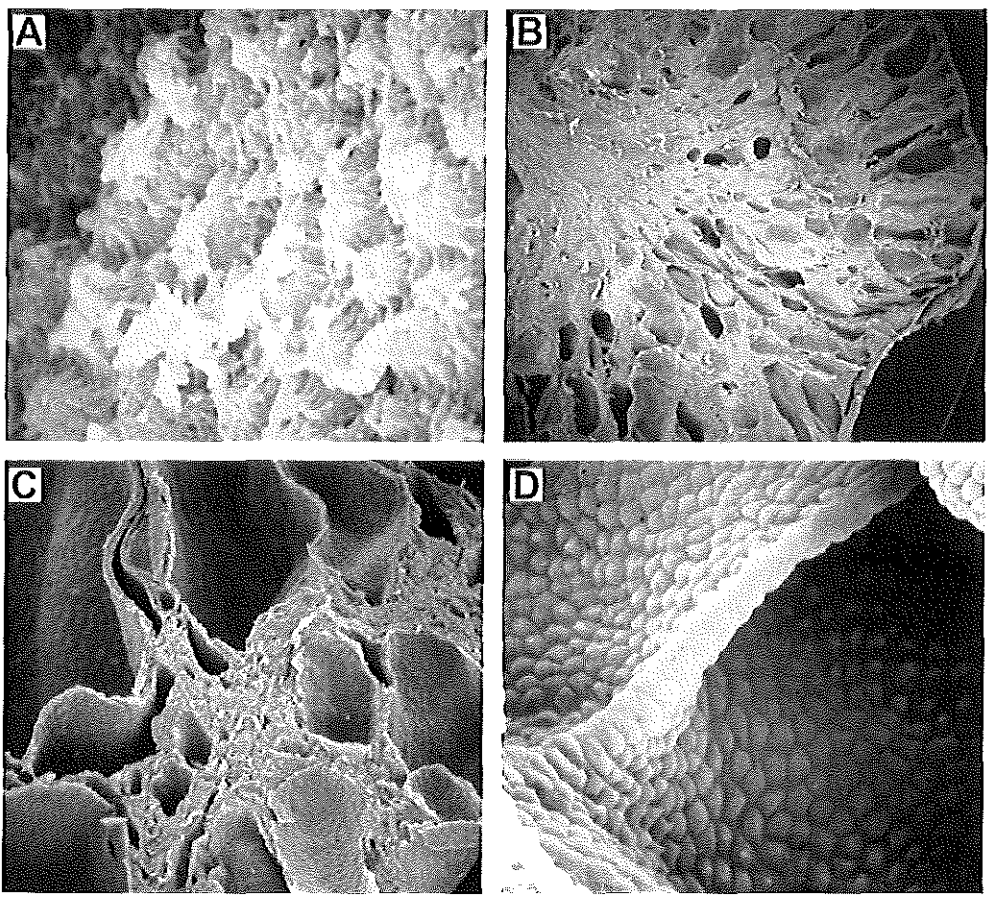


Fig. 3. Scanning electron microscopy (SEM) of renal cysts on neonatal (A) and 3 weeks old (B-F) affected wpk/wpk rats and of an end stage human ARPKD kidney (G-H). (**A**): A neonatal wpk/wpk cyst lined by a brushborder baring celltype, suggestive of a proximal tubular celltype. (**B**): A 3 weeks old wpk/wpk kidney. At low magnification radially alligned fusiform cysts can be observed in the cortex and more rounded cysts in the medulla. (**C**): At this stage the cysts are densily packed and the epithelia of adjacent cysts lay often juxtaposed, with little interstitial tissue in between. Normal renal structures can hardly been identified. To be continued on next page.

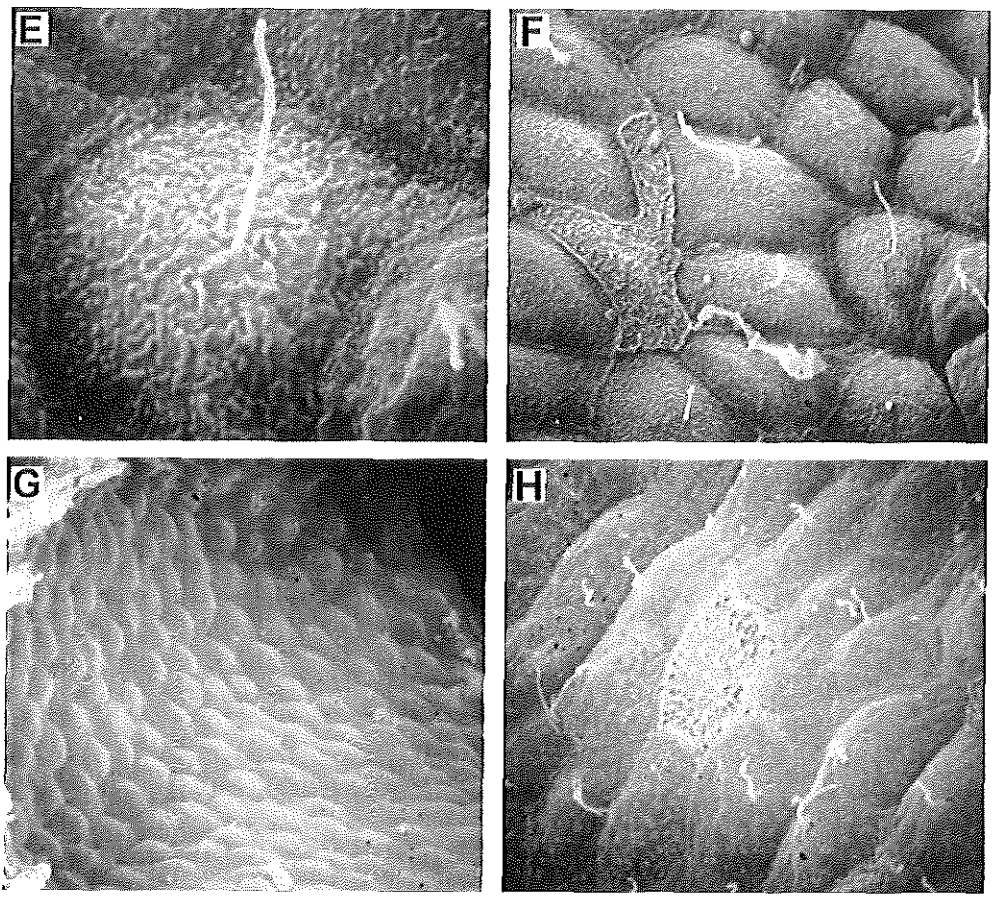


Fig. 3. (Continued from previous page) **(D)**: The vast majority of cysts are lined by a homogeneous cobblestone-like cell pattern. **(E)**: Most cells have the typical characteristics of principle cells including a relatively smooth, rounded surface and a single cilium. **(F)**: Other cells have the characteristics of intercalated cells, including a relatively rough and angular surface and no cilium. The microprojections are moderate in both size and number, as described for type B intercalated cells (78) **(G)**: Human ARPKD cyst typically lined by a homogenous cobblestone-like cell pattern and **(H)** a mix of principle and intercalated cells. Magnification A x 6.500, B x 10, C x 100, D x 450, E x 6.500, F x 2.000, G x 650, H x 2.000.

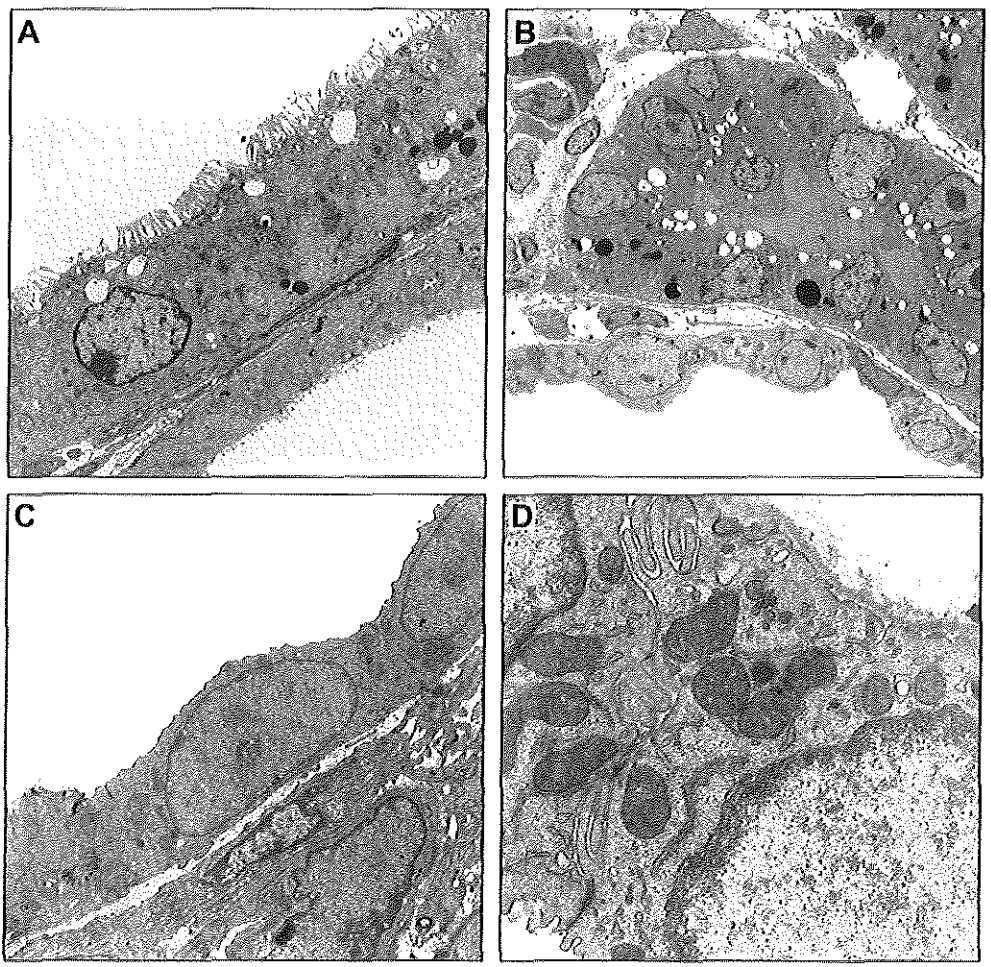


Fig. 4. Transmission electron microscopy of renal lesions in neonatal (A,B) and 3 week old (C,D) wpk/wpk rats. A: Neonatal cyst lined by proximal tubular epithelium. The cells are characterized by a well differentiated brushborder, an apical endosomal-lysosomal apparatus, a dense network of microtubules, normal intracellular organelles including peroxisomes and many mitochondria which are short and randomly distributed. The basal lamina are regular and intact (x 4.000). B: Cortical cyst lined by collecting tubular cells featuring a relatively smooth cell surface, apical tubulovesicular profiles and a regular basement membrane of normal thickness (x 3.000). C: Cyst-lining cells of a 3 weeks old affected rat. Characteristics of principle cells include a fairly smooth apical surface, few and short microvilli, basolateral folds, a tight cytoplasmic network of tubulovesicular profiles below the apical membrane and sparse mitochondria. The basal lamina are intact and regular (x 5.000). D: The intercellular junctions of these cells are relatively deep and tight and the cells have narrow folds of cytoplasm that interlock with counterparts of adjacent cells (x 25.000).

Nonrenal tissues

Multi-organ autopsy of 3-week old Wistar-*wpk/wpk* pups as well as of adult Wistar-+/*wpk* heterozygotes did not reveal any non-renal structural abnormalities. Of particular note, no evidence ductal plate malformation or biliary cysts was evident by light microscopy in multiple liver sections from six 3-week old *wpk/wpk* rats. Morphometric analysis of standardized liver sections revealed no evidence of portal triad abnormalities or bile ductule proliferation in *wpk/wpk* homozygotes as compared to unaffected controls (Table 2). Similarly pancreatic tissue had normal ducts and normal endocrine and exocrine structures in both mutant animals and controls (data not shown).

Table 2. Intrahepatic biliary profiles

	Wistar *wpk / wpk*	Wistar + / +
Portal area's / 100 veins	16.6 ± 2.7	14.9 ± 2.2
Bile ductules / portal area	1.27 ± 0.13	1.19 ± 0.07

Intrahepatic biliary profiles in 3 week old *wpk/wpk* rats. The development of the biliary system is expressed as the percentage of recognizable portal areas relative to the total number of vein profiles. Bile ductules per portal area is a measure of biliary proliferation. Numbers are given as means ± SD. The differences are statistically not significant.

(Wistar-+/wpk X BB-DP)F1 wpk/+ intercross

In our (Wistar-*wpk*/+ X BB-DP)F1 intercross, F1 +/*wpk* hybrids were identified by progeny testing. Evaluation of aged F1 male and female rats revealed no manifestations of renal cystic disease and both F1 males and females bred in a robust fashion.

Of the 225 F2 progeny generated to date, 55 (24.4%) have recessive PKD. The number of F2 *wpk/wpk* pups is consistent with that expected for the Mendelian inheritance of a single recessive trait. Detailed histologic analysis of F2 mutants was not performed.

Genetic mapping of wpk

To test whether *wpk* is allelic with the *cy* locus on rat Chr 5, we crossed test-proven Wistar-+/*wpk* and SPRD-+/*cy* heterozygotes. None of the 35 F1 pups manifest the severe, early-onset phenotype evident in either *wpk/wpk* or *cy/cy* rats. As expected, 17 of the 35 (48.5%), expressed a phenotype consistent with that described in SPRD-+/*cy* heterozygotes. These data exclude allelism between the *wpk* and *cy* loci.

We then performed a whole genome scan using interval haplotype analysis as described by Neuhaus and Beier (73). In effect, by typing markers at the ends of each chromosome, we generated a series of 20 chromosomal intervals for the 35 affected F2 progeny of the (Wistar-+/*wpk* X BB-DP)F1 intercross.

Among the progeny of an intercross, a proportion of the F2 pups will inherit chromosomes that are apparently non-recombinant (NR), that is, the alleles of markers along these chromosomes will correspond to a single parental strain, in this case either

Wistar or BB-DP. In the analysis of a recessive mutation like *wpk*, a rapid genome scan can be performed by analyzing each chromosome for the distribution of NR intervals. The fewer the number of NR chromosomes which correspond to the unaffected parental strain (BB-DP), the less likely it is that the loci along that chromosome are randomly distributed. Accordingly, this chromosome interval is more likely to carry the mutation.

The distribution of chromosomal intervals is evaluated by Chi-square analysis. The maximum inferred Chi-square is calculated for each chromosomal interval and expressed as a percentage of the maximum possible Chi-square (%max Chi-square). Previous modeling experiments have established a % max Chi-square value > 0.75 as the threshold for linkage (73). Therefore, this strategy provides an efficient method to identify a candidate chromosome for more detailed analysis using standard recombinational mapping techniques.

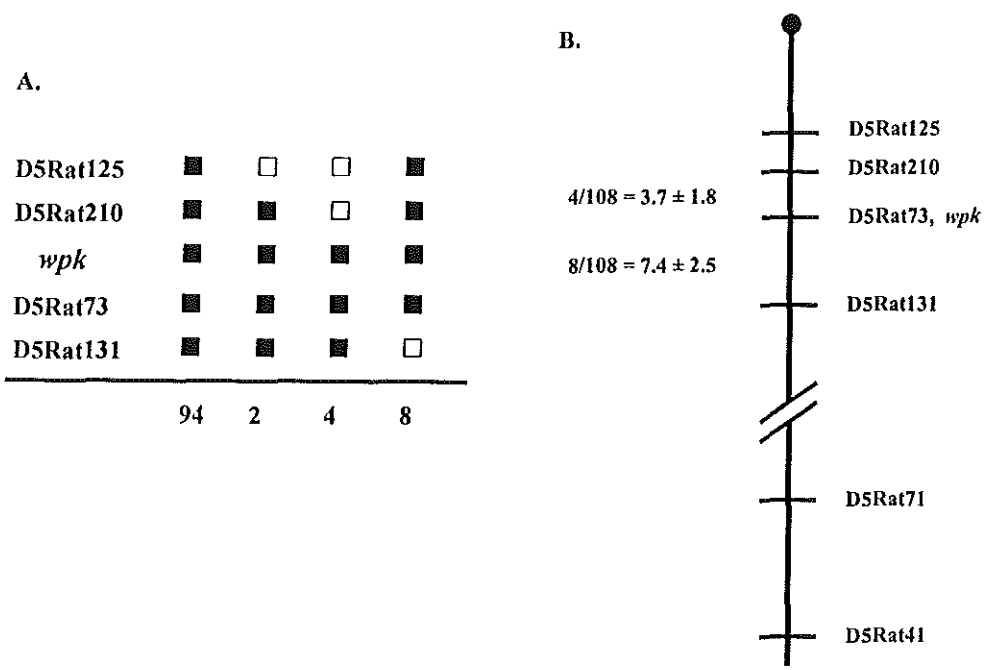


Fig.5 Genetic localization of the *wpk* locus on Chromosome 5. (**A**) Haplotype distribution among 108 test chromosomes from the (Wistar- +/*wpk* x BB-DP) F1 intercross. For each locus, black boxes represent the inheritance of Wistar-derived alleles and white boxes represent the inheritance of BB-DP-derived alleles. The number of chromosomes for each haplotype is shown below the columns. (**B**) The markers, *D5Rat125* and *D5Rat41*, used in the initial interval haplotype are indicated. The map distances, in centimorgans (cM), were calculated from the recombination frequencies observed for each interval and are expressed with the standard errors.

Table 3. Haplotype analysis

		Single point analysis		Haplotype analysis			
Chr.	Markers	n	Chi-sq	Max P Chi-sq	exp M Chi-sq	% max Chi-sq	Nr of H-types
1	D1Rat8-D1Rat84	35	4.8-0.5	70	14.6	20	19
2	D2Rat1-D2Rat103	35	3.9-0.5	70	27.6	39	13
3	D3Rat3-D3Rat7	35	2.3-6.9	70	44.8	64	7
4	D4Rat149-D4Rat67	34	0.3-1.2	68	28.4	41	12
5	D5Rat125-D5Rat41	34	66-1.4	68	60.2	88	2
6	D6Rat105-D6Rat1	34	3.8-2.2	68	28.4	41	12
7	D7mit20-D7Rat115	32	1.5-2.1	64	30.2	47	10
8	D8Rat56-D8Rat12	35	4.7-2.8	68	46.1	67	6
9	D9Rat43-D9Rat1	35	2.8-0	70	22.8	32	15
10	D10Rat2-D10Rat47	35	2.8-0	70	22.8	32	15
11	D11Rat52-D11Rat37	29	7.6-11.6	58	39.7	68	5
12	D12Rat2-D12Rat22	34	3.3-0.7	68	23.5	34	14
13	D13Rat4-D13Rat157	35	4.8-8	70	30.2	43	12
14	D14Rat5-D14Rat51	35	1.4-2.3	70	22.8	32	15
15	D15Rat5-D15Rat29	35	2.3-1.9	70	22.8	32	15
16	D16Rat16-D16Rat21	35	8.3-1.6	70	35.7	51	10
17	D17Rat6-D17Rat51	35	1.6-0.2	70	38.6	55	9
18	D18Rat29-D18Rat13	35	1.6-1.2	70	30.2	43	12
19	D19Rat28-D19Rat2	34	4.7-1.4	68	46.1	67	6
20	D20Rat21-D20Rat29	35	1.6-3.5	70	20.6	29	16

Table 3: Haplotype analysis. A whole genome scan was performed with 20 chromosomal intervals flanked by a proximal and distal marker, e.g. *D1Rat8* and *D1Rat84*, respectively. N is the number of animals in the dataset. Chi-square values for each marker are indicated. Max P Chi-sq is the maximum possible Chi-square for the indicated n; exp M Chi-sq is the maximum inferred experimental Chi-square; %max Chi-sq is the percentage of the maximum possible Chi-square; NR haplotypes is the number of non-recombinant haplotypes inherited from the unaffected parental strain.

Analysis of our dataset revealed a %max Chi-square value = 88% for Chr 5 and % max Chi-square value < 75% for all other chromosome intervals (Table 3). These data provided presumptive evidence for linkage to Chr 5. We typed the initial cohort of 35 affected F2 pups with a series of anonymous DNA microsatellite markers spaced at ~20 cM intervals along this chromosome. These pups and an additional 19 F2 pups (total 54 pups/108 meioses) were typed with markers within 4 cM of *D5Rat73*. These data, summarized in Figure 5, position *wpk* within a 11.1 cM interval centered on *D5Rat73*.

Discussion

Similarities between the rat wpk/wpk phenotype and human ARPKD

The rat *wpk* model and human ARPKD share numerous similarities in their clinical phenotype and renal histopathology. In *wpk/wpk* homozygotes, renal cysts develop *in utero* and enlarged kidneys are palpable within the first few weeks of life. Disease progression is associated with continued renal enlargement, systemic hypertension, significant reduction in urinary concentrating capacity, progressive renal insufficiency, and death by 4-6 weeks of age.

In comparison, the majority of human ARPKD patients are identified either *in utero* or at birth. The most severely affected fetuses have enlarged echogenic kidneys and oligohydramnios due to poor fetal renal output. At birth, these neonates often have a critical degree of pulmonary hypoplasia that is incompatible with survival. Renal function, though frequently compromised, is rarely a cause of neonatal death. For those infants who survive the perinatal period, systemic hypertension, and progressive renal failure usually evolve (5). In addition, ARPKD patients have defects in both urinary diluting capacity and concentrating capacity. There is often hyponatremia, presumably resulting from defects in free water excretion (5).

In both the rat *wpk* model and human ARPKD, renal cysts develop in both proximal and distal nephron segments (80). With disease progression, the cystic lesion in both the rat model and human ARPKD predominantly involves the cortical and medullary collecting ducts.

Systemic hypertension

Hypertension is a common finding among human PKD patients and contributes to both morbidity and mortality. Longitudinal observations indicate that hypertension is a major contributant to disease progression in ADPKD (81). Among affected children, hypertension occurs in both dominant and recessive PKD, but tends to be more severe in ARPKD (63,82-85).

Stimulation of the renal-angiotensin-aldosterone axis (RAAS) appears to be a major mechanism causing hypertension in ADPKD (81). Whether and to what degree increased activity of the RAAS contributes to hypertension in ARPKD remains unclear. The limited available data are conflicting. On the one hand, histopathologic observations indicate that with progressive disease, the glomeruli are compressed in the septae between expanding collecting duct cysts. Mechanical compression of the glomeruli and the intrarenal vasculature could, at least theoretically, stimulate RAAS activity. However, clinical data from affected neonates indicate that ARPKD is actually a low renin state with expansion of total body volume and occasional hyponatremia (61).

Given the extensive body of work regarding blood pressure regulation in the rat and the striking phenotypic similarities between the rat *wpk* model and human ARPKD, we propose that the *wpk* rat may provide a new, powerful model system to investigate the physiologic and genetic mechanisms that contribute to hypertension in recessive PKD.

Absence of biliary abnormalities

In this study, we found no evidence for biliary histopathology in Wistar-*wpk/wpk* homozygotes. Since we did not examine the liver histology of F2 *wpk/wpk* homozygotes of the Wisatr x BB-DP intercross, we can not comment as to whether genetic background affects the expression of a biliary lesion in the wpk model. However, we note a possible precedence with the mouse cpk mutation, another murine model of ARPKD. In the C57BL/6J mouse strain, cpk/cpk homozygotes do not have biliary disease. However, when the cpk mutation is outcrossed to several different strains, e.g. AKR, BALB/c, DBA/2J, and CAST/Ei strains, cpk/cpk homozygotes express a biliary lesion which varies in severity and closely resembles the ductal plate malformation evident in human ARPKD (86-87).

Cellular morphology of wpk/wpk renal cysts

Characterization of the cystic epithelia in the *wpk* model has yielded a number of interesting insights. First, the initial cysts appear to be derived from functioning, non-obstructed nephrons and the cystic epithelia maintain their segment-specific phenotype. These observations are consistent with data from the mouse *bpk* and *cpk* models and suggest that the disease-susceptibility genes in these models do not disrupt the early stages of nephrogenesis, e.g. induction of mesenchymal-to-epithelial transformation, acquisition of stem-cell character, fate determination, epitheliogenesis, and polarization (88).

In more advanced disease, cysts in the Wistar-*wpk/wpk* kidneys are lined by well-differentiated collecting duct cells, as evidenced by segment-specific marker profiling and electron microscopy. Although the cystic epithelia appear to have escaped the normal mechanisms controlling tubular diameter, they retain specific epithelial phenotypes with a heterogeneous population of principal cells and intercalated cells and maintain their organization as a monolayer. Interestingly, the cyst epithelium is not uniformly proliferative, with focal areas of increased proliferation in contiguous areas of adjacent cysts. This observation suggests that cell proliferation is influenced by local environmental factors as well as by the defective gene.

Interestingly, no abnormalities of the extracellular matrix or the basal lamina were associated with the renal cyst initiation or progression in *wpk/wpk* kidneys.

The wpk as a new model of recessive PKD

The mutant phenotype of the rat *wpk* model closely resembles human ARPKD and the mouse *cpk*, *bpk*, and *orpk* models. However, the *wpk* model is genetically distinct from all other previously described PKD loci. The *wpk* locus maps to proximal Chr 5. While the rat *cy* locus also maps to Chr 5 (37), we have demonstrated that *wpk* and *cy* are not allelic. Based on homology mapping, the human *wpk* orthologue maps to chr 8q11 (*http://www.ncbi.nlm.nih.gov/Homology*). These data exclude the *wpk* locus as a candidate for the human ARPKD gene, *PKHD1*, which maps to human Chr 6p21-p12 (89). Similarly, the mouse *wpk* orthologue maps to proximal mouse chromosome 4 and thus is genetically distinct from mouse *bpk* on Chr 10 (75), *cpk* on Chr 12 (90), or *orpk* on Chr 14 (91). It is interesting to note, however, that a principal modifying gene for both the mouse *cpk* and *pcy* models also maps to proximal Chr 4 (67-68). This observation raises the provocative possibility that the mouse *wpk* orthologue may be a candidate PKD modifying gene.

Conclusion:

We have characterized the clinical and histopathologic phenotype of a new rat PKD model. Like the mouse models, *cpk* and *bpk*, the rat *wpk* model strongly resembles human ARPKD. Homology mapping indicates that the rat *wpk* gene is distinct from the human ARPKD gene, *PKHD1*, as well as the mouse *cpk* and *bpk* genes. In addition to identifying a new gene involved in the pathogenesis of recessive PKD, this new rat mutation provides the first experimental model to study the renal pathophysiology associated with recessive PKD.

Acknowledgment

We thank D.R. Beier, MD, PhD for helpful discussions and C.E. Klein, R.N. Brigoos and J. Mahabier for tender animal care. Preliminary reports of these findings were presented at the Annual meetings of the American Society of Nephrology in '97 and '99 and have appeared in abstract form (39). The locus symbol, *wpk*, has been submitted to RATMAP (Rat Genome Database) for approval and reservation. These studies were supported by the Dutch Kidney Foundation (JN) and by the NIH (DK51034 tp LGW).

References

1. Fick G, Gabow P: Hereditary and acquired cystic disease of the kidney. Kidney Int 46: 951-964, 1994
2. Gabow P, Grantham J: Polycystic kidney disease.In: Diseases of the Kidney, edited by R. Schrier and C. Gottschalk. Boston, Little, Brown, 1997, pp 521-560
3. Guay-Woodford L, Bernstein J: (in press). Other cystic kidney diseases.In: Principles of Nephrology. R. Johnson and J. Feehally,.London, Mosby International.
4. Antignac C, Kleinknecht C, Habib R: Toward identification of a gene for familial nephronophthisis (autosomal recessive medullary cystic kidney disease). Adv Nephrol 24: 379-393, 1995
5. Guay-Woodford L: Autosomal recessive disease: clinical and genetic profiles. In: Polycystic Kidney Disease, edited by M. Watson and V. Torres, Oxford, Oxford University Press, 1996, pp 237-267
6. Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, et al.: Identification of the Von Hippel-Lindau disease tumor suppressor gene. Science 260: 1317-1320, 1993
7. The European Chromosome 16 Tuberous Sclerosis Consortium Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 75: 1305-1315,1993
8. The European Polycystic Kidney Disease Consortium: The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. Cell 77: 881-894, 1994
9. The American PKD1 Consortium: Analysis of the genomic sequence for the autosomal dominant polycystic kidney disease (PKD1) gene predicts the presence of a leucine-rich repeat. Hum Mol Genet 4: 575-582, 1995
10. Sandford R, Sgotto B, Aparicio S, Brenner S, Vaudin M, Wilson RK, Chissoe S, Pepin K, Bateman A, Chothia C, Hughes J, Harris: Polycystin: Comperative analysis of the polycystic kidney disease 1 (PKD1) gene reveals an integral membrane glycoprotein with multiple evolutionary conserved domains. Hum Mol Genet 6:1483-1489, 1997
11. The International Polycystic Kidney Disease Consortium Polycystic kidney disease: the complete structure of the PKD1 gene and its protein. Cell 8: 289-298, 1995
12. Mochizuki T, Wu G, Hayashi T, Xenophontos SL, Veldhuizen B, Saris JJ, Reynolds DM, Cai Y, Gabow PA, Pierides A, Kimberling WJ, Breuning MH, Deltas CC, Peters DJ, Somlo S: PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. Science 272:1339-42, 1996
13. Hildebrandt F, Otto E, Rensing C, Nothwang H, Vollmer M, Adolphs J, Hanusch H, Brandis M: A novel gene encoding an SH3 domain protein is mutated in nephronophthisis type 1. Nature Genet 17: 149-153, 1997
14. Saunier S, Calado J, Heilig R, Silbermann F, Benessy F, Morin G, Konrad M, Broyer M, Gubler M, Weissenbach J, et al.: A novel gene that encodes a protein with a putative src homology 3 domain is acandidate gene for familial juvenile nephronophthisis. Hum Mol Genet 6: 2317-2323, 1997
15. Van Slegtenhorst M, Hoogt R d., Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, Ouweland A v. d., Halley D et al: Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science 277: 805-808, 1997
16. Zerres K, Mucher G, Bachner L., Deschennes G., Eggermann T, Kaariainen H, Knapp M, Lenner T, Misselwitz J, Muhlendahl K v., et al.: Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. Nat Genet 7: 429-32, 1994
17. Guay-Woodford L, Muecher G, Hopkins S, Avner E, Germino G, Guillot A, Herrin J, Holleman R, Irons D, Primack W, et al.: The severe perinatal form of autosomal recessive polycystic kidney disease (ARPKD) maps to chromosome 6p21.1-p12: Implications for genetic counseling. Amer J Hum Genet 56: 1101-1107, 1995

18. Winyard P, Feather S: Genetics of human kidney malformations. Nephrol Dial Transplant 11: 976-978, 1996
19. Qian F, Watnick T, Onuchic L, Germino G: The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type I. Cell 87: 979-987, 1996
20. Torres V: Tuberous sclerosis complex. In: Polycystic Kidney Disease. Ed. by Torres V and Watson M, Oxford, Oxford University Press, 1996, pp 283-308
21. Brasier J, Henske E: Loss of the polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss-of-function model for cyst pathogenesis. J Clin Invest 99: 194-199, 1997
22. Lu W, Peissel B, Babakhanlou H, Pavlova A, Geng L, Fan X, Larson C, Brent G, Zhou J: Perinatal lethality with kidney and pancreas defects in mice with a targetted PKD1 mutation. Nature Gen 17: 179-181, 1997
23. Neumann H, Zbar B: Renal cysts, renal cancer, and von Hippel-Lindau disease. Kidney Int 51: 16-26, 1997
24. Wu G, D'Agati V, Cai Y, Markowitz G, Park JH, Reynolds DM, Maeda Y, Le TC, Hou H Jr, Kucherlapati R, Edelmann W, Somlo S: Somatic inactivation of Pkd2 results in Polycystic kidney disease. Cell 93: 177-188, 1998
25. Watnick TJ, Torres VE, Gandolph MA, Qian F, Onuchic LF, Klinger KW, Landes G, Germino GG: Somatic mutation in individual liver cysts supports a two-hit model of cystogenesis in autosomal dominant polycystic kidney disease. Mol Cell 2:247-251, 1998
26. Koptides M, Hadjimichael C, Koupepidou P, Pierides A, Constantinou Deltas C: Germinal and somatic mutations in the PKD2 gene of renal cysts in autosomal dominant polycystic kidney disease. Hum Mol Genet 8:509-513, 1999
27. Torra R, Badenas C, San Milan JL, Perez-Oller L, Estevill X, Darnell A: A loss-of-function model for cystogenesis in human autosomal dominant polycystic kidney disease type 2. Am J Hum Genet 65:345-52, 1999
28. Qian F, Germino F, Cai Y, Zhang X, Somlo S, Germino G: PKD1 interacts with PKD2 through a probable coiled-coil domain. Nat Genet 16: 179-183, 1997
29. Sampson J, Maheshwar M, Aspinwall R, Thompson P, Cheadle J, Ravine D, Roy S, Haan E, Bernstein J, Harris P: Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene. Am J Hum Genet 61: 843-851, 1997
30. Tsiokas L, Kim E, Arnould T, Sukatme V, Walz G: Homo- and heterodimeric interacrtions between the gene products of PKD1 and PKD2. Proc Nat Acad Sci USA 94: 6965-6970, 1997
31. Preminger G, Koch W, Fried F, McFarland E, Murphy E, Mandell J: Murine congenital polycystic kidney disease: a model for studying development of cystic disease. J Urol 127: 556-560, 1982
32. Werder A, Amos M, Nielsen A, Wolfe G: Comparative effects of germfree and ambient environments on the development of cystic kidney disease in CFWwd mice. J Lab Clin Med 103: 399-407, 1984
33. Kelly C, Nielson E: Medullary cystic disease: an inherited form of autoimmune interstitial nephritis. Am J Kidney Dis 10: 389-395, 1987
34. Takahashi H, Calvet J, Dittemore-Hoover D, Yoshida K, Grantham J, Gattone V: A hereditary model of slowly progressive polycystic kidney disease in the mouse. J Am Soc Nephrol 1: 980-989, 1991
35. Nauta J, Ozawa Y, Sweeney W, Rutledge J, Avner E: Renal and biliary abnormalities in a new murine model of autosomal recessive polycystic kidney disease. Pediatr Nephrol 7: 163-172, 1993
36. Iakoubova O, Dushkin H, Beier D: Localization of a murine recessive polycystic kidney disease mutation and modifying loci that affect disease severity. Genomics 26: 107-114, 1995
37. Bihoreau M, Ceccherini I, Browne J, Kranzlin B, Romeo G, Lathrop G, James M, Gretz N: Location of the first genetic locus, PKDr1, controlling autosomal dominant polycystic kidney disease in Han:SPRD cy/+ rat. Hum Mol Genet 6: 609-613, 1997

38. Janaswami P, Birkenmeier E, Cook S, Rowe L, Bronson R, Davisson M: Identification and genetic mapping of a new polycystic kidney disease on mouse chromosome 8. Genomics 40: 101-107, 1997
39. Nauta J, Goedbloed M, Herck H v., Hoogeveen A, Hasselink D, Wright C, Guay-Woodford L: A new rat model that phenotypically resembles autosomal recessive polycystic kidney disease (ARPKD). J Am Soc Nephrol 8: 1737A, 1997
40. Schäfer K, Gretz N, Bader M, Oberbaumer I, Eckardt K, Kriz W, Bachmann S: Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 46: 134-152, 1994
41. Inage Z, Inage Z, Kikkawa Y, Minato M, Owada M, Kitagawa T, Ohno K, Kondo K, Ueda Y, Iidaka K: Autosomal recesive polycystic kidney disease in rats. Nephron 59: 637-640, 1991
42. Feather SA, Woolf AS, Donnai D, Malcolm S, Winter RM: The oral-facial-digital syndrome type 1 (OFD1), a cause of polycystic kidney disease and associated malformations, maps to Xp22.2-Xp22.3. Hum Mol Genet 6:1163-7, 1997
43. Flaherty L, Bryda E, Collins D, Rudofsky U, Montgomery J: New mouse model for polycystic kidney disease with both recessive and dominant gene effects. Kidney Int 47: 552-558, 1995
44. MacKay K, Striker L, Pinkert C, Brinster R, Striker G: Glomerulosclerosis and renal cysts in mice transgenic for the early region of SV40. Kidney Int 32: 827-837, 1987
45. Kelley K, Agarwal N, Reeders S, Herrup K: Renal cyst formation and multifocal neoplasia in transgenic mice carrying the simian virus 40 early region. J Am Soc Nephrol 2: 84-97, 1991
46. Trudel M, D'Agati V, Costantini F: C-myc as an inducer of polycystic kidney disease in transgenic mice. Kidney Int 39: 665-671, 1991
47. Schaffner D, Barrios R, Massey C, Banez E, Ou C, Rajagopalan S, Aguilar-Cordova E, Lebovitz R, Overbeek P, Lieberman M: Targeting of the rasT24 oncogene to the proximal convoluted tubules in transgenic mice results in hyperplasia and polycystic kidneys. Am J Pathol 142: 1051-1060, 1993
48. Veis D, Sorenson C, Shutter J, Korsmeyer S: Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75: 229-240, 1993
49. Keller S, Jones J, Boyle A, Barrow L, Killen P, Green D, Kapousta N, Hitchcock P, Swank R, Meisler M: Kidney and retinal defects (Krd), a transgene-induced mutation with a deletion of mouse chromosome 19 that includes the Pax2 locus. Genomics 23: 309-320, 1994
50. Lowden D, Lindemann G, Merlino G, Barash B, Calvet J, Gattone V n.: Renal cysts in transgenic mice expressing transforming growth factor-α. J Lab Clin Med 124: 386-394, 1994
51. Moyer J, Lee-Tischler M, Kwoη H-Y, Schrick J, Avner E, Sweeney W, Godfrey V, Cacheiro N, Wilkinson J, Woychik R: Candidate gene associated with a mutation causing recessive polycystic kidney disease in mice. Science 264: 1329-1333, 1994
52. Lo SH, Yu QC, Degenstein L, Chen LB, Fuchs E: Progressive kidney degeneration in mice lacking tensin. J Cell Biol 136:1349-61, 1997
53. Lu W, Fan X, Basora N, Babakhanlou H, Law T, Rifai N, Harris PC, Perez-Atayde AR, Rennke HG, Zhou J: Late onset of renal and hepatic cysts in Pkd-1 targeted heterozygotes. Nat genet 21: 160-161, 1999
54. Moser M, Pscherer A, Roth C, Becker J, Mucher G, Zerres K, Dixkens C, Weis J, Guay-Woodford L, Buettner R, Fassler R: Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2β. Genes Dev 11: 1938-48, 1997
55. Boulter CA, Aguzzi A, Evans MJ, Affara N: A chimeric model for ADPKD. In: Breuning MH, Devoto M, Romeo G eds. Polycystic kidney disease. Karger, Basel, Contrib Nephrol 97:60-70, 1992
56. Nguyen HQ, Danilenko DM, Bucay N, DeRose ML, Van GY, Thomason A, Simonet WS: Expression of keratinocyte growth factor in embryonic liver of transgenic mice causes changes in epithelial growth and differentiation resulting in polycystic kidneys and other organ malformations. Oncogene 12:2109-19, 1996

57. Takayama H, LaRochelle WJ, Sabnis SG, Otsuka T, Merlino G: Renal tubular hyperplasia, polycystic disease, and glomerulosclerosis in transgenic mice overexpressing hepatocyte growth factor/scatter factor. Lab Invest 77:131-8, 1997
58. Wanke R, Hermanns W, Folger S, Wolf E, Brem G: Accelerated growth and visceral lesions in transgen mice expressing foreign genes of the growth hormone family: an overview. Pediatr Nephrol 5:513-2 1991
59. Hocher B, Thone-Reineke C, Rohmeiss P, Schmager F, Slowinski T, Burst V, Siegmund F, Quertermous T, Bauer C, Neumayer HH, Schleuning WD, Theuring F J: Endothelin-1 transgenic mice develop glomerulosclerosis, interstitial fibrosis, and renal cysts but not hypertension. Clin Invest 99:1380-9, 1997
60. Schieren G, Pey R, Bach J, Hafner M, Gretz N: Murine models of polycystic kidney disease. Nephrol Dial Transplant 11((Suppl 6)): 38-45, 1996
61. Kaplan B, Fay J, Shah V: Autosomal recessive polycystic kidney disease. Pediatr Nephrol 3: 43-49, 1989
62. Gretz N, Kranzlin B, Pey R, Schieren G, Bach J, Obermuller N, Ceccherini I, Kloting I, Rohmeiss P, Bachmann S, et al.: Rat models of autosomal dominant polycystic kidney disease. Nephrol Dial Transplant 11: 46-51, 1996
63. Zerres K, Rudnik-Schoneborn S, Deget F, Holtkamp U, Brodehl J, Geisert J, Scharer K, Nephrologie A. f. P.: Autosomal recessive polycystic kidney disease in 115 children: clinical presentation, course and influence of gender. Acta Paediatr 85: 437-445, 1996
64. Iakoubova O, Dushkin H, Beier D: Genetic analysis of a quantitative trait in a mouse model of polycystic kidney disease. Am J Respir Crit Care Med 156: S72-S77, 1997
65. Wright C, Guay-Woodford L: Genetic analysis of loci that modify renal cystic disease in the mouse bpk model. J Am Soc Nephrol 8: 1781A, 1997
66. Upadhya P, Churchill G, Birkenmeier E, Barker J, Frankel W: Genetic modifiers of polycystic kidney disease in intersubspecific KAT2J mutants. Genomics 58: 129-137, 1999
67. Woo D., Miao S, Tran T: Progression of polycystic kidney disease in cpk mice is a quantitative trait under polygenic control. J Am Soc Nephrol 6: 731A, 1995
68. Woo D, Progression of polycystic kidney disease in pcy mice is a quantitative trait under polygenic control. J Clin Invest 100: 1934-1940, 1998
69. Dokkum RPE van, Jacob HJ, Provoost AP: Genetic differences define severity of renal damage after L-NAME-induced hypertension. J Am Soc Nephrol 9:363-71, 1998
70. Leer EHG van, Roo GM de, Bruijn JA, Hoedemaeker PJ, Heer E de, Synergistic effects of anti-gp330 and anti-dipeptidyl peptidase type IV antibodies in the induction of glomerular damage. Exp Nephrol 48:1-13, 1993
71. Demarchez M, Hatmann DJ, Herbage D, Ville G, Prunieras M: Wound healing of human skin transplanted onto the nude mouse. An immunohistological and ultrastructural study of the epidermal basement membrane zone reconstruction and connective tissue reorganization. Dev Biol 121:119-129, 1987
72. Havenith MG, Dingemans KP, Cleutjens JPM, Wagenaar SS, Bosman FT: Basement membranes in bronchogenic squamous cell carcinoma. An immunohistochemical and ultrastructural study. Ultrastr Pathol 14:51-63, 1990
73. Neuhaus I, Beier D: Efficient localization of mutations by interval haplotype analysis. Mamm Genome 9: 150-154, 1998
74. Szpirer C, Szpirer J, Vooren P v., Tissir F, Simon J, Koike G, Jacob H, Lander E, Helou K, Kinga-Levan K, et al.: Gene-based anchoring of the rat genetic linkage and cytogenetic maps: new regional localizations, orientation of the linkage groups, and insights into mammalian chromosome evolution. Mamm Genome 9: 721-734, 1998
75. Guay-Woodford L, Bryda E, Christine B, Lindsey J, Collier W, Avner E, D'Eustachio P, Flaherty L: Evidence that two phenotypically distinct mouse PKD mutations, bpk and jcpk, are allelic. Kidney Int 50: 1158-1165, 1996

76. Welling LW, Grantham JJ: Cystic diseases of the kidney. In: Renal Pathology, ed. by Tisher CC and Brenner BM, Philadelphia, Lippingcott, 1989, pp 1233-77
77. Trudel M, Barisoni L, Lanoix J, D'Agati V: Polycystic kidney disease in SBM transgenic mice: role of c-myc in disease induction and progression. Am J Pathol 152(1): 219-29, 1998
78. Verlander VJ, Madsen KM, Tisher CC: Effect of acute respiratory acidosis on two populations of intercalated cells in the rat cortical collecting duct. Am J Physiol 253:F1142-56, 1987
79. Venkatachalam MA, Kriz W: Anatomy in: Heptinstall's pathology of the kidney, 5th Ed., ed. by Jennette JC, Olson JL, Schwartz MM, Silva FG. Philadelphia, Lippincott, 1998, pp 3-66
80. Sweeney W, Avner E: Proximal tubular cysts in fetal human ARPKD. J Am Soc Nephrol 9: 1953A, 1998
81. Chapman A, Gabow P: Hypertension in autosomal dominant polycystic kidney disease. Kidney Int 52 (Suppl 61): S71-S73, 1997
82. Kääriäinen H, Koskiemis O, Norio R: Dominant and recessive polycystic kidney disease in children: evaluation of clinical features and laboratory data. Pediatr Nephrol 2:296-302, 1988
83. Roy S, Dillon MJ, Trompeter RS, Barratt TM: Autosomal recessive polycystic kidney disease: long-term outcome of neonatal survivors. Pediatr Nephrol 11:302-6, 1997
84. Gabow PA, Johnson AM, Kaehny WD, Kimberling WJ, Lezotte DC, Duley ET, Jones RH: Factors affecting the progression of renal disease in autosomal dominant polycystic kidney disease. Kidney Int 41:1311-9, 1992
85. Watson ML: Hypertension in polycystic kidney disease. In: Oxford textbook of clinical nephrology, ed. by Watson ML, Torres VE, Oxford, Oxford Press, 1996, pp 407-429
86. Gattone V n., MacNaughton K, Kraybill A: Murine autosomal recessive polycystic kidney disease with multiorgan involvement induced by the cpk gene. Anat Rec 245: 488-499, 1996
87. Guay-Woodford L, Lindsay J, Beier D: The ductal plate malformation (DPM) in the cpk mouse model: histopathologic and genetic analyses. J Am Soc Nephrol 8: 1910A, 1998
88. Davies J: Mesenchyme to epithelial transition during development of the mammalian kidney tubule. Acta Anat 156: 187-201, 1996
89. Muecher G, Becker J, Knapp M, Buttner R, Moser M, Rudnik-Schoneborn S, Somlo S, Germino G, Onuchic L, Avner E, et al.: Fine mapping of the autosomal recessive polycystic kidney disease locus (PKHD1) and the genes MUT, RDS, CSNK2 β, and GSTA1 at 6p21.1-p12. Genomics 48(1): 40-45, 1998
90. Simon E, Cook S, Davisson M, D'Eustachio P, Guay-Woodford L: The mouse congenital polycystic kidney (cpk) locus maps within 1.3 cM of the chromosome 12 marker D12Nyu2. Genomics 21: 415-418, 1994
91. Onuchic L, Schrick J, Ma J, Hudson T, Guay-Woodford L, Zerres K, Woychik R, Reeders S: Sequence analysis of the human hTg737 gene and its polymorphic sites in patients with autosomal recessive polycystic kidney disease. Mamm Genome 6: 805-808, 1995

3

Cell survival and Polycystin-1 identification in Human Kidneys

2 studies

3.1

Deregulation of cell survival in cystic and dysplastic renal development

Winyard PJ, Nauta J, Lirenman DS, Hardman P, Sams VR, Risdon RA, Woolf AS.

Developmental Biology Unit, Institute of Child Health, London WC1E IEH, UK; Department of Paediatric Nephrology, Sophia Childrens Hospital, 3015 GJ Rottterdam, The Netherlands; Department of Histopathology, Hospital for Sick Children, Great Ormond Street, London WCIN 1JH, UK; Department of Histopathology, University College Medical School, London WC1E 6JJ, UK

Kidney Int 49:135-146, 1996

Deregulation of cell survival in cystic and dysplastic renal development

Abstract

Various aberrations of cell biology have been reported in polycystic kidney diseases and in cystic renal dysplasias. A common theme in these disorders is failure of maturation of renal cells which superficially resemble embryonic tissue. Apoptosis is afeature of normal murine nephrogenesis, where it has been implicated in morphogenesis, and fulminant apoptosis occurs in small cystic kidneys which develop in mice with null mutations of *bcl-2*. Therefore we examined the location and extent of apoptosis in pre- and postnatal samples of human polycystic and dysplastic kidneys using propidium iodide staining, in situ end-labeling and electron microscopy. In dysplastic kidneys cell death was prominent in undifferentiated cells around dysplastic tubules and was occasionally found in cystic epithelia. The incidence of apoptosis was significantly greater than in normal controls of comparable age both pre- and postnatally. In the polycystic kidneys there was widespread apoptosis in the interstitium around undilated tubules distant from cysts, in undilated tubules between cysts and in cystic epithelia. The level of apoptosis compared to controls was significantly increased postnatally. A similar increase of cell death was also noted in the early and late stages of renal disease in the polycystic *cpk/cpk* mouse model. We speculate that deregulation of cell survival in these kidneys may reflect incomplete tissue maturation, and may contribute to the progressive destruction of functional kidney tissue in polycystic kidneys and the spontaneous involution reported in cystic dysplastic kidneys.

Introduction

Organogenesis involves an increase in cell numbers, cell differentiation and morphogenesis. In normal development the increase in cell number is determined by the balance between proliferation and death, which in this context has been called 'programmed cell death'. This term is often used interchangably with 'apoptosis', first used by Kerr *et al.* [1] to describe death in a variety of normal and pathological contexts when accompanied by nuclear condensation and fragmentation with cell shrinkage. These changes are striking on electron microscopy but can also be detected by light microscopy as pyknotic nuclei [2]. A common biochemical correlate of apoptosis is DNA digestion by endonucleases into nucleosome sized fragments which can be visualised as a 'ladder' on electrophoresis [3] and detected *in situ* by end-labeling [4]. Exceptionally some

programmed cell death during development is not accompanied by these morphological events [5] and DNA laddering is not always detected in cells dying by morphological criteria of apoptosis since the DNA is sometimes cleaved into larger fragments (50-300 kbp) [6]. In the worm *Caenorhabditis elegans* apoptosis is developmentally regulated by the expression of specific genes and some of their mammalian homologues have been defined [7]. 'Growth factors' can not only control proliferation and differentiation but can also enhance apoptosis [8] or act as survival factors [9]. In contrast to apoptosis, cells dying by necrosis swell with destruction of organelles and loss of integrity of cell membranes [10]. DNA laddering does not occur in necrosis, although some models show single-strand DNA breaks [6].

In nephrogenesis cell proliferation, differentiation and morphogenesis occur during the branching of the ureteric bud and in nephron formation [11-13]. Apoptosis has been demonstrated during normal murine renal development in the nephrogenic zone and also in the papilla; cell death can be reduced by epidermal growth factor (EGF) [2]. Koseki and colleagues showed that the uninduced renal mesenchyme died by apoptosis when isolated from the ureteric bud, and that this process was ameliorated by EGF [14]. Similarly, hepatocyte growth factor (HGF) has been implicated in survival of renal mesenchymal cells in serum-free organ culture [15].

The pathogenesis of some human kidney diseases can be understood in the context of aberrant development. In polycystic kidney diseases (PKD) there is evidence of enhanced epithelial proliferation [16] and altered polarity [17-18] leading some authors to suggest that the epithelial cells are "locked" in an immature, dedifferentiated state [19]. Similarly, in dysplastic kidneys there is a failure of differentiation of renal mesenchyme into nephrons and decreased branching of the ureteric bud [11, 20, 21]. We therefore considered it pertinent to examine the location and quantity of apoptosis in normal human nephrogenesis and in kidney diseases which can be viewed as aberrations of normal nephrogenesis. In this study we demonstrate that the distribution and incidence of renal programmed cell death is increased in children with polycystic and dysplastic kidneys.

Methods

Experimental Strategy

The aim of the study was to define the tissue location and quantify the extent of apoptosis during normal kidney development and in fetal and childhood polycystic and dysplastic kidneys. The methodologies we used included both histological and molecular techniques together with quantitative statistical analysis.

Classification of Specimens

This research was approved by the hospitals' research ethics committees. During human development the metanephros can first be identified in the sixth week after fertilisation and sequential layers of nephrons are formed until 36 weeks after fertilisation [11]. We therefore arbitrarily divided our specimens into experimental prenatal groups before 40 weeks gestation and postnatal groups after 40 weeks as follows:

i) Normal prenatal kidneys (n = 6).
ii) Polycystic prenatal kidneys (n = 4).
iii) Dysplastic prenatal kidneys (n = 6).
iv) Normal postnatal kidneys (n = 6).
v) Polycystic postnatal kidneys (n = 4).
vi) Dysplastic postnatal kidneys (n = 4).

Details of these patients and specimens are listed in Tables 1 and 2, including their sex, the gestational or chronological age, associated abnormalities in other organ systems, indications for termination of pregnancy or for removal of a kidney, plus the side affected by renal disease and the plasma creatinine in the childhood samples. There is no significant difference in age and sex distribution. within the prenatal and postnatal groups. Kidney pathology was classified by gross morphology, routine histopathology and, in the cases of polycystic kidneys, by liver histology and family history. All of the polycystic kidneys were classified as autosomal recessive polycystic kidney disease (ARPKD) apart from one postnatal sample which had histology consistent with dominant disease (ADPKD), although ultrasound scans of the parents (aged 21 and 23) were normal. Two children with ARPKD had elevated plasma creatinines. The dysplastic samples all had classical histological criteria of this disorder, namely dysplastic tubules, "undifferentiated mesenchymal" tissue and metaplastic cartilage.

Collection of Specimens

For the normal prenatal kidney group we studied both spontaneous miscarriages and phenotypically normal kidneys from abortions performed for severe abnormalities in other organ systems which would have compromised the survival of the fetus or infant. These samples were compared with the polycystic and dysplastic kidneys which were collected under similar conditions. In these three groups the parents were given time to mourn the loss of the child. Then the fetus was stored at $4^{o}C$ until autopsy when organs were fixed in 10% formalin. All specimens were processed within 24 hours.

The postnatal polycystic and dysplastic samples were harvested surgically. Therefore, as a comparable normal surgical group we used kidney tissue adjacent to, but unaffected by, Wilms' tumors. These kidneys were chosen to control for any potential changes in tissue morphology which may have been induced by general anaesthesia or surgery. Since both WT1 mutations and chemotherapy may cause apoptosis [6,22], we excluded children

Table 1. Prenatal kidneys

Gestational age weeks	Sex	Renal and associated pathology
Normal		
17	M	Abortion for neural tube defect
19	M	Spontaneous miscarriage
20	F	Abortion for trisomy 21 with atrial septum defect
20	F	Spontaneous miscarriage
21	M	Abortion for cephalocoele
22	F	Abortion for major skeletal malformations
Polycystic		
20	M	Abortion for ARPKD
34	F	Spontaneous premature labor, ARPKD
35	F	Spontaneous premature labor, ARPKD and liver fibrosis
35	M	Spontaneous premature labor, ARPKD and liver fibrosis
Dysplastic		
17	M	Abortion for multicystic dysplastic kidney with contralateral renal agenesis
19	F	Abortion for multicystic dysplastic kidney with contralateral renal agenesis
20	M	Abortion for bilateral renal cystic dysplasia
22	M	Abortion for bilateral renal cystic dysplasia, bladder dilated without anatomical urethral obstruction, atrial septum defect
24	F	Abortion for bilateral renal cystic dysplasia
34	M	Spontaneous premature labor, kidneys dysplastic, bladder dilated with partial urethral obstruction

* Children born at 34 to 35 weeks died within hours because of respiratory failure due to hypoplastic lungs. Chromosomes were normal in all fetuses assessed except for the case of trisomy 21. Parents were normal by history in all cases.

who had received chemotherapy, and none of our patients had mutations in the WT1 gene (Personal communication - Dr. Richard Grundy, Dept. of Haematology and Oncology, Hospital for Sick Children, Great Ormond Street, London, U.K.). Surgical specimens were placed on ice, examined by a pathologist and immediately fixed in 10% formalin for histology. A limited number of dysplastic samples (n=3) were snap frozen in liquid nitrogen for DNA extraction and fixed in glutaraldehyde for electron microscopy.

Detection of apoptosis by propidium iodide staining

All chemicals were supplied by Sigma (Poole, Dorset, UK.) unless otherwise stated. Propidium iodide is a fluorescent dye which intercalates with nucleic acids. Apoptotic cells can be identified in tissue sections by their small, fragmented (pyknotic) nuclei

Table 2. Postnatal kidneys

Age *months*	Side	Sex	Creat. umol/L	Renal Pathology
Normal				
5	R	F	60	Wilms tumor before chemotherapy
8	R	M	65	Wilms tumor before chemotherapy
28	L	M	35	Wilms tumor before chemotherapy
30	R	M	48	Wilms tumor before chemotherapy
38	R	M	34	Wilms tumor before chemotherapy
72	L	F	38	Wilms tumor before chemotherapy
Polycystic				
10	R	F	98	Diagnostic open surgical biopsy, ARPKD
11	L	M	14	Diagnostic open surgical biopsy, histologically ADPKD
48	R	M	322	Nephrectomy at transplantation; ARPKD
72	R	M	50	Surgical biopsy for infections, ARPKD
Dysplastic				
8	R	F	50	Cystic dysplastic upper pole with obstruction of the pelvi-ureteric junction
15	R	M	33	Non-functioning multicystic dysplasia
16	R	M	32	Non-functioning multicystic dysplasia
60	L	F	38	Unilateral duplex ureters with non-functioning dysplastic upper pole

Plasma creatinine is the immediate preoperative plasma value. Two children in the polycystic group had elevated creatinine values, but this not appear to correlate with the pyknotic index in these specimens (data not shown). No kidney disease was reported in any of the parents including the histologically defined ADPKD.

which stain brightly when they are visualised under fluorescence miscoscopy. We used the technique of Coles *et al.* [2] with minor modifications. Five micrometer paraffin sections were dewaxed through Histo-Clear (National Diagnostics, Atlanta, USA.) twice for 10 minutes, followed by dehydration through 100% alcohol (Hayman Ltd., Witham, Essex, UK.) twice for 5 minutes and then stepwise through 95%, 90%, 75%, 50% and 30% alcohol for 3 minutes each. After washing in phosphate buffered saline (PBS, pH 7.4) for 5 minutes they were incubated in propidium iodide (4 mg/l) with RNase A (100 mg/l; Unipath, Basingstoke, Hampshire, UK.) in PBS at 37^{o}C for 30 minutes. After one further wash in PBS they were mounted in Citifluor^{TM} (Chemical Labs, University of Kent, Canterbury, Kent, UK.). Specimens were examined under fluorescence (wavelength 568nm) on a Zeiss Axiophot microscope (Carl Zeiss, 7082 Oberkochen, Germany) and on a Leica confocal laser scanning microscope (CLSM Aristoplan-Leica, Heidleberg, Germany). The precise tissue location of apoptosis was determined in ARPKD samples

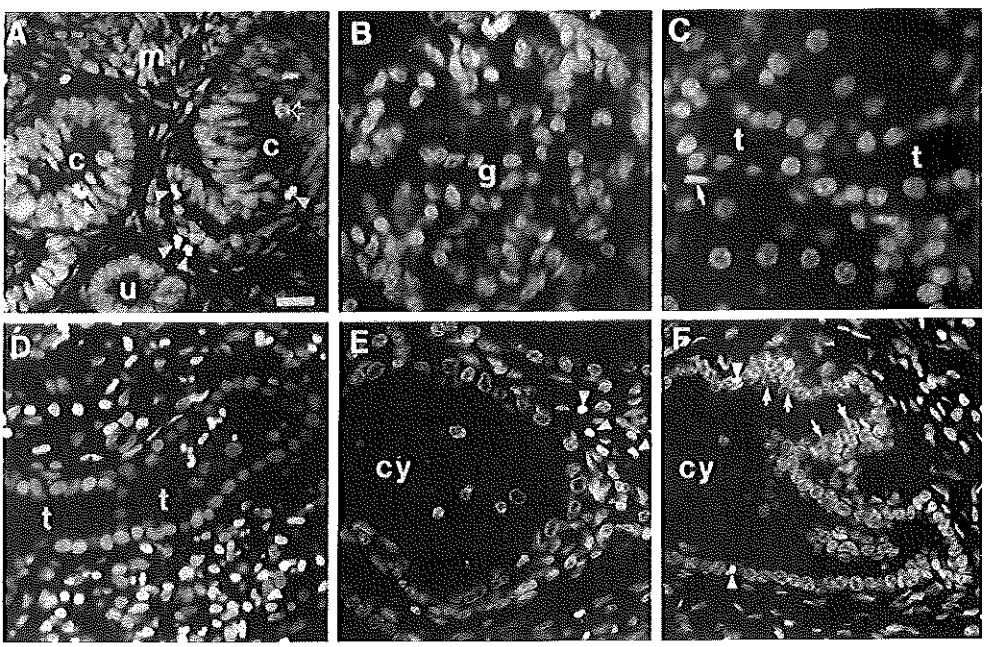


Figure 1. *Location of apoptosis in propidium iodide stained sections of normal and polycystic kidneys.* A) Nephrogenic zone from normal developing kidney, 20 weeks gestation. Note apoptotic nuclei in comma shaped bodies, which are primitive nephrons. B) Glomerulus and C) proximal tubule from normal mature kidney , aged 30 months. Note absence of apoptosis, although nuclei in the glomerulus appear generally brighter. A fibroblast is seen adjacent to the tubule epithelium in (C). It has a bright elongated nucleus but should not be confused with the smaller irregular pyknotic nuclei. D) Undilated tubule from polycystic kidney harvested at 34 weeks of gestation surrounded by numerous apoptotic nuclei in interstitial tissue. E) is from a child with ARPKD and shows a group of apoptotic nuclei in the interstitium between cysts. F) is from an infant with dominant disease showing two apoptotic nuclei in the epithelium of a cyst close to a multilayered region of epithelium suggestive of hyperproliferation. Key: comma shaped body (c), cyst (cy), glomerulus (g), undifferentiated renal mesenchyme (m), tubule (t) and ureteric bud (u). Arrowheads indicate apoptotic nuclei, open arrow in A) indicates a mitotic figure and closed arrows in C) indicates a fibroblast and closed arrows in F) point to multilayered epithelium. Bar is 20 micrometer.

by counterstaining with FITC conjugated *Tetragonolobus lotus* or *Arachis hypogaea* lectins. These bind to proximal tubules and distal segments (distal tubule and collecting ducts) respectively [23,24]. After the propidium iodide staining the lectins were applied to the sections at 1:50 dilution in PBS at room temperature for 4 hours, mounted in Citifluor^{TM} and examined under fluorescence (wavelength 488 nm). Apoptotic nuclei

detected by the propidium iodide method were quantified by generating a pyknotic index as described and by calculating the percentage of pycnotic nuclei as described below.

Detection of apoptosis by* in situ *end-labeling

During apoptosis the nuclear DNA is digested by endonucleases leaving free 3' ends. These ends can be tagged using terminal deoxytransferase to add labeled nucleotides which can be visualised on tissue section using secondary detection systems. We modified the technique of Gavrelli *et al.* [4] by using an Apoptag^{TM} kit (Oncor, Gaithersburg, Maryland, USA.). Paraffin sections were dewaxed and rehydrated, treated with proteinase K (20 mg/l) for 15 minutes and washed in PBS. Slides were then covered with equilibration buffer for 30 seconds, followed by terminal deoxytransferase and digoxigenin conjugated UTP from the Apoptag^{TM} kit as recommended by the manufacturer. The reaction was terminated using the Apoptag^{TM} stop buffer. Labeled nuclei were detected either i) by light microscopy after incubation with a peroxidase conjugated anti digoxigenin antibody and diamino benzidine (these sections were quenched with 3% hydrogen peroxide for 15 minutes at room temperature as an initial step) or ii) by fluorescent microscopy (wavelength 568 nm) after applying an anti-digoxigenin antibody conjugated with rhodamine (Boehringer Mannheim, Lewes, East Sussex, UK.).

Quantitation of apoptosis in human kidneys

Preliminary experiments with both propidium iodide and *in situ* end-labeled specimens showed that the number of apoptotic cells could vary between different areas of the same section, although there was good correlation between the two techniques. Therefore, in order to integrate the quantity of apoptosis in each specimen we photographed 10 random fields from throughout each sample stained with propidium iodide at 20 x magnification on a Zeiss Axiophot microscope. The number of pyknotic nuclei was then counted by 3 'blinded' observers and we derived the pyknotic index for each specimen by taking the mean number of pyknotic nuclei per 10 fields. Results from each observer were also compared to ensure that there was no observer bias [25]. In this study we have also expressed the quantity of apoptosis as a percentage of the total number of nuclei. Since the pyknotic indices and the percentage of apoptotic cells in each experimental group were not normally distributed we transformed the data by converting to $\log_{10}$ values, thus allowing analysis by Stident's t-test. A probability value of $p<0.05$ was considered to be statistically significant.

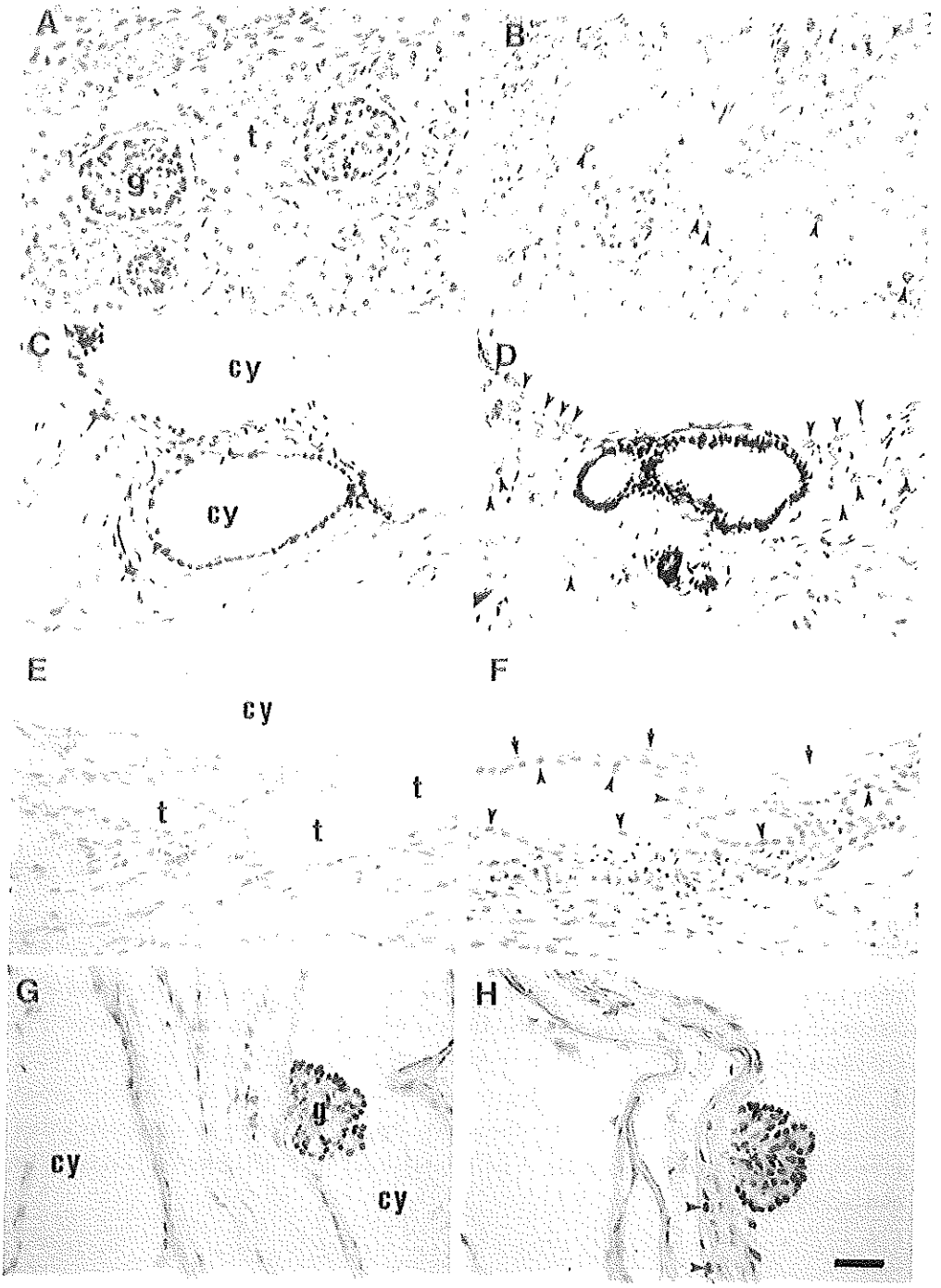


Fig. 2 (Legends on next page)

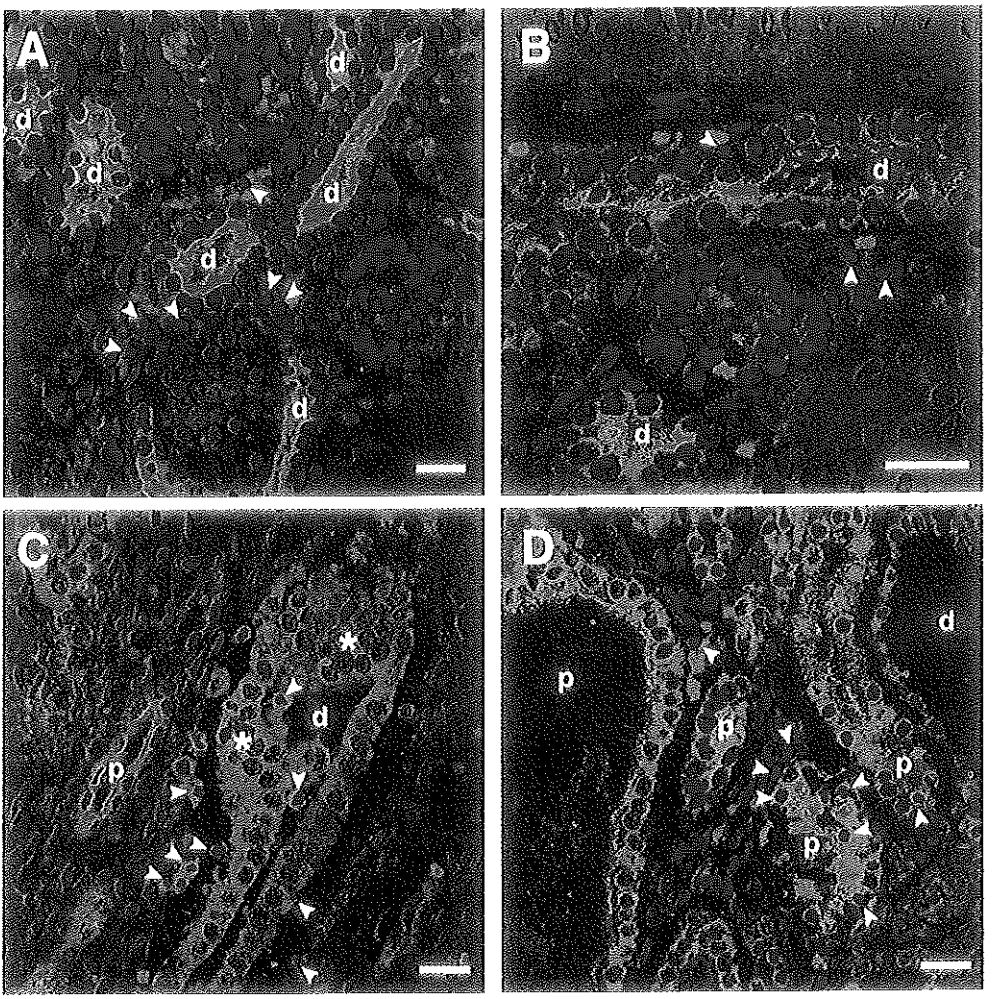


Fig. 3. *Location of apoptotic nuclei assessed by propidium iodide and lectin staining in polycystic kidneys.* All sections stained with propidium iodide (red) and FITC-conjugated lectin (green) are from ARPKD. (**A** and **B**) (higher power): Area of undilated distal tubules stained with *Arachis hypogaea* lectin with several adjacent pyknotic nuclei. (**C** and **D**): Tetragonolobus lotus lectin staining shows proximal tubules. (C) Undilated proximal tubule next to distal tubule with adjacent pyknotic cells and hyperproliferative epithelium. Two apoptotic cells also seen in the lumen of the distal tubule. (D): Dilated proximal tubule on the left and dilated distal segment on the right. Three proximal tubules between cysts. One of these (right) contains a single apoptotic cell within its epithelium whereas the degenerating central tubule contains many apoptotic cells. Key: distal segment (d), hyperproliferative area (*), and proximal tubule (p). Arrowheads indicate apoptotic nuclei. Bar is 20 micrometer.

Figure 2. (Previous page) *Location of apoptotic nuclei assessed by in situ end labeling in sections of normal and polycystic kidneys.* **A), C), E)** and **G)** are control sections in which the terminal deoxytransferase enzyme was omitted. Note that labeled nuclei, which appear brown, are absent. **B), D), F)** and **H)** are adjoining sections which have been subjected to the complete end labeling procedure using digoxigenin UTP as described in the Methods. All sections counterstained with methyl green. A) and B): Normal mature kidney showing apoptotic nuclei in tubular epithelial cells. This section together with Figure 1C. demonstrates the range of apoptosis in normal postnatal kidneys. C) to F) show sections from patients with ARPKD harvested before 36 weeks of gestation . There is a wide distribution of apoptotic nuclei in the cyst epithelium and interstitial cells in D) and in the epithelial cells of relatively undilated tubules adjacent to a large cyst in F). Note that in F) the nuclei of the flattened epithelial cells lining the large cyst are not apoptotic, demonstrating the specificity of the technique. G) and H): Glomerulus from a child with dominant disease showing a cyst of Bowman's space with apoptotic nuclei in both the cyst wall and the glomerulus. Key: cyst (cy), glomerulus (G) and tubule (t). Arrowheads indicate apoptotic nuclei which appear brown. Bar is 40 micrometer.

Detection of apoptosis by electron microscopy

Apoptotic cells have a characteristic appearance on electron microscopy with nuclear condensation around the periphery of the nucleus, cell shrinkage and budding off of both the nuclear and cell membranes. Samples of 3 dysplastic kidneys were fixed initially in 2% glutaraldehyde in 0.05M cacodylate buffer, then in 1% Osmium tetroxide and finally embedded in epoxy resin. Ultrathin sections contrasted with uranyl acetate and lead citrate were examined with a Joel 100 CX transmission electron microscope.

Detection of apoptosis by DNA electrophoresis

During apoptosis the endonuclease DNA digestion produces nucleotide fragments which are multiples of 180 - 200 bases (180, 360, 540, 720 etc.) [3]. These can be visualised as a ladder on agarose gel electrophoresis. Fresh surgical specimens of 3 dysplastic kidneys were thawed to room temperature (after storage at -70^{o}C), homogenised in a lysis buffer (10 mM Tris HCl (pH 8.0), 10 mM EDTA, 0.1% SDS, 100 ug/ml proteinase K) and incubated overnight at 52^{o}C. DNA was then extracted using the phenol/chloroform technique [26]. Briefly, equal volumes of phenol (equilibrated pH 8.0) and chloroform are added to the specimen which was then centrifuged at 5,000 x g. The aqueous layer is separated and the process is repeated. DNA was then precipitated with an equal volume of ice cold 100% alcohol, washed in 70% ethanol and resuspended in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). Two micrograms of DNA was subjected to electrophoresis through a 1.5% agarose gel containing ethidium bromide and visualised under UV illumination. This extraction technique should be specific for DNA but any potential

RNA contamination was eliminated by incubating the samples with 1ug/ul of RNAse A for 30 minutes before electrophoresis.

Detection of apoptosis in mice with polycystic kidneys

Human ARPKD is a relatively heterogeneous condition. Therefore, we examined apoptosis in *cpk/cpk* mice which phenotypically ressemble human ARPKD, but in which the kidney histology is more homogeneous and the progressive course of the disease is highly predictable [18,27,28]. Homozygous *cpk/cpk* mice (Jackson Laboratory, USA) were sacrificed at birth when there is mild dilatation of the proximal tubules and at day 14 when there is gross cystic changes in the collecting ducts. The extent of apoptosis was compared with phenotypically normal littermates (*cpk/+* and +/+) as discussed in the Quantitation section above.

Effects of storage on apoptosis in fetal mice kidneys

To assess the possible effects of storage on the quantity of renal apoptosis we performed the following experiment. Pregnant CD1 mice (Charles River UK Ltd.)) were sacrificed by cervical dislocation at the 16th day of gestation; a stage when the fetal kidney contains a spectrum of nephrogenesis broadly equivalent to our human prenatal samples. Embryos were killed by cervical dislocation. In one group kidneys were immediately removed and fixed in 4% PFA, whilst in the other group the whole fetus was left at 4^{o}C for 24 hours before removing and processing the kidneys. Subsequently, apoptosis was quantitated on histological sections.

Effects of anaesthesia and renal artery clamping on apoptosis in mice

We assessed the potential effects of anaesthesia and renal artery clamping on apoptosis by performing these procedures on mice. Adult CD1 mice (25g) were anaesthatized with nitrous oxide / halothane and underwent laparotomy. The renal vessels were identified using a dissecting microscope and the renal arteries were clamped for 15 minutes. Mice were then sacrificed. Control mice underwent cervical dislocation with immediate dissection and processing of the kidneys. Apoptosis was quantitated as above.

Results

Location of apoptosis in normal human kidneys

In prenatal kidneys apoptosis was detected in nephron precursors in the nephrogenic cortex, typically in comma shaped bodies (Figure 1A) and in interstitial cells in the medulla (not shown). Apoptosis was not found in either the undifferentiated mesenchyme (Figure 1A) or in more mature nephrons towards the center of the organ. In the postnatal

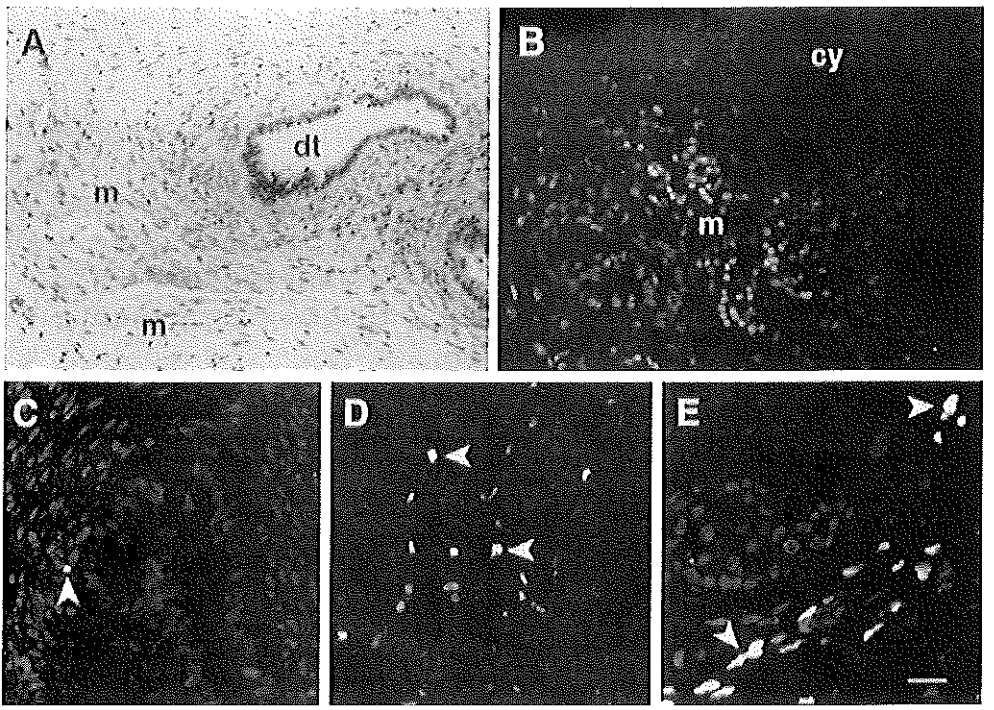


Figure 4. *Location of apoptotic nuclei in dysplastic kidneys.* A) Low power view of dysplastic kidney from a 16 month old child counterstained with toluidine blue to show dysplastic tubules immediately surrounded by fibromuscular collarettes and adjacent areas of undifferentiated cells of mesenchymal appearance. B) Propidium iodide labeling showing pyknotic nuclei in the interstitium between cysts. C) to E) are in situ end labeled sections using an anti digoxigenin antibody conjugated to rhodamine. C) Large dysplastic tubule with surrounding collarette containing one apoptotic nucleus. D) Numerous apoptotic nuclei in area of undifferentiated cells, similar to the area in B). E) Small dysplastic tubule with many adjacent apoptotic nuclei. Key: collarette (ct), dysplastic tubule (dt) and undifferentiated mesenchyme-like cells (m). Arrowheads indicate apoptotic cells. Bar is 40 micrometer in A and B and 20 micrometer in all other sections.

samples rare apoptotic nuclei were detected in the epithelial cells of proximal tubules (Figure 2B) but were never noted in glomeruli (Figures 1B and 2B), interstitial cells (Figures 1C and 2B) or loops of Henle.

Location of apoptosis in polycystic human kidneys

The distribution of apoptosis within the kidney was similar in both pre- and postnatal samples. In ARPKD apoptosis was detected (i) in the interstitium around undilated proximal and distal tubules (Figs. 1D, 3A-C); (ii) within the epithelial lining of cysts,

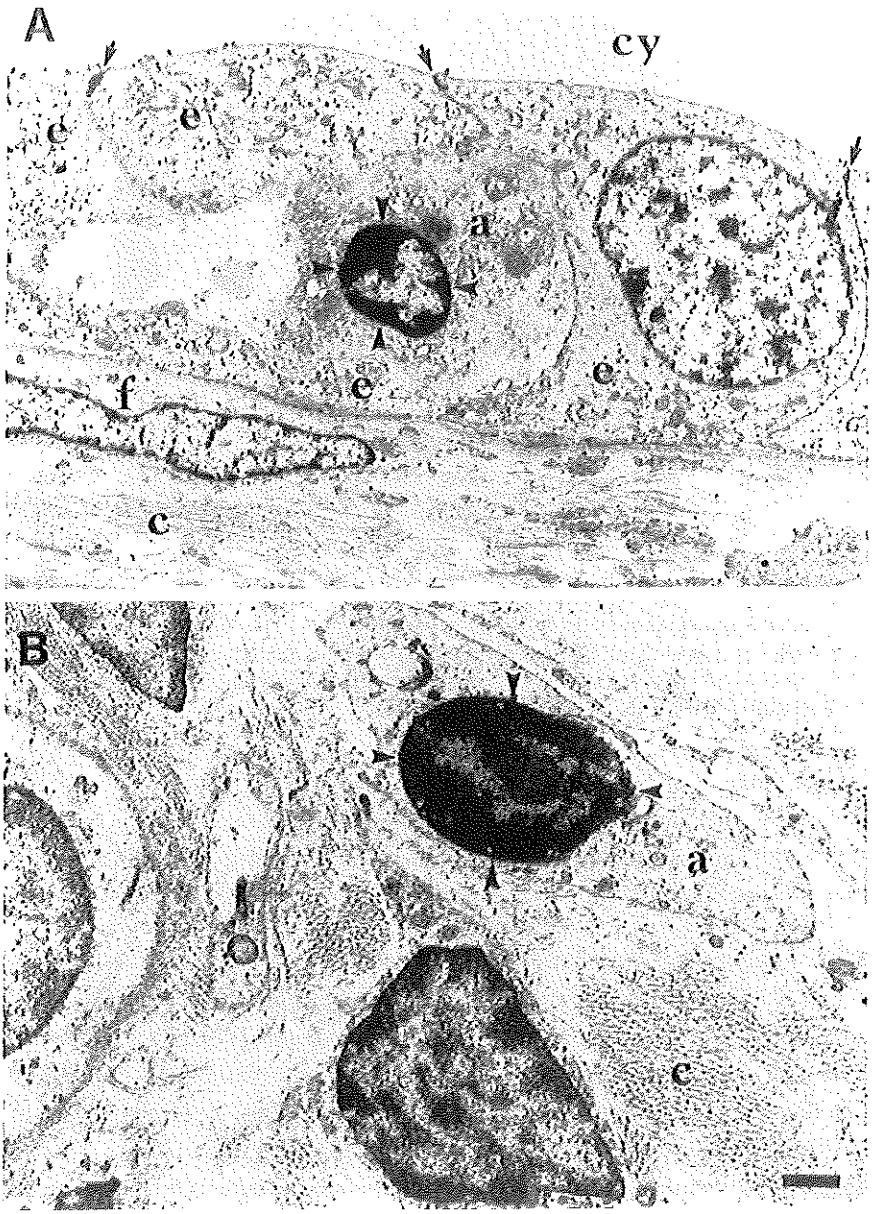


Figure 5. *Electron microscopy of dysplastic kidneys* A) Cyst wall showing apoptotic cell with condensed pyknotic nucleus surrounded by normal epithelial cells. The apoptotic cell appears to be shrinking. B) Poorly differentiated mesenchyme showing both normal cells and one cell undergoing apoptosis with nuclear condensation. Key: apoptotic cells (a), collagen (c), cyst (cy), epithelial cells (E), fibroblast (F). Arrows indicate the tight junctions between epithelial cells, arrowheads indicate pyknotic nuclei. Bars are 1mm.

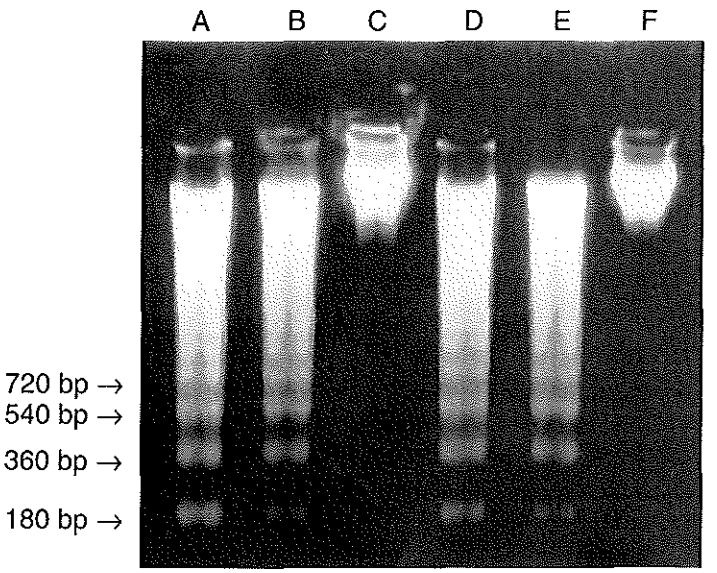


Figure 6. *DNA laddering in dysplastic kidneys.* DNA was extracted from dysplastic kidneys from infants and children, then electrophoresed through a 1.5 % agarose gel as described in the Methods. Lane A and lane B: Dysplastic kidney showing laddering. Lane C: Dysplastic kidney with no detectable ladder. Lanes D,E and F: Samples from the same kidney as A, B and C treated with RNAse A rule out RNA contamination.

often adjacent to areas of epithelial hypercellularity (Figs. 1E, 1F, 2D, 3C); (iii) and in the epithelium of undilated proximal tubules between large cysts (Fig 3D). In the ADPKD sample apoptosis was additionally observed within glomeruli with cystic dilatation of Bowman's capsule (Fig 2H).

Location of apoptosis in dysplastic human kidneys

A similar histological pattern of cell death was seen in prenatal and postnatal specimens. Apoptosis was prominent in areas of undifferentiated cells (Figure 4B, 4D and 5B) but was also, on occasion, observed in close proximity to dysplastic tubules (Figure 4C and 4E). In the multicystic dysplastic kidneys apoptosis was rarely detected in cystic epithelia and areas of cartilage did not contain apoptotic cells (data not shown). These sites of apoptosis were confirmed on electron microscopy: Figure 5A shows an electron micrograph of an apoptotic cell adjacent to normal epithelial cells in the wall of a cyst. Figure 5B shows an apoptotic cell in an undifferentiated area which ressembles uninduced mesenchyme. We found DNA laddering in two out of the three dysplastic samples (Figure 6) although histology revealed apoptotic nuclei in all samples; this might be explained by the finding that DNA is sometimes broken down into large fragments during apoptosis which cannot be visualised by the laddering technique [6].

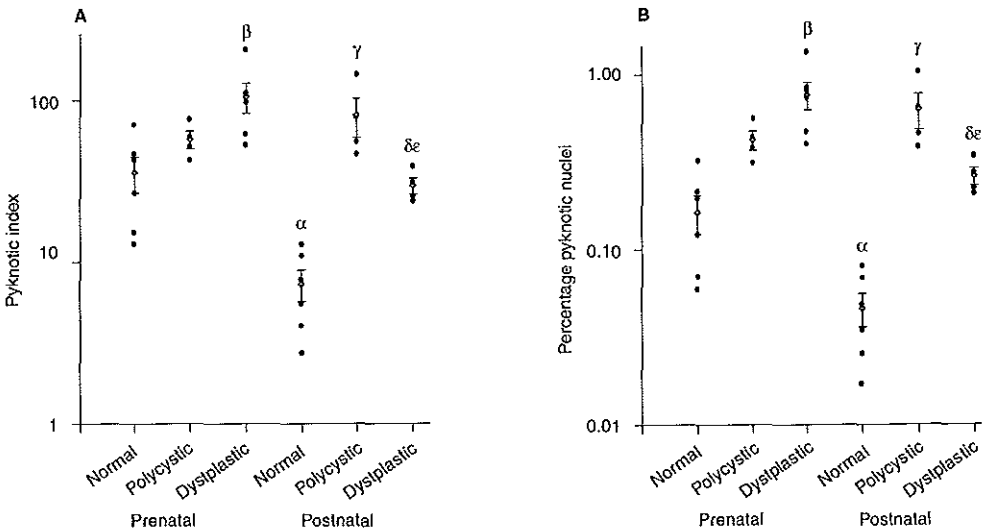


Figure 7. *Quantitation of apoptosis.* Apoptosis was quantified in tissue sections using the propidium iodide method described in the text and expressed as (A) Pyknotic Index corresponding to the mean number of apoptotic cells per 10 microscope fields from throughout the tissue and (B) Percentage of pyknotic nuclei. Groups were: normal prenatal kidneys, polycystic prenatal kidneys, dysplastic prenatal kidneys, normal postnatal kidneys, polycystic postnatal kidneys and dysplastic postnatal kidneys. Mean and SEM is shown. Significant differences were: α – normal prenatal versus normal postnatal ($p < 0.01$), β – dysplastic prenatal versus normal prenatal ($p < 0.001$), γ - polycystic postnatal versus normal postnatal ($p < 0.001$) and δ – dysplastic postnatal versus normal postnatal ($p < 0.002$) and ε – dysplastic prenatal versus dysplastic postnatal ($p<0.002$).

Quantification of apoptosis in human kidneys

Apoptotic ells were rare in normal postnatal kidneys, but significantly more death was seen in normal prenatal organs (Fig. 7; $p<0.01$). This difference provided the rationale for analyzing the quantity of apoptosis in dysplastic and polycystic patient samples in separate prenatal and postnatal groups. Dysplastic kidneys had more apoptosis than normal kidneys of a comparable age (Fig.7; prenatally $P<0.001$; postnataaly $P<0.002$), and the absolute levels of apoptosis fell significantly between the prenatal and postnatal period (Fig.7; $P<0.005$). In prenatal polycystic kidneys, apoptosis was not significantly greater than in the normal group. In the postnatal period, however, a high level of cell death was maintained, and this value was significantly higher than normal controls (Fig. 7; $P<0.001$). All postnatal polycystic kidneys contained high levels of apoptosis irrespective of the level of plasma creatinine (Table 2).

Apoptosis in animal experiments

Cpk/cpk mice had significantly more apoptosis than their phenotypically normal littermates both on the first day of life, when there was only mild dilation of the proximal tubules (Table 3; $P<0.01$), and at postnatal day 14 when the kidneys were grossly distende by distal cysts (Table 3; $P<0.01$). The location of apoptosis was similar to that seen in the human samples (data not shown). There was no significant difference in the level of apoptosis between mouse fetal kidneys which were either processed immediately or left in situ for 24 hours at 4°C before removal and fixation (Table 3). Similarly, no significance in the quantity of apoptosis was observed in mice kidneys which had been subjected to anaestesia and renal artery clamping versus organs which were immediately removed and processed (Table 3).

Table 3. Pyknotic index and percentage pyknotic nuclei in kidneys from animal experiments outlined in the text

Experimental group	Number	Pyknotic index *mean (sd)*	% Pyknotic nuclei *mean (sd)*
A			
Normal newborn	4	0.16 (0.10)	0.02 (0.004)
Polycystic newborn	4	1.58 (0.14)	0.07 (0.007)
Normal day 14	4	0.41 (0.29)	0.02 (0.008)
Polycystic day 14	4	1.37 (0.26)	0.08 (0.008)
B			
Control fetal mice	10	1.04 (0.76)	0.09 (0.06)
Storage for 24 hours	5	1.12 (0.84)	0.10 (0.07)
C			
Control adult mice	6	0.44 (0.25)	0.04 (0.02)
Operative group	8	1.08 (0.79)	0.09 (0.07)

Group A: Significantly more apoptosis in both cpk/cpk polycystic groups compared to age matched controls ($P<0.001$). Group B: No significant difference between fetal kidneys processed immediately or stored in situ for 24 hours. Group C: No significant difference in renal apoptosis between controladult mice and the operative group, which underwent anaestesia and renal artery clamping.

Discussion

In the adult kidney apoptosis has been described in animal models of glomerulonephritis [29] and in experimental reperfusion injury [30]. The results from the current study suggest that apoptosis also occurs in dysplastic renal malformations and childhood polycystic kidney diseases.

Apoptosis in normal kidneys

In normal human fetal kidneys apoptosis was detected in the nephrogenic cortex and the medulla. In view of the high level of apoptosis in comma-shaped bodies (Fig. 1A) we speculate that it my be involved in both nephron morphogenesis and controlling the number of cells within a nephron. Apoptosis also occurs between branches of the collecting ducts [2], an area where Potter described regression of the first generations of nephrons [11]. It could be argued that a delay of processing human specimens may have altered the degree of apoptosis, but we did not find any difference in the level of cell death in embryonic mice kidneys after prolonged storage at 4°C (Table 3). In addition, the distribution of apoptosis was similar to that described in a study in rats where developing kidneys were perfusion fixed immediately before harvesting [2]. We found a low level of apoptosis in proximal tubules of normal postnatal specimens (Figs. 2 and 7), but apoptosis was never observed in glomeruli, Henle loops or interstitial cells (Figs. 1 and 2). Conflicting studies have suggested that hypoxia / ischaemia causes apoptosis in renal epithelia in *ex vivo* experiments [31], but has no effect in cell culture [32]. In the current study, however, we found that anaestesia and surgery did not increase apoptosis in an animal model (Table 3). Other workers have also shown that apoptosis *in vivo* is only enhanced in the reperfusion phase after renal artery clamping [30,33]. We therefore suggest that there is balanced cell turnover in the mature human kidney with lost cells either dying by apoptosis or shed into the urine [34], and these are replaced by a low level of epithelial proliferation [35].

Deregulation of apoptosis in polycystic kidneys

We have made a number of original observations regarding apoptosis in polycystic kidneys obtained predominantly from patients with ARPKD. Firstly, in the postnatal period, pyknotic nuclei were observed in the interstitial tissue around undilated proximal and distal tubules (Figs. 1 to 3), but we never observed apoptosis in this location in normal postnatal kidneys. Secondly, apoptosis occurred in hyperproliferative epithelium of distal cysts (Figs. 1 and 3), and we also found undilated proximal tubules dying by apoptosis between cysts (Fig. 3). Lastly, the incidence of apoptosis was raised in postnatal polycystic kidneys (Table 3 and Fig. 7). Next we sought evidence of apoptosis in the *cpk/cpk* mouse ; here the kidney phenotypically resembles human ARPKD, but the histology is more homogeneous and the disease progression is highly predictable [18,27]. We found that apoptosis was increased, compared to phenotypically normal littermates, in both an early and a late stage of renal disease (Table 3). The animal data support our findings in human ARPKD and are also in accord with a study reporting apoptosis in human ADPKD [36]. Thus, enhanced apoptosis is found in different types of polycystic kidney disease. It is unknown how apoptosis relates to the genetic defects in human polycystic kidney diseases [37-40]. Evidence for a primary genetic derulation of renal

survival is, however, provided by mice with *bcl-2* null mutations which develop cystic kidneys [41,42]. Moreover, transgenic mice which overexpress c-myc develop renal cysts [43], and enhanced expression of this cellular protooncogene occurs in *cpk/cpk* mice [44,45]. *C-myc* cuses proliferation or apoptosis depending on the concentrations of ambient growth factors [46], and thus the imbalance of EGF [47,48] and HGF [49] reported in polycystic kidney diseases may affect cell survival. We speculate that proliferation and fluid secretion in polycystic kidney epithelia [16,19,50] outweigh the high levels of cell death reported in this study, hence causing incresed size of the kidneys with concomitant destruction of functional renal tissue.

Deregulation of apoptosis in dysplastic kidneys

Most apoptosis in dysplastic kidneys occurred in cells located around dysplastic tubules. Cells in this area superficially remble undifferentiated mesenchyme which, in the normal metanephros, will differentiate into nephrons [11-13]. We suggest that enhanced apoptosis in dystplastic organs may prevent the differentiation of these precursors. Moreover, we hypothesize that enhanced cell death may contribute to the well recognized phenomenon of of spontaneous involution of dysplastic kidneys [51-53]. During normal nephrogenesis, bcl-2 is highly expressed in nephron precursors [54,55], and this protein may be required to prevent the death of cells during the mesenchymal to epithelial transition. Preliminary data from our laboratory [54] suggest that bcl-2 is highly expressed in dysplastic tubules where apoptosis is rare, but is only weakly expressed in undifferentiated cells where apoptosis is found. In humans, renal dysplasias may occur in conjunction with renal obstruction (such as posterior urethral valves) and some, but not all, animal studies suggest that prenatal obstruction causes dysplasia [56-58]. Increased apoptosis is seen in experimental urinary obstruction in the postnatal period [59,60] and is associated with increased renal TGF-β [61], a molecule implicated in epithelial apoptosis [8]. However, only a minority of our patients had obstructed kidneys (Tables 1 and 2). Renal malformations occur in mice with null mutations for *ret* [62], *wnt-4* [63] and *RARs* [64], but apoptosis has not been studied in these models, and none of them faithfully mimic human multicystic renal dysplasia.

Conclusions

In summary, we have found that apoptosis occurs during normal human kidney development in locations similar to those reported in animals, and continues at a very low level after birth. More importantly, apoptosis occurs at a high level in ARPKD and dysplastic kidneys, which are important causes of end-stage renal failure in infancy and childhood [21]. The fact that apoptosis can be reduced by various molecular means both *in vitro* and *in vivo* [2,14,65] may suggest novel therapeutic strategies for these diseases.

Acknowledgments

This work is supported by grants from Action Research to P. J. D. W.; from the National Kidney Research Fund to A. S. W.; and from the Wellcome Trust to P. H. We would like to thank Drs Philip Ransley, Patrick Duffy, Jeeta Dhillon, Fergal Quinn and Helen Pardoe for their help in collecting the surgical specimens and Glenn Anderson, Dianne Rampling, and Sue Howard for their technical assistance. Part of this work was presented in abstract form at the 27 th American Society of Nephrology meeting in Orlando in October 1994. This manuscript was reprinted by permission of Blackwell Science, Inc.

References

1 **Kerr** JFR, Wyllie AH, Currie AR: Apoptosis: A basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 26:239-57, 1972

2 **Coles** HSR, Burne JF, Raff MC: Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development 118 :777-784, 1993

3 **Wyllie** AH: Glucocorticoid-induced thymocye apoptosis is associated with endogenous endonuclease activation. Nature 284:555-556, 1980

4 **Gavrelli** Y, Sherman Y, Ben-Sassoon SA: Identification of programmed cell death in situ via specific labeling of DNA fragmentation. J Cell Biol 119:493-501, 1992

5 **Schwartz** LM, Smith SW, Jones ME, Osborne BA: Do all programmed cell deaths occur via apoptosis ? Proc Soc Natl Acad Sci USA 90:980-984, 1993

6 **Bortner** CD, Oldenburg NBE, Cidlowski JA: The role of DNA fragmentation in apoptosis. Trends in Cell Biology 5:22-27, 1995

7 **Vaux** DL: Towards an understanding of the molecular mechanisms of physiological cell death. Proc Soc Natl Acad Sci USA 90:786-789, 1993

8 **Oberhammer** F, Wilson JW, Dive C, Morris ID, Hickmann JA, Wakeling AE, Walker PR, Sikorska M: Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 12:3679-3684, 1993

9 **Collins** MKL, Perkins GR, Rodriguez-Tarduchy G, Nieto MA, Lopéz-Rivas A: Growth factors as survival factors: regulation of apoptosis. Bio Essays 16:133-138, 1994

10 **Wyllie** AH, Kerr JFR, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 68:251-306, 1980

11 **Potter** EL: Normal and abnormal development of the kidney. Chicago USA, Year Book Medical Publishers Inc, 1972

12 **Saxen** L: Organogenesis of the Kidney. Cambridge UK, Cambridge University Press, 1987

13 **Hardman** P, Kolatsi M, Winyard PJ, Towers PR, Woolf AS: Branching out with the ureteric bud. Exp Nephrol 2:211-219, 1994

14 **Koseki** C, Herzlinger D, Al-Awqati Q: Apoptosis in metanephric development. J Cell Biol 119:1322-1333, 1992

15 **Woolf** AS, Kolatsi-Joannou M, Hardman P, Andermarcher E, Moorby C, Fine LG, Jat PS, Noble MD, Gherardi E: Roles of hepatocyte growth factor/scatter factor (HGF/SF) and met in early development of the metanephros. J Cell Biol 128:171-184, 1995

16 **Wilson** PD, Hreniuk D, Gabow PA: Abnormal extracellular matrix and excessive growth of human adult polycystic kidney disease epithelia. J Cell Physiol 150:360-369, 1992

17 **Avner** ED, Sweeney WE, Nelson WJ: Abnormal sodium pump distribution during renal tubulogenesis in congenital murine polycystic kidney disease. Proc Soc Natl Acad Sci USA 89:7447-7451, 1992

18 **Woo** DDL, Miao S, Pelayo J, Woolf AS: Taxol inhibits congenital polycystic kidney disease progression. Nature 368:750-753, 1994

19 **Grantham** JJ: 1992 Homer Smith Award. Fluid secretion, cellular proliferation, and the pathogenesis of renal epithelial cysts. J Am Soc Nephrol 3:1841-1857, 1992

20 **Risdon** RA: Renal dysplasia. Part I. A clinico-pathological study of 76 cases. J Clin Path 24:57-71, 1971

21 **Woolf** AS: Clinical impact and biological basis of renal malformations. Seminars in Nephrology. 15:361372, 1995

22 **Kreidberg** JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R: WT-1 is required for early kidney development. Cell 74:679-691, 1991

23 **Verani** R, Walker P, Silva FG: Renal cystic disease of infancy: results of histochemical studies. Ped Nephrol 3:37-42, 1989

24 **Holthoffer** H, Virtanen I, Pettersson E, Tornroth T, Alfthan O, Linder E, Miettinen A: Lectins as fluorescence microscopic markers for saccharides in the human kidney. Lab Inv 45:391-399, 1981

25 **Bland** JM, Altmann DG: Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 8476:307-310, 1986

26 **Sambrook** J, Fritsch EF, Maniatis T: Molecular cloning: A laboratory manual. Cold Spring Harbor, Cold spring Harbor Laboratory Press, 1989

27 **Avner** ED, Studnecki FE, Young MC, Sweeney WE, Jr, Piesco NP, Ellis D, Fetterman GH: Congenital murine polycystic kidney disease. Pediatric Nephrol 1:587-596, 1987

28 **Gattone** II VH, Calvet JP, Cowley Jr. BD, Evan AP, Shaver TS, Helmstadter K, Grantham JJ: Autosomal recessive polycystic kidney disease in a murine model. A gross and microscopic description. Lab Invest 59:231-238, 1988

29 **Savill** J, Smith J, Sarraf C, Ren Y, Abbott F, Rees A: Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. Kidney Int 42:924-936, 1992

30 **Schumer** M, Colombel MC, Sawczuk IS, Gobe G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R: Morphological, biochemical and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischaemia. Am J Pathol 140:831-838, 1992

31 **Beeri** R, Symon Z, Brezis M, Ben-Sasson SA, Baehr PH, Rosen S, Zager RA: Rapid DNA fragmentation from hypoxia along the thick ascending limb of rat kidneys. Kidney Int 47:1806-1810, 1995

32 **Iwata** M, Torok-Storb B, Zager RA: An evaluation of Renal Tubular DNA laddering in response to Oxygen deprivation and Oxidant injury. J Am Soc Nephrol 5:1307-1313, 1994

33 **Fuerstenberg** SM, Torok-Straub B, Zager RA: Evidence against apoptosis as a dominant mechanism for post-ischemic/post-hypoxic proximal tubular necrosis. (abstract) J Am Soc Nephrol 4:735, 1993

34 **Racusen** LC, Fivush BA, Andersson H, Gahl WA: Culture of renal tubular cells from the urine of patients with nephropathic cystinosis. J Am Soc Nephrol 1:1028-1033, 1991

35 **Laurent** G, Toubeau G, Heuson-Stiennon JA, Tulkens P, Maldague P: Kidney tissue repair after nephrotoxic injury: Biochemical and Morphological characterisation. CRC Crit Rev Toxicol 19:147-183, 1988

36 **Woo** DDL: Apoptosis and loss of renal tissue in polycystic kidney diseases. N Eng J Med 333:18-25, 1995

37 **Zerres** K, Mucher G, Bachner L, Deschennes G, Eggerman T, Kaariainen H, Knapp M, Lennert T, Misselwitz J, von Munlendahl KE, Neumann HPH, Pirson Y, Rudnik-Schoneborn S, Steinbicker V, Wirth B, Scharer K: Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. Nature Genetics 7:429-432, 1994

38 **European** polycystic kidney disease consortium: The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. Cell 77:881-894, 1994

39 **European** chromosome 16 tuberous sclerosis consortium: Identification and characterisation of the tuberous sclerosis gene on chromosome 16. Cell 75:1305-1315, 1993

40 **Latif** F, Tory K, Gnarra J, Yao M, Duh F-M, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, Schmidt L, Zhou F, Li H, Wei MH, Chen F, Glenn G, Choyke P, Walther MM, Weng Y, Duan D-SR, Dean M, Glavac D, Richards FM, Crossey PA, Ferguson-Smith MA, Le Paslier D, Chumakov I, Cohen D, Chinault AC, Maher ER, Linehan WM, Zbar B, Lerman MI: Identification of the von Hippel-Lindau disease tumor suppressor gene. Science 260:1317-1320, 1993

41 **Veis** DJ, Sorenson CM, Shutter JR, Korsmeyer SJ: bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys and hypopigmented hair. Cell 75:229-240, 1994

42 **Sorenson** CM, Rogers SA, Hammerman MR, Korsemeyer SJ: Fulminant metanephric apoptosis in abnormal kidney development in bcl-2 deficient mice. Am J Physiol 268:73-81, 1995

43 **Trudel** M, D'Agati V, Costantini F: C-myc as an inducer of polycystic disease in transgenic mice. Kidney Int 39:665-671, 1991

44 **Cowley** Jr. BD, Smardo Jr. FL, Grantham JJ, Calvet JP: Elevated c-myc proto-oncogene expression in autosomal recessive polycystic kidney disease. Proc Soc Natl Acad Sci USA 84:8394-8398, 1987

45 **Harding** MA, Gattone II VH, Grantham JJ, Calvet JP: Localization of overexpressed c-myc mRNA in polycystic kidneys of the cpk mouse. Kidney Int 41:317-325, 1992

46 **Harrington** EA, Bennett MR, Fanidi A, Evan GI: c-Myc induced apoptosis in fibroblasts is inhibited by specific cytokines. EMBO J 13:3286-3295, 1994

47 **Gattone** II VH, Andrews GK, Fu-wen N, Chadwick LJ, Klein RM, Calvet JP: Defective Epidermal Growth Factor Gene Expression in Mice with Polycystic Kidney Disease. Dev Biol 138:225-230, 1990

48 **Horikoshi** S, Kubota S, Martin GR, Yamada Y, Klotman PE: Epidermal growth factor (EGF) in the congenital polycystic mouse kidney. Kidney Int 39:57-62, 1991

49 **Horie** S, Higashihara E, Nutahara K, Mikami Y, Okubo A, Kano M, Kawabe K: Mediation of renal cyst formation by hepatocyte growth factor. Lancet 344:789-791, 1994

50 **Ye** M, Grantham JJ: The secretion of fluid by renal cysts from patients with autosomal dominant polycystic kidney disease. N Engl J Med 329:310-313, 1993

51 **Dungan** JS, Fernandez MT, Abbitt PL, Thiagarajah S, Howards SS, Hogge WA: Multicystic dysplastic kidney: natural history of prenatally detected cases. Prenat Diag 10:175-182, 1990

52 **Mesrobian** H-GJ, Rushton HG, Bulas D: Unilateral renal agenesis may result from in utero regression of multicystic dysplasia. J Urol 150:793-794, 1993

53 **Al-Khaldi** N, Watson AR, Zucollo J, Twining P, Rose DH: Outcome of antenatally detected cystic dysplastic kidney disease. Arch Dis Child 70:520-522, 1994

54 **Winyard** PJD, Risdon RA, Woolf AS: WT1 expression in the absence of bcl-2 may explain excessive apoptosis which occurs in human kidney malformations. (abstr) J Am Soc Nephrol 6:729, 1995

55 **LeBrun** DP, Warnke RA, Cleary ML: Expression of bcl-2 in Fetal Tissues suggests a Role in Morphogenesis. Am J Pathol 142:743-753, 1993

56 **Berman** D, Maizels M: The role of urinary obstruction in the genesis of renal dysplasia. J Urol 128:1091-1100, 1982

57 **Steinhardt** GF, Vogler G, Salinas-Madrigal L, LeRegina M: Induced renal dysplasia in the young pouch opossum. J Ped Surg 23:1127-1130, 1988

58 **Gonzalez** R, Reinberg Y, Burke B, Wells T, Vernier RL: Early bladder outlet obstruction in fetal lambs induces renal dysplasia and the prune-belly syndrome. J Ped Surg 25:342-34, 1990

59 **Connor** J, Buttyan R, Olsson CA, D'Agati V, O'Toole K, Sawczuk IS: SGP-2 expression as a genetic marker of progressive cellular pathology in experimental hydronephrosis. Kidney Int 391098-1103, 1991

60 **Kennedy** II WA, Sternberg A, Lackgren G, Hernsle TW, Sawczuk IS: Renal tubular apoptosis after partial ureteral obstruction. J Urol 152:658-664, 1994

61 **Pimentel** JL, Kopp JB, Sunden JL, Martinez-Maldonado M: TGF-b induction in unilateral ureteric obstruction. J Am Soc Nephrol 4:474, 1993

62 **Schuchardt** A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V: Defects in kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367:380-383, 1994

63 **Stark** K, Vainio S, Vassileva G, McMahon AP: Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372:679-683, 1994

64 **Mendelsohn** C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P, Mark M: Function of the retinoic acid receptors (RAR) during development. Development 120:2749-2771, 1994

65 **Kennedy** II WA, Buttyan R, Sawczuk IS: Epidermal Growth Factor (EGF) suppresses renal tubular apoptosis following ureteral obstruction. J Am Soc Nephrol 4:738, 1993

3.2

Immunological detection of polycystin-1 in human kidney

Jeroen Nauta, Miriam A. Goedbloed,
Ans M.W. van den Ouweland, Mark Nellist, André T. Hoogeveen

Departments of Pediatric Nephrology and Clinical Genetics
Erasmus Medical Center Rotterdam, The Netherlands

Immunological detection of polycystin-1 in human kidney

Abstract

Polycystin-1 is the protein product of the PKD1 gene. Mutations in this gene are responsible for most cases of polycystic kidney disease, but little is known about how these mutations lead to the development of cysts. Indeed, even less is known about the normal role of polycystin-1 in the kidney. The cellular localisation of polycystin-1 has been the subject of intense investigation by many groups, including ours. In this report we describe our results and compare our data with those of others. We generated 14 different polyclonal antisera against fragments of the predicted 462 kD polycystin-1 molecule to enable us to investigate the expression of polycystin-1 in cells and tissues by immunocytochemistry, Western blotting and immunoprecipitation. Our antibodies readily recognised a 134 kD polycystin-1 fragment overexpressed in COS cells and stained the epithelial cells of fetal, adult and cystic kidney sections with the same pattern as reported by others. However, further investigations revealed that this pattern was not specific for polycystin-1. We could not unequivocally detect polycystin-1 in vivo, either by immunoblotting or immunocytochemistry. Therefore our studies do not support the reported pattern of polycystin-1 expression in the kidney.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited diseases, affecting approximately 1 in 1000 individuals. Cystic destruction of the kidneys causes renal failure in approximately half of the patients, usually by the age of 50 (Welling and Grantham 1996). In most cases cysts are also formed in the biliary tract and, less frequently, in the pancreas, spleen, ovary and testis. In addition to lesions in epithelial tissue, ADPKD is also associated with connective tissue abnormalities including colonic diverticula, arterial aneurysms and dysfunctional cardiac valves.

Mutations in the PKD1 gene are responsible for approximately 85% of cases (Welling and Grantham 1996), while most of the remaining cases result from mutations to the PKD2 gene (Mochizuki et al. 1996).

PKD1 encodes polycystin-1, a 4302 amino acid (462kD) protein. Polycystin-1 is most likely a membrane-spanning glycoprotein, featuring multiple extracellular domains that may be involved in cell-cell or cell-matrix interactions and several intracellular phosphorylation sites that could be involved in signal transduction (Hughes et al. 1995; Sandford et al. 1997; The International PKD Consortium 1995). At least three other

transcripts that are approximately 97% identical to PKD1, are expressed from multiple copies of the genomic region containing PKD1 exons 1 - 31. It is not known whether these transcripts code for protein.

We have developed and tested a large number of antibodies against polycystin-1. Here we show that our antisera recognise specifically a large fragment of polycystin-1 when this is over-expressed in COS cells. The renal epithelial staining obtained with our antisera is in good agreement with published reports (Aguiari et al. 1997; Geng et al. 1996; Geng et al. 1997; Griffin et al. 1996; Griffin et al. 1997a; Griffin et al. 1997b; Ibraghimov-Beskrovnaya et al. 1997; Ong et al. 1999a; Ong et al. 1999b; Palsson et al. 1996; Peters et al. 1996; Peters et al. 1999; Van Adelsberg et al. 1997; Ward et al. 1996; Weston et al. 1997; Wilson 1997; Wilson and Burrow 1999). However, we have been unable to demonstrate that this staining pattern is polycystin-1 specific. In view of our experience we propose that additional work is required before polycystin-1 can be reliably detected by immunocytochemistry .

Methods

Animals and tissues

Three month old New Zealand White rabbits were obtained from a commercial breeder (Charles River, Someren, The Netherlands). The animals were selected for immunization based on low immunoreactivity of their preimmune sera against either adult or fetal human renal tissue, when tested on Western blots and/or tissue sections. Sera from chicken, goat, guinea pig and mice were obtained from the same breeder.

Normal renal tissue was obtained from tumor nephrectomy specimens and from a donor kidney that could not be used for transplantation (a kind gift from Eurotransplant, Leiden). Fetal renal tissue was obtained at 14-16 weeks. Cystic kidneys were surgically obtained from three ADPKD patients with end stage renal disease.

Constructs, peptides and proteins

Sequences of peptides and fusion proteins used during the course of this work are given in Table 1. Figure 1 shows their relative positions in the putative polycystin-1 molecule. Two peptides corresponding to the aminoacid sequence of polycystin-1 were produced using solid phase peptide synthesis in a continuous flow manner, using Fmoc strategy on a Novasyn-Crystal automated peptide synthesizer (Erickson 1976), conjugated to Keyhole-Limpet Hemocyanin (KLH) and purified by reverse phase high performance liquid chromatography. Purity was confirmed by amino acid sequencing.

cDNA clones AH4, 3A3 and AH3 (The European PKD Consortium 1994) were trimmed to smaller fragments by restriction enzyme digestion and cloned into either the

pGEX (GST) or the pQE30 (HIS) expression vectors. GST and HIS-tagged fusion proteins were expressed in E.Coli (XL-1 blue). HIS3A3-protein (aminoacids (aa): 3725-3871) was purified on a Ni-NTA column according to the manufacturers instructons (Diagen in Hilden, Germany). GSTAH4-protein (aa: 4275-4302) was purified on a glutathione sepharose 4B column (Pharmacia) as recommended by the manufacturer. GSTAH4-protein (aa: 4095-4302), GSTAH3-protein (aa: 3133-3322) and HISAH3-protein (aa: 3133-3322) were purified on SDS-PAGE preparative gels.

The AH8-cDNA encodes the C-terminal 1225 aminoacids of polycystin-1, including all the epitopes recognised by our antisera. AH8-cDNA, cloned into the pcDNA3 expression vector was a kind gift of P.C.Harris and C.J.Ward, Oxford.

Antiserum production

Rabbits were immunized using 100-250 µgram of protein in PBS, mixed as a 1:1 emulsion with Freund's complete adjuvant. Booster emulsions in Freund's incomplete adjuvant were injected at 3 weeks and then at 6 weeks. Sera were obtained 9-14 days following injection. The immune reactivity of the antisera was tested against the corresponding immunogen, using either ELISA (anti-peptide sera) or Western blot analysis (anti-fusion protein sera). The antisera were affinity purified, using the antigens GSTAH4 (aa: 4275-4302 and aa: 4095-4302), GSTAH3 (aa: 3133-3322), HIS3A3 (aa: 3725-3871) and GST alone, either spotted on blotting paper or coupled to a CNBR-activated sepharose 4B column (Pharmacia). The unbound and purified fractions were subsequently tested against the corresponding antigens (dot blots) and on AH8 transfected COS cells.

Expression of AH8

AH8-cDNA was transcribed and translated in vitro in the presence of ^{35}S-methionine, according to the manufacturers instructions (TNT kit, Promega, USA). This construct was also used to transfect COS cells, using Lipofectamine™ according to manufacturers instructions (Life Technologies, Gaithersburg, MD, USA). Transfected cells were grown for 2 days and then used for immunocytochemistry or immunoblotting experiments.

Western blotting and immunoprecipitation

AH8 transfected COS cells were sonicated in PBS in the presence of protease inhibitors, electrophoresed through a 8% SDS/polyacrylamide gel, and blotted onto Immobilon-P (Millipore). Homogenates from renal tissues in 50mM Tris-EDTA and cultured cells in either PBS, Tris or RIPA buffer (150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50mM Tris) were similarly sonicated in the presence of protease inhibitors, including leupeptin (ICN), pepstatinA (ICN), antipain (Sigma) and Pefabloc (Boehringer).

Membrane fractions were obtained according to Geng (Geng et al. 1997). For the detection of polycystin-1, the extracts were electrophoresed in 5% gels or 5-15% gradient gels and blotted. Antisera were diluted 1:1.000-1:5.000. Binding of antibody was visualized by enhanced chemiluminescence (ECL, Amersham), using horseradish peroxidase conjugated swine-anti-rabbit IgG as a secondary antibody (DAKO).

For immunoprecipitation protein extracts from in vitro translation products and detergent lysates of AH8 transfected COS cells were incubated with antibodies and with protein A sepharose beads. The precipitates were washed extensively in RIPA buffer and incubated in SDS sample buffer, prior to electrophoresis. After electrophoresis, gels containing ^{35}S methionine labeled proteins were fixed and immersed in Amplify (Amersham) for 15 minutes, dried and exposed to X-ray film for several days. Gels containing precipitates of unlabeled protein were immunoblotted as before.

Immunocytochemistry

Tissue for immunocytochemistry was fixed in paraformaldehyde (4%) prior to embedding in paraffin. Sections were incubated in 1% H_2O_2 to block endogenous peroxidase activity and aspecific binding was blocked with Protifar (0.5%) (Nutricia, The Netherlands), glycine (0.15%). For the identification of polycystin-1 we explored a number of antigen unmasking methods, including incubation in 0.1% pronase for 5 minutes, or in 0.2% SDS for 5 minutes, or in 0.1% or 1.0% Triton X-100 for 5 minutes, or in a microwave for 3 minutes in 10mM citrate. Antigen unmasking had no significant effect on the staining. The sections were subsequently immunostained using an indirect immunoperoxidase protocol including primary antibodies (dilution 1:100-500), horseradish peroxidase conjugated swine-anti-rabbit immunoglobulin as a secondary antibody at 1:100 (DAKO) and 3,3'diaminobenzidine.HCl as substrate. Affinity purified antibodies were used at concentrations four times higher than used for the corresponding crude sera. Controls included replacement of the primary antiserum by both the corresponding preimmune serum and by PBS. Immunocytochemistry with affinity purified sera was additionally controled using the unbound fraction as a primary step.

Cultured cells and fresh frozen tissue sections were fixed in either 4% paraformaldehyde for 10 minutes, followed by methanol for 20 minutes and 1% H_2O_2, or with methanol for 5 minutes at –20°C, or with acetone at –20°C for either 2 seconds or 2 minutes. Since the results with these protocols were not significantly different, we used the first protocol as a standard. Blocking aspecific binding and subsequent immunostaining was as described for paraffin embedded sections.

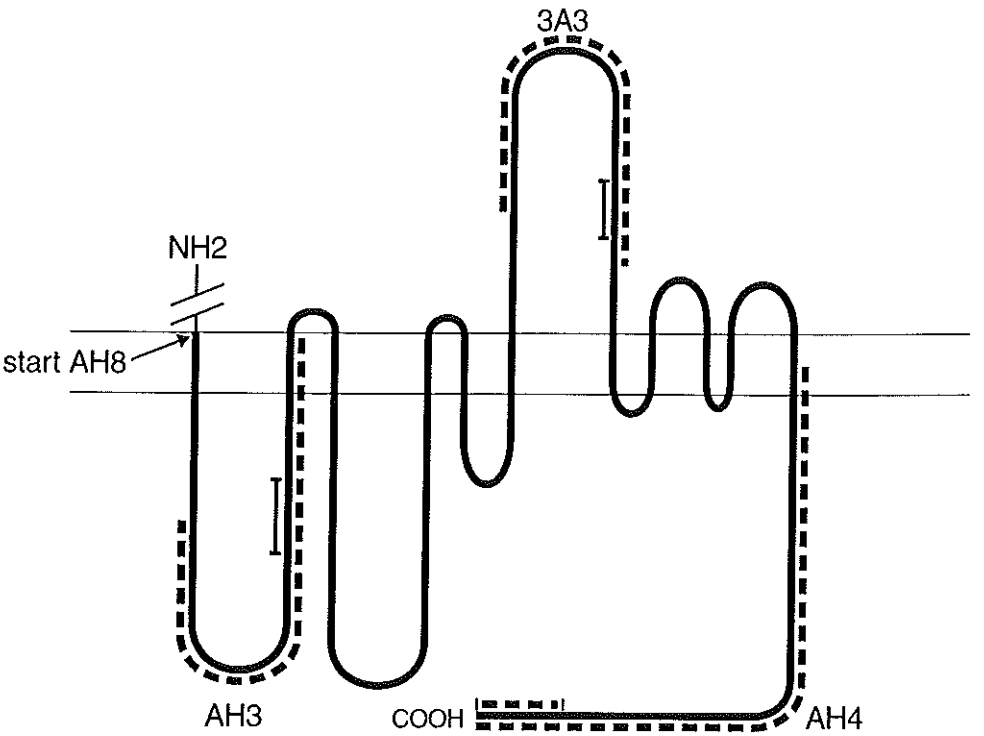


Figure.1 Schematic representation of the intracellular and membrane spanning domains of polycystin-1 based on amino acid sequence and domain analysis (Sandford et al. 1997, Hughes et al. 1995). This region corresponds to the AH8 encoded polycystin-1 fragment (aa 3078-4302) used to test the antisera. Also indicated are the polycystin-1 fragments corresponding to the fusion proteins (interrupted lines) and peptides (solid bars) used to generate domain specific antisera. AH3 includes amino acids 3133-3322, 3A3 includes aa 3725-3871 and AH4 includes aa 4095-4302.

Results

Characterisation of anti-polycystin-1 antisera

Polyclonal antibodies against peptides and fusion proteins derived from different parts of the C-terminal domain of polycystin-1 were raised in 14 New Zealand White rabbits (Figure 1, Table 1.). The immunoreactivity of each serum was tested against the corresponding immunogen and against AH8, a 134kDa protein containing the entire C-terminal domain of polycystin-1 (Ward et al. 1996). The best results were obtained with antiserum 2226. This was raised against AH4, a GST fusion protein containing the 208 most C-terminal amino acids of polycystin-1. Serum 2226 was affinity purified using the same fusion protein.

Table 1. Immunoreactivity of 14 antisera tested against AH8

		AH8 In vitro translation	AH8 COS transfection	
Anti serum	Raised against Epitopes [a] :	Immuno precipitation	Western blot	Immuno Cytochem.
1633	3232-3255	±	-	+
1805	3133-3322-GST fusion	-	nd	nd
1932	3133-3322-HIS fusion	+	+	±
2225	"	nd	+	+
1635	3847-3865	-	nd	nd
1923	3725-3871-HIS fusion	++	+	+
1931	"	++	+	±
1818	4275-4302-GST fusion	+	+++	+
2118	"	nd	+++	+
2224	"	nd	+++	+
1815	4095-4302-GST fusion	+	++	nd
1930	"	++	+++	+
2226	"	+	+++	++
1630	'1590-1614' [b]	nd	-	-

[a] Polycystin-1 fragments used for immunization. Aminoacid sequence numbers correspond to reference: Hughes 1995. [b] Sequence corresponds to amino acids 1590-1614 of the initially reported PKD1 sequence (The Eur PKD Cons 1994), later shown not to be part of the PKD1 coding region (Hughes 1995). nd: not done.

In vitro translation of an expression construct encoding the C-terminal domain of polycystin-1 gave the expected 134kDa AH8 protein product. This protein was immunoprecipitated by the 2226 antiserum, but not by the corresponding preimmune serum. Next, COS cells were transfected with the AH8 expression construct. Again, 2226 detected a single protein band of the expected size (134kDa), both by immunoprecipitation and directly on a Western blot (Figure 2.). Immunocytochemical analysis of the transfected COS cells with 2226 clearly indicated strong expression of the AH8 protein. The staining was localised to the intracellular compartment and had a meshwork like pattern (Figure 4A.). 2226 did not react with untransfected COS cells nor with cells over-expressing other, unrelated proteins. The reactivity of the affinity purified

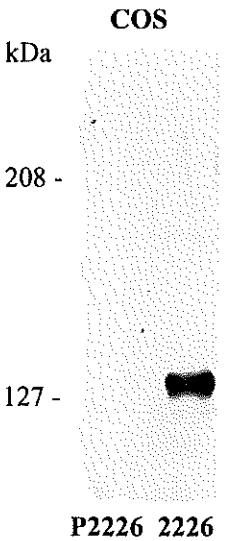


Figure.2. Western blot of AH8 transfected COS cell homogenates stained with serum 2226 and with the corresponding preimmune serum (P2226). No proteins other than AH8 were recognised.

preparation was comparable with the crude 2226 immune serum, while the 2226 preimmune serum and the unbound fraction after affinity purification were negative (Figure 4.).

In addition to 2226 several other antisera (Table 1.) were also strongly immunoreactive to AH8. However, unlike 2226, these antisera not only recognised additional protein bands on Western blots of transfected and untransfected COS cells but also weakly stained untransfected cells in the immunocytochemical experiments (not shown). None of the additional (non-AH8) protein bands were recognised on Western blots by more than one antiserum. In each case the corresponding preimmune serum was less reactive. That is, it recognised fewer protein bands on Western blots (or none at all) and did not stain untransfected COS cells in immunocytochemichal experiments.

Since we could show that 2226 was specific for polycystin-1, we performed Western blot analysis of normal human kidney tissue and human fibroblast and ureteric bud cells in culture with the crude 2226 immune serum (Figure 3.). We were unable to detect a protein band corresponding to polycystin-1, suggesting that the protein is either not expressed at detectable levels, or lost during sample preparation. Interestingly, we noticed that the AH8 protein was unstable, when over-expressed in COS cells. This 130 kD polycystin fragment was not easy to detect unless the lysates were analysed immediately after preparation.

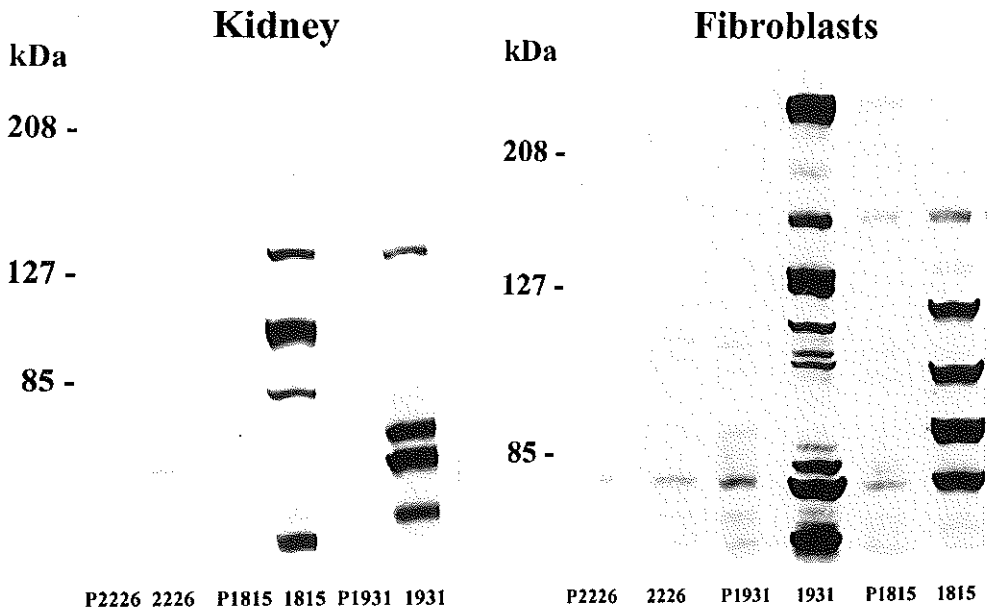


Figure. 3. Western blots of homogenates of normal adult renal tissue and of normal human fibroblasts, stained with three different antisera and with the corresponding preimmune sera, (P)2226, (P)1931 and (P)1815. Serum 2226 did not recognise any protein. The 2 other antisera illustrate the induction of immunoreativity during the immunisation procedure. However, the stained protein bands most likely did not represent polycystin-1 or polycystin-1 breakdown products since they were either not recognised by more than one antiserum or also recognised by one or more preimmune sera. Moreover these bands were usually not of the expected molecular weight.

The same analysis was carried out using the other antisera raised against polycystin-1. Several different protein bands were detected by the different antisera (Figure 3). However, in addition to not being recognised by 2226, these bands were either not of the expected size, not recognised by more than one antiserum or were also recognised by one or more preimmune sera. It is therefore unlikely that they represent polycystin-1 or polycystin-1 breakdown products.

Immunocytochemical analysis of human renal tissues using 2226

Immunocytochemical analysis of adult, fetal and cystic kidneys with the crude 2226 immune serum revealed specific staining of renal tubular epithelia (Figure 4.). In fetal kidneys robust staining of the ureteric bud and medullary collecting tubules was observed. This staining was either cytoplasmic or confined to basal cell surfaces. Moderate staining was seen in some of the cortical tubules and occasionally in the parietal epithelium of Bowman's capsule. In adult kidneys a weak cytoplasmic staining of all tubular segments, but not of Bowman's capsule, was noted. In end stage kidneys from three ADPKD

patients many cysts were stained. The staining intensity varied between cysts and cellular distribution was cytoplasmic. No immunoreactivity was detected against glomeruli or interstitium. Cultured human fibroblasts or human renal epithelial cells were not stained by 2226.

Although the 2226 preimmune serum did not stain adult or cystic kidneys, it stained the fetal ureteric bud and collecting tubules even more intensely than the crude 2226 immune serum did.

Affinity purification of 2226 abolished completely the reactivity against both fetal and adult tissue. The unbound fraction, however, stained the ureteric bud and collecting tubules similar to the crude 2226 immune serum. Taken together, these results indicate that the observed staining of the fetal uteric bud and collecting tubules is very unlikely to represent polycystin-1.

Immunocytochemical analysis of human renal tissues using other antisera against polycystin-1

In addition to 2226, we generated 13 other polyclonal antisera against polycystin-1 (see above). In contrast to their corresponding preimmune sera which were unreactive, most antisera stained renal tubular epithelia similar to 2226. In each case, the staining was cytoplasmic or confined to basal cell surfaces. The fetal ureteric bud and branches were stained by all antisera and by one preimmune serum (preimmune 2225). In addition, some antisera stained more proximal fetal tubular segments and, occasionally, the parietal cells of Bowman's capsule. Adult kidneys were stained by some, but not all antisera. If present, this staining was either confined to distal tubules and collecting ducts or localised to all tubular epithelia. Kidney sections from patients with ADPKD also stained positive with a number of antisera. This consisted of intracellular staining of normal tubules and of approximately 70-90% of the cysts. Some of the immunoreactive cysts were more robustly stained than the unaffected tubules.

Figure.4. (next page) Immunocytochemistry of AH8 transfected COS cells (COS-AH8), a 14 week old human fetal kidney, a normal adult kidney and an end stage cystic kidney from one of the patients with ADPKD. Staining with antiserum 2226 (A-D), affinity purified serum 2226 (E-H), the unbound fraction of this purification (I-L), the corresponding preimmune serum P2226 (M-P) and a polycystin-1-unrelated antiserum (1630), raised against a nonsense peptide (Q-T). The efficiency of the affinity purification is demonstrated by the COS-AH8 experiments. Purified 2226 did not stain any renal structures in either normal, fetal or cystic kidneys. Note that most other serum samples gave considerable polycystin-1-unrelated background staining of human renal epithelia. The unrelated antiserum 1630 gave a similar staining pattern as 2226 on fetal and cystic kidneys. In adult kidneys relative intense staining with 1630 was noted in distal tubules and collecting ducts, when compared to proximal tubules (panel S). Interestingly, serum from a later bleeding of the same rabbit (1630) stained adult kidney (not shown) more like 2226 (panel C), manifesting the same intensity in all tubular elements. The preimmune serum p1630 was unreactive (not shown).

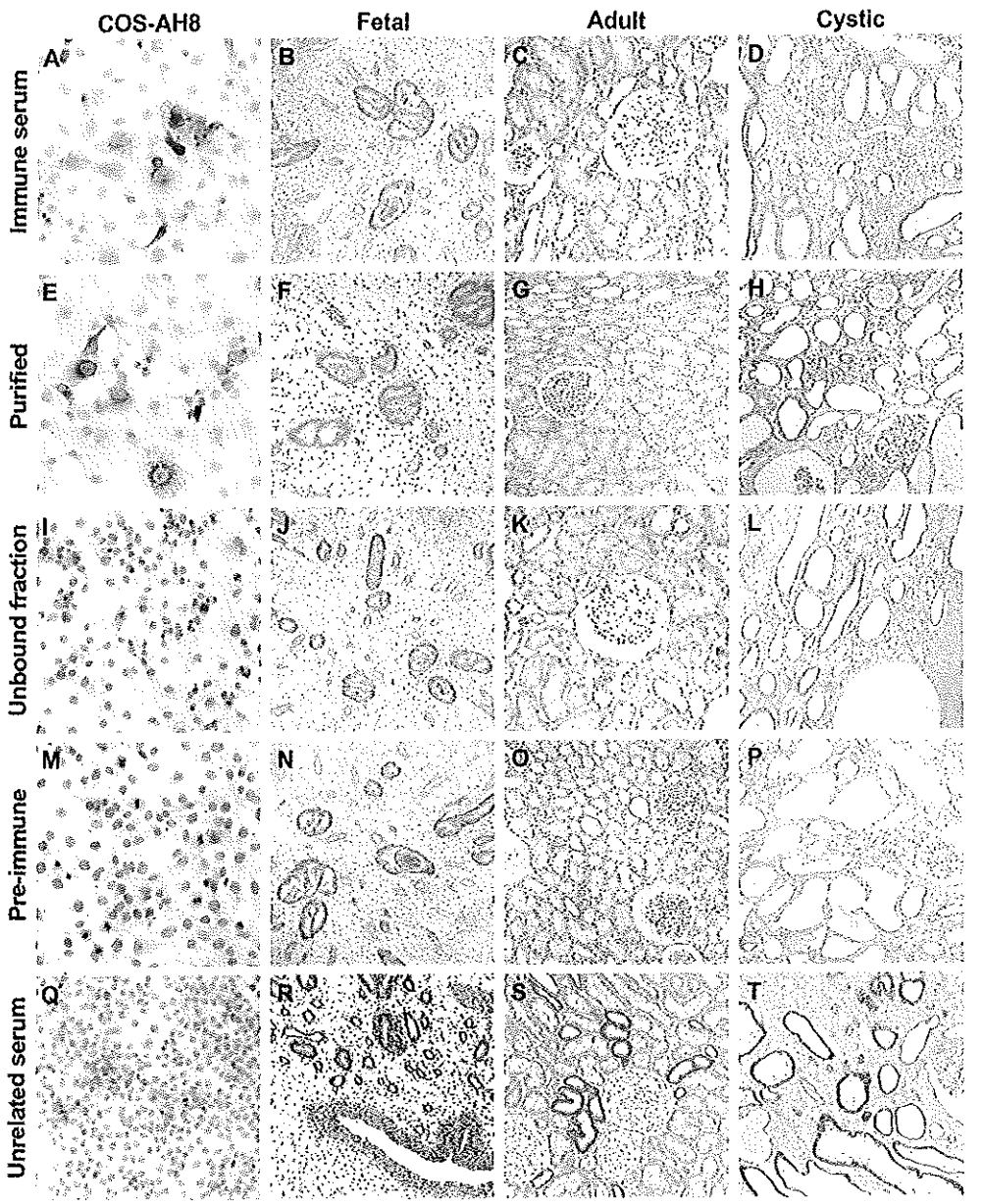


Fig. 4. (legends on previous page)

A number of antisera raised against non-polycystin-1 epitopes also stained tubular epithelia of fetal, adult and cystic kidneys, in a pattern similar to the one described for our anti-polycystin-1 sera. In these cases the corresponding preimmune sera were unreactive (unpublished results). One of these antisera (#1630) was raised against a peptide from the polycystin-1 C-terminal as originally described (The European PKD Consortium 1994), (Table 1; Figure 4Q-T). This peptide was subsequently found to correspond to non-coding sequences in the 3'UTR of the PKD1 gene and is therefore not translated (Hughes et al. 1995).

Further, the same staining pattern was observed in either fetal or adult kidneys using sera from 15 out of 26 unimmunised rabbits and also with sera from chicken, goat, guinea pig and mouse, confirming that non-specific staining can be a significant obstacle to the detection of polycystin-1 in renal epithelial structures.

Discussion

Reported expression of polycystin-1 in renal tissues

Expression of the PKD1 transcript in human and murine tissues and whole organ homogenates has been described (Geng et al. 1997; Griffin et al. 1997a; The European PKD Consortium 1994; Ward et al. 1996). These studies indicate that mRNA is expressed in all tissues examined.

Many reports describe the cellular localisation of the PKD1 protein, polycystin-1 (Aguiari et al. 1997; Geng et al. 1996; Geng et al. 1997; Griffin et al. 1996; Griffin et al. 1997a; Griffin et al. 1997b; Ibraghimov-Beskrovnaya et al. 1997; Ong et al. 1999a; Ong et al. 1999b; Palsson et al. 1996; Peters et al. 1996; Peters et al. 1999; Van Adelsberg et al. 1997; Ward et al. 1996; Weston et al. 1997; Wilson 1997; Wilson and Burrow 1999). The antisera used in these studies were raised against over 25 different peptides and fusion proteins, all derived from the predicted polycystin-1 amino acid sequence. In each report the renal expression of polycystin-1 was localized predominantly to tubular epithelia of adult, fetal and cystic kidneys. The most intense staining was usually observed in the ureteric bud of fetal kidneys and in cyst-lining epithelia from ADPKD patients. However, despite this broad agreement, the exact pattern of polycystin-1 expression is not the same in all cases. While a tubular segment specific distribution has been reported by many authors, no uniform pattern has yet emerged. Some studies indicate renal tubular expression in proximal and distal convoluted tubules and to a lesser extent in collecting ducts (Weston et al. 1997), while others localised polycystin-1 exclusively to collecting tubules (Palsson et al. 1996; Wilson 1997). Glomerular expression other than in Bowman's capsule is absent according to most studies. However

significant staining of glomerular visceral epithelia was observed by some investigators (Palsson et al. 1996; Peters et al. 1996; Weston et al. 1997). The renal vasculature and endothelium were unstained in most, but not all studies (Ibraghimov et al. 1997; Peters et al. 1996). The intracellular distribution is similarly unclear. Most authors report a cytoplasmic distribution in most structures. Additional plasma membrane expression has been observed in some studies (Aguiari et al. 1997; Geng et al. 1996; Ibraghimov et al. 1997; Palsson et al. 1997; Van Adelsberg et al. 1997), but not in others. This plasma membrane staining has been either unpolarised (Ibraghimov et al. 1997; Van Adelsberg et al. 1997) or polarised to the apical cell membrane (Geng et al. 1996; Palsson et al. 1996). The intracellular distribution of polycystin-1 has also been studied in cultured human renal epithelial cells. Using the same cell lines, two groups made different observations, localising polycystin-1 to either the peripheral cytoplasm (Griffin et al. 1996) or to the cell membrane of human renal proximal tubular epithelial cells (Ibraghimov et al. 1997). It is as yet unclear which of these reports describe the true expression pattern.

The expression of polycystin-1 in renal cysts from patients with ADPKD has also been studied. The mechanism by which a mutation of the PKD1 gene leads to the formation of these cysts is not clear. Recent evidence indicates that cysts are lined by cells where a second hit mutation of the wild type PKD1 allele has occurred, suggesting that cyst formation is caused by the absence of functional polycystin-1. Loss of heterozygosity (LOH) in cyst-lining epithelia was demonstrated for PKD1 as well as for PKD2, and in both renal and hepatic cysts (Brasier et al. 1997; Koptides et al. 1999; Qian et al. 1996; Watnick et al. 1998; Wu et al. 1998). However, in conflict with this concept is the robust staining of most cysts by anti-polycystin-1 antisera, described by most authors. Absence of staining in cyst-lining cells, as predicted by the LOH hypothesis, has been demonstrated for PKD2 in a murine model, but not for PKD1 (Wu et al. 1998).

Characterisation of the anti-polycystin-1 antisera

We raised 14 different polyclonal antisera against human polycystin-1. To thoroughly test and characterise the specificity of the immune sera we used a transfection-based system whereby we could over-express a 134kDa protein, consisting of the unique C-terminal domain of polycystin-1, in cultured mammalian cells. We called this protein AH8. Using this approach, we could perform a range of different immunological assays with our antisera.

We demonstrated that most of our antisera could detect AH8 by immunocytochemistry, immunoprecipitation and Western blot analysis. One antiserum (1630), raised against the same epitopes described by Van Adelsberg, did not recognise AH8 (Van Adelsberg and Frank 1995). Re-analysis of the PKD1 transcript has shown that these epitopes are in fact not part of the translated polycystin-1 molecule (Hughes et al. 1995; The International PKD Consortium 1995).

With the exception of serum 2226 (detailed below) we noted that on Western blots our antisera recognised other proteins, in addition to AH8. These proteins were detected in both untransfected COS cells and in cells transfected with AH8 and were generally not detected by the corresponding preimmune sera. This indicates that the reactivity may have been caused by the immunisation procedure itself, perhaps by either a general enhancement of the overall immune response or by the extended exposure of immunised rabbits to human (epithelial) antigens. Alternatively, the extra bands may represent polycystin-1 homologues. Examples of human proteins with homology to the domain recognised by our antisera include the PKD2 protein (Mochizuki et al. 1996) and the PKDREJ protein (Hughes et al. 1999).

Serum 2226, was superior to the other antisera raised against polycystin-1 since there were no appreciable background signals. No additional protein species were recognised by this serum on Western blots or by immunoprecipitation, and untransfected COS cells were not stained.

Immunocytochemical detection of polycystin-1 in human renal tissues using our antisera

Our initial observations suggested that all our antisera recognised polycystin-1 in renal tissue sections. The observed pattern of immune staining was the same for antisera raised against different polycystin-1 epitopes and was not observed with most of the corresponding preimmune sera. Our data were also in broad agreement with the other published reports, as described above. However, since we subsequently discovered that this epithelial staining pattern was also seen when using polycystin-1 unrelated sera, we could not be certain that the observed staining was due to polycystin-1. This view was supported by our observations that sera from unimmunised rabbits and from rabbits immunised with antigens unrelated to polycystin-1, including serum 1630, had a similar pattern of immunoreactivity against human renal epithelia. Further, many of our antisera, but not antiserum 2226, recognised additional proteins on Western blots of COS cells transfected with AH8, suggesting that these antisera were not absolutely specific for polycystin-1. Supporting this conclusion was the background staining of untransfected COS cells by most antisera, but not 2226, in immunocytochemical experiments.

The strongest evidence that the observed epithelial expression pattern was unlikely to be specific for polycystin-1 came from our experiments with serum 2226. In each of our test assays using the over-expressed AH8 protein, 2226 produced the strongest signal with the least background. Immunocytochemical analysis of adult and fetal kidney sections with the crude 2226 immune serum revealed the characteristic epithelial staining pattern, similar to our other antisera. However, affinity purification of 2226 completely abolished this staining, while the avidity for AH8 was unaffected. Conversely, the non-binding fraction from this purification procedure did not recognise AH8, but did stain renal

epithelium. Therefore, on the basis of these results, we conclude that the epithelial staining observed by us, and possibly others, was not specific for polycystin-1 and that we could not detect polycystin-1 in either paraffin-embedded or frozen renal tissue sections. Supporting this conclusion, with (crude or affinity purified) 2226 we were also unable to detect any protein band on Western blots of either human renal tissue or cells. Several of our other antisera did detect proteins in the expected size range (400 - 700kDa). However, no single band was detected by more than one antiserum, indicating that these protein bands were unlikely to represent polycystin-1. Since the antisera were raised against the same polycystin-1 domain, these additional bands are also unlikely to represent polycystin-1 breakdown products.

There are several possible explanations for our results. Firstly, polycystin-1 may not be expressed, or expressed at vanishingly low levels in the tissues examined. Alternatively, it may not survive the sample preparation procedures for either Western blotting or the immunocytochemical analysis of tissue sections. This conclusion is supported by our observation that the over-expressed AH8 protein was also unstable. Further, the epitopes recognised by our antibodies may be obscured by their immediate environment, such as the plasma membrane where polycystin-1 is thought to reside, and/or the binding of other renal proteins. It is also possible that conformational changes during sample preparation may destroy or obscure the polycystin-1 epitopes recognised by our antibodies.

In summary

We have generated a large number of polyclonal antisera against human polycystin-1 and characterised these as far as possible in transfected cells over-expressing the C-terminal domain of this important protein. Our antibodies show a similar epithelial expression pattern as described by others. However, we have strong evidence that the pattern we found is aspecific and results from unrelated immunoreactivity. Our data suggest that endogenously expressed polycystin-1 is very difficult to detect, either because it is masked by its immediate environment and/or extremely labile, or because it is expressed at very low levels in adult and fetal human kidney. The value of the currently available data on the cellular and tissue distribution of polycystin-1 may be considerably enhanced by exchange of antisera between independent research groups, as well as by the use of true negative control tissue to rigorously test the specificity of all the different antisera described to date. Such control tissue may potentially be derived from PKD1-knockout animal models (Lu 99), as well as from human PKD1 cysts in which loss of heterozygosity has directly been demonstrated. Until these resources become available, the renal localisation of polycystin-1 may remain uncertain.

Acknowledgments

We thank Arnold J. Reuser and Rob Willemsen for stimulating discussions and advice. The Dutch kidney foundation is kindly acknowledged for their support (grant 95.1400).

References

Agulari GL, Bozza A, Piva R, Vilinia S, Reali E, Maestri I, Magri E, Cavazzinin L, del Senno L (1997) Expression of protein fragments from the human PKD1 gene and production of rabbit polyclonal antibodies to the recombinant proteins. Contr Nephrol 122: 49-52

Brasier JL, Henske EP (1997) Loss of polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss of function model for cyst pathogenesis. J Clin Invest 99:194-199

Erickson BW, Merrifield RB (1976) In The proteins (3rd ed), edited by Neurath H and Hill RL, New York, Academic Press, pp 255-527

Geng L, Segal Y, Peissel B, Deng N, Pei Y, Carone F, Rennke HG, Glucksmann-Kuis AM, Schneider MC, Ericsson M, Reeders ST, Zhou J (1996) Identification and localization of polycystin, the PKD1 gene product. J Clin Invest 98:2674-2682

Geng L, Segal Y, Pavlova A, Barros EJ, Lohning C, Lu W, Nigam SK, Frischauf AM, Reeders ST, Zhou J (1997) Distribution and develomentally regulated expression of murine polycystin. Am J Physiol 272:F451-F459

Griffin MD, Torres VE, Grande JP, Kumar R (1996) Immunolocalization of polycystin in human tissues and cultured cells. Proc Assoc Am Physicians 108:185-197

Griffin MD, O'Sullivan DA, Torres VE, Grande JP, Kanwar YS, Kumar R (1997a) Expression of polycystin in mouse metanephros and extra-metanephric tissues. Kidney Int 52:1196-1205

Griffin MD, Torres VE, Grande JP, Kumar R: Vascular expression of polycystin (1997b). J Am Soc Nephrol 8:616-626

Hughes J, Ward CJ, Peral B, Aspinwall R, Clark K, San Millán JL, Gamble V, Harris PC (1995) The polycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. Nature Genetics 10: 151-160

Hughes J, Ward CJ, Aspinwall R, Butler R, Harris PC (1999) Identification of a human homologue of the sea urchin receptor for egg jelly: a polycystic kidney disease – like protein. Hum Mol Genet 8:543-549

Ibraghimov-Beskrovnaya O, Dackowski WR, Foggensteiner L, Coleman N, Thiru S, Petry LR, Burn TC, Connors TD, Van Raay T, Bradley J,Qian F, Onuchic LF, Watnick TJ, Piontek K, Hakim RM, Landes GM, Germino GG, Sandford R, Klinger KW (1997) Polycystin: In vitro synthesis, in vivo tissue expression, and subcellular localization identifies a large membrane-associated protein. PNAS 94: 6397-6402

Koptides M, Hadjimichael C, Koupepidou P, Pierides A, Constantinou Deltas C (1999) Germinal and somatic mutations in the PKD2 gene of renal cysts in autosomal dominant polycystic kidney disease. Hum Mol Genet 8:509-513

Lu W, Peissel B, Babakhanlou H, Pavlova A, Geng L, Fan X, Larson C, Brent G, Zhou J (1997) Perinatal lethality with kidney and pancreas defects in mice with a targetted PKD1 mutation. Nat Genet 17:179-181

Lu W, Fan X, Basora N, Babakhanlou A, Law T, Rifai N, Harris PC, Perez-Atayda AR, Rennke HG, Zhou J (1999) Late onset of renal and hepatic cysts in PKD1-targeted heterozygotes. Nat Genet 21:160-161

Mochizuki T, Wu G, Hayashi T, Xenophontos SL, Veldhuizen B, Saris JJ, Reynolds DM, Cai Y, Gabow PA, Pierides A, Kimberling WJ, Breuning MH, Constantinou Deltas CD, Peters DJM,

Somlo S (1996) PKD2, a gene for polycystic kidney disease that encodes an integral membrane protein. Science 272: 1339-1342

Ong ACM, Ward CJ, Butler RJ, Biddolph S, Bowker C, Torra R, Pei Y, Harris PC (1999a) Coordinate expression of the autosomal dominant polycystic kidney disease proteins, Polycystin-2 and Polycystin-1, in normal and cystic tissue. Am J Pathol 154:1721-1729

Ong ACM, Harris PC, Davies DR, Pritchard L, Rossetti S, Biddolph S, Vaux DJT, Migone N, Ward CJ (1999b) Polycystin-1 expression in PKD1, early onset PKD1 and TSC2/PKD1 cystic tissue: implications for understanding cystogenesis. Kidney Int 56: 1324-1333

Palsson R, Sharma CP, Kim K, McLaughlin M, Brown D, Arnaout MA (1996) Characterization and cell distribution of polycystin, the product of autosomal dominant polycystic kidney disease gene 1. Mol Med 2: 702-711

Peters DJ, Spruit L, Klingel R, Prins F, Baelde Hj, Giordano PC, Bernini LF, de Heer E, Breuning MH, Bruijn JA (1996) Adult, fetal and polycystic kidney expression of polycystin, the polycystic kidney disease-1 gene product. Lab Invest 75:221-230

Peters DJM, Wal A van de, Spruit L, Saris JJ, Breuning MH, Bruijn JA, Heer E de (1999) Cellular localisation and tissue distribution of polycystin-1. J Pathol 188: 439-446

Qian F, Watnick TJ, Onuchic LF, Germino GG (1996) The molecular basis of focal cyst formation in human autosomal polycystic kidney disease type 1. Cell 87:979-987

Sandford R, Sgotto B, Aparicio S, Brenner S, Vaudin M, Wilson RK, Chissoe S, Pepin K, Bateman A, Chothia C, Hughes J, Harris (1997) Polycystin: Comperative analysis of the polycystic kidney disease 1 (PKD1) gene reveals an integral membrane glycoprotein with multiple evolutionary conserved domains. Hum Mol Genet 6:1483-1489

The European Polycystic Kidney Disease Consortium (1994) The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. Cell 77:881-894

The International Polycystic Kidney Disease Consortium (1995) Polycystic kidney disease: The complete structure of the PKD1 gene and its protein. Cell 81: 289-298

Van Adelsberg JS, Frank D (1995) The PKD1 gene produces a developmentally regulated protein in mesenchyme and vasculature. Nat Med 1:359-364

Van Adelsberg J, Chamberlain S, d'Agati V (1997) Polycystin expression is temporally and spatially regulated during renal development. Am J Physiol 272: F602-F609

Ward CJ, Turley H, Ong ACM, Comley M, Biddolph S, Chetty R, Ratcliffe PJ, Gatter K, Harris PC (1996) Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult, and polycystic kidney. PNAS 93: 1524-1528

Watnick TJ, Torres VE, Gandolph MA, Qian F, Onuchic LF, Klinger KW, Landes G, Germino GG (1998) Somatic mutation in individual liver cysts supports a two-hit model of cystogenesis in autosomal dominant polycystic kidney disease. Mol Cell 2:247-251

Welling LW, Grantham JJ (1996) Cystic and developmental diseases of the kidney, in: The Kidney (vol 2 chapter 38), edited by Brenner BM, Philacelphia, Saunders, pp 1828-1863

Weston BS, Jeffery S, Jeffrey I, Sharaf SFA, Carter N, Saggar-Malik A, Price RG (1997) Polycystin expression during embryonic development of human kidney in adult tissues and ADPKD tissue. Histochemical Journal 29: 847-856

Wilson PD (1997) Epithelial cell polarity and disease. Am J Physiol 272:F434-42

Wilson PD, Burrow CR (1999) Cystic diseases of the kidney: Role of adhesion molecules in normal and abnormal tubulogenesis. Exp Nephrol 7: 114-124

Wu G, D'Agati V, Cai Y, Markowitz G, Park JH, Reynolds DM, Maeda Y, Le TC, Hou H Jr, Kucherlapati R, Edelmann W, Somlo S (1998) Somatic inactivation of PKD2 results in polycystic kidney disease. Cell 93: 177-88

4

General Discussion

Future Perspectives

General discussion and future perspectives

Ultimate goals of medical research are the cure and prevention of disease. The development of new treatment strategies is often triggered by novel insight in specific biological events that underlie the disease process. Here, we will summarize the current knowledge on PKD and discuss options for further investigations in view of the development of new treatment strategies. Then, we will suggest how experimental models may contribute to these investigations.

Genetic defects in PKD

The genes of the predominant forms of ADPKD (PKD1 and PKD2) and the gene associated with one of the mouse models of ARPKD (Tg737) have recently been cloned and sequenced (The European Consortium '94, Mochizuki '96). Many other genes, including the human ARPKD gene (PKHD1) and a number of genes involved in murine PKD have also been localized, but not yet characterized. In addition, several genetic loci were associated with disease severity in murine PKD, suggesting that genetic factors ('modifying genes') other than the primarily affected PKD-gene, modulate disease expression in PKD.

The mutational mechanism underlying PKD1 is controversial. Evidence is accumulating that germline PKD1 mutations are probably inactivating. The relatively few mutations described to date are mainly predicted to terminate the protein prematurely (Peral '96, '97, Roelfsema '97). The observation that large deletions, disrupting PKD1 and the adjacent TSC2 gene, are associated with severe early onset PKD indicates that a PKD1 null allele can result in cyst formation (Sampson '97). Moreover, the severe cystic phenotype of fetal homozygous pkd1-knockout mice indicates that the complete absence of polycystin-1 is associated with cyst formation (Lu '97).

Additional insight into the mutational mechanism of PKD1 comes from the observation that epithelial cells from ADPKD cysts are clonal in origin (Qian '96) and that approximately 20% of cysts exhibit loss of heterozygosity (LOH) at PKD1, apparently due to a mutation of the wild type allele (Qian '96, Brasier '97). A 'two-hit' model is an attractive explanation for the focal nature of cyst formation and for the variability of cystic disease in PKD1. It is however unclear why LOH was demonstrated in only a minority of the cysts. It is possible that this reflects a limited ability to detect mutations, rather than a lack of somatic mutations. An alternative explanation may be that second-hit mutations are not required for cyst formation.

Further exploration of the mechanism by which the initial germline mutation leads to the focal development of cysts in ADPKD may guide the development of treatment strategies. If, as an example, ADPKD is based on 'loss of function' of polycystin-1 or

polycystin-2, targeted gene complementation might be an option to interrupt the cystic process (Moullier '94).

The gene-products of the PKD1 (polycystin-1), PKD2 (polycystin-2) and Tg737 genes have been predicted from the DNA sequences and the respective protein functions are the focus of intense investigations. Polycystin-1 is thought to be a membrane glycoprotein involved in cell adhesions (Hughes '95 The International Consortium '95), whereas polycystin-2 has homology to a family of voltage gated sodium/calcium channels (Mochizuki '96). The two proteins can interact in experimental systems, suggesting cooperation in vivo (Qian '97, Tsiokas '97), consistent with the phenotypic similarities of PKD1 and PKD2. Further in vitro studies indicate that polycystin-1 may also co-associate and potentially interact with other proteins including Tg737, G-proteins and cell adhesion molecules, suggesting that polycystin-1 plays a role in multiprotein cell-cell and cell-matrix interactions (Wilson '99b, '99c). Such interactions may regulate different cellular programs such as proliferation, differentiation and apoptosis through the activation of specific transcription factors (Arnould '99).

It is likely that further investigations will reveal even more proteins involved in the normal cellular functions of the polycystins and disclose whether the observations in experimental systems reflect the situation in vivo. Understanding the normal function of these proteins may contribute to the understanding of the pathophysiology of cystformation.

It is to be expected that many more genes involved in renal cystic diseases, other than PKD1 and PKD2, will be identified and characterized within the near future. These include the ARPKD gene, the PKD3 gene, the nephronophthisis and medullary cystic disease genes and a number of genes responsible for spontaneous animal models of PKD, including primary disease genes and disease modifying genes. It is tempting to speculate that some or all of these genes are involved in a common cystogenic pathway and that their characterization may provide extra opportunities to study the complex processes of cyst formation and cyst expansion. Such studies may also reveal the mechanisms that determine the high variability of disease expression, associated with both ADPKD and ARPKD.

The cellular phenotype of PKD epithelia potential targets for treatment

Cellular pathophysiology

Cyst lining epithelia have been studied extensively by many investigators. These studies revealed a vast number of cellular abnormalities, as reviewed by many authors

(Wilson '96) as well as in Chapter 1 of this thesis. The causal relationship of these abnormalities with the underlying genetic defects of PKD is unclear and mandates further investigations. Irrespective of the complete understanding of their pathophysiology, cellular phenomena may serve as targets for the treatment of PKD.

Following an early stage of normal nephrogenesis, PKD cysts develop as dilations of normally developed renal tubules. The initiation of cyst formation is highly variable among patients as well as among individual nephrons. It has become evident that the focal loss of control on tubular diameter and the subsequent formation of cysts is associated with abnormal epithelial cell proliferation and cell survival, altered transtubular fluid transport and abnormal composition and accumulation of extracellular matrix constituents.

In addition to these abnormal cell functions, a large number of abnormalities of the structure and composition of the cystic cell have been described. These include abnormalities of the cytoskeleton, the Golgi complex, the plasmamembrane composition, the basement membranes, and many different proteins including growth factor receptors and transcription factors. Particularly interesting has been the observation in human ADPKD and ARPKD, as well as in some of the murine models of PKD, that cyst lining epithelia manifest abnormal cell polarity with regard to a number of proteins, including the EGF receptor and Na-K ATPase. Abnormal cellular distribution of these proteins may theoretically play a role in both the proliferation and abnormal transtubular fluid transport of cystic epithelia. It should be noted however, that most of these data are derived from either end stage ADPKD kidneys or from murine models of PKD. Whether they also reflect the situation in early cystogenesis in human disease is unclear.

It also remains to be determined how the various cellular abnormalities of the cystic phenotype relate to each other and contribute to either the formation of cysts and/or to the subsequent progression of the lesions.

The cellular phenotype as a target for treatment

The current knowledge of the cellular phenotype of cystic epithelia may present new opportunities for treatment. To target therapies to the abnormal extracellular matrix composition and matrix metabolism or to the altered tubular fluid transport will be difficult until more is known about the pathophysiology of these processes. The most appealing target for therapeutic interventions at present appears to be the abnormal cell proliferation in cyst-lining epithelia.

Cumulative data indicate that epithelial cell proliferation plays a primary role in cyst development and cyst growth. Epithelial hyperplasia is present in all human renal cystic diseases and appears to precede the formation of cysts (Gabow '91, Nadasdy '95). At the molecular level, PKD has been associated with increased expression of a number of proto-oncogenes. Moreover, overexpression of proto-oncogenes and growth factors in experimental animals has resulted in renal cyst formation.

A particular role in the proliferation of cystic epithelia appears to be played by the EGF-EGFR axis. EGF is one of the most potent stimulators of epithelial cells, has been detected in mitogenic concentrations in ADPKD cystfluid and is capable of inducing cysts in organ culture models. Moreover, functionally active EGF-receptor was found to be overexpressed and mislocated to the luminal cell membranes of ADPKD cysts, as opposed to the basolateral distribution in normal renal tubules. Similar results were obtained with tissues from three genetically different mouse models of ARPKD (Sweeney '98). Taken together, these data suggest that an abnormal autocrine or paracrine EGF loop may be a common cystogenic pathway, downstream from a number of different primary gene mutations. Two recent studies indicate that interference with this pathway may ameliorate the cystic process. Richards et al. reported that mice homozygous for the *orpk* mutation have less cystic lesions and a longer life span when intercrossed with EGF-receptor mutants (Richards '98). Sweeney et al. demonstrated that pharmacological inhibition of EGF receptor activity can reduce progression of cystic lesions in a murine organ culture model of ARPKD (Sweeney '99). From these studies it can be hypothesized that inhibition of the EGF-EGFR axis in human PKD may reduce the cystic damage. Such treatments will only be applicable to human patients if specific targeting to the cells of interest can be guaranteed, in order to avoid serious side effects.

It is of interest in this regard, that succesful targeting of vectors to tubular-segment specific sites, has recently been reported. Moullier et al. demonstrated in mice that vectors brought into the renal arterial circulation would target proximal tubules, while retrograde introduction from the renal pelvis would target collecting tubules (Moullier '94, Lai '98). This is particularly relevant in ARPKD where cysts arise predominantly in collecting tubules. In addition, Zhu et al. reported that genes can also be transferred succesfully into normal as well as cystic rat kidneys (Zhu '96). Selective targeting may be enhanced by taking advantage of the apically expressed EGFR in cystic epithelia. Methods to target delivery through receptor mediated endocytosis of foreign genes, coupled to ligands such as EGF, have already been reported (Wu '89).

An alternative approach to target cystic epithelia and inhibit cell proliferation specifically in collecting tubular cysts has been proposed by Gattone et al. (Gattone '99). Collecting tubules are the predominant site of cyst development in human ARPKD as well as in most recessive mouse models. One of the biological systems specifically localized to collecting tubules is the vasopressin-aquaporin axis. It was hypothesized that this axis might be involved in the progression of PKD. This was based on the observations that the vasopressin type-2 receptor (V_2R) was overexpressed in cystic kidneys of *cpk* mice and that activation of V_2R is known to stimulate the production of cAMP, a substance capable to promote cyst enlargement in vitro. Gattone et al. found that inhibition of the vasopressin-aquaporin system, using a relatively specific V_2R antagonist, was associated with a marked reduction of cyst enlargement and with delay of renal failure. This approach

may be an attractive option for human ARPKD patients, if a similar effect can be demonstrated. V_2R antagonist have already been used succesfully in human cardiac patients participating in clinical trials (Martin '99).

We conclude that the cellular phenotype of PKD presents targets for therapeutic interventions. The various options need to be explored in more detail. Further understanding of the cellular pathophysiology of PKD may identify even more and better potential targets for treatment.

The clinical phenotype of PKD potential targets for treatment

This discussion deals with renal aspects of PKD. Non-renal manifestations of PKD, including severe complications such as cardiovascular disease, cerebral aneurysms and hepatobiliary disease, are beyond the scope of this discussion and have been reviewed elsewhere (Welling 96). The clinically most serious renal symptoms are hypertension and renal failure.

Hypertension

Hypertension is a common complication of PKD and contributes to its morbidity and mortality. Longitudinal observations indicate that hypertension is a major contributant to disease progression in ADPKD (Chapman '97, see also the following section 'renal failure'). Among affected children, hypertension occurs in both ADPKD and ARPKD, but tends to be more severe in ARPKD.

The mechanisms causing hypertension in PKD are unclear. Expansion of the intravascular volume is thought to play an important role in both recessive and dominant PKD (Kaplan '89, d'Angelo '75). Activation of the renin-angiotensin-aldosteron system (RAAS) appears to play an additional role in ADPKD and has been associated with increased renal vascular resistence, increased sensitivity to angiotensin II, renal sodium retention and volume expansion (Watson '92, Wang '97). Whether activation of RAAS is also present in ARPKD is unclear because the reported data are controversial. It is thought that increased activity of RAAS may result from intrarenal angiotensin II production, due to local ischaemic conditions and may directly contribute to the observed vascular damage and renal interstitial fibrosis. Such vascular lesions, including glomerular sclerosis, are a prominent finding in ADPKD. Zeier et al. described that, in advanced ADPKD, arteriolar sclerosis was much more marked in the kidney than in other viscera (Zeier '91). Although the progression of renal damage in PKD is clearly multifactorial, extrapolation from other progressive renal disorders such as diabetic nephropathy, in association with the observed increased activity of RAAS, indicates that glomerular hypertension may be an important factor.

In view of these considerations, angiotensin converting enzyme (ace) inhibitors have been advocated as drug of choice in ADPKD induced hypertension (Watson '92). In addition to lowering systemic blood pressure (Ecder '99), these drugs are thought to reduce glomerular hypertension and thus may reduce the progression of renal damage in PKD.

Renal failure

Although convincing hypotheses have been advanced to explain the development of cysts, the mechanisms leading to renal failure in cystic kidneys are less understood. It is noted that even in advanced cases of ADPKD, cysts develop in only a minority of the total nephron population. Most nephrons appear to be lost without forming a cyst, either as a consequence or in parallel with, cystformation elsewhere. Histological features associated with end stage renal failure (ESRF) in ADPKD include advanced renal vascular sclerosis, marked interstitial fibrosis and focal tubular atrophy (Zeier '91). Vascular changes are assumed not to be just the consequence of hypertension since these are also present in normotensive patients. Moreover vascular changes in ADPKD are kidney specific and more severe than in patients with comparable renal dysfunction who suffered from glomerular disease. Severe interstitial fibrosis was associated with scarce infiltrates of lymfocytes and macrophages. Pathophysiologic mechanisms that might explain the loss of functioning nephrons include glomerular hyperfiltration, vascular sclerosis and mechanical compression by cysts and / or interstitial fibrosis. Further studies on these mechanisms are hampered by the lack of human tissues from early disease states.

End stage renal failure (ESRF) complicates the disease course in all patients with ARPKD and about half the patients with ADPKD. However, the rate of progression to ESRF is highly variable, even among family members, who are affected by the same genetic defect. In ADPKD, a number of factors have been associated with progression, including genotype, gender, ethnicity, hypertension, age at clinical presentation, pregnancies and urinary tract infections. The pathophysiologic mechanisms underlying these associations are poorly understood. Similar data on ARPKD patients are not available.

The effect of genetic heterogeneity on the cystic phenotype has been well recognized in ADPKD. Although the clinical and histopathological manifestations of PKD1 and PKD2 are indistinguishable in individual patients, these are markedly different when comparing groups of patients. Patients with PKD1 have on average a more severe disease, including more hypertension and the development of renal failure at a higher frequency and at younger age than patients with PKD2. Genetic heterogeneity also contributes to clinical variations within the subgroups of PKD1 and PKD2 patients. It has, for example, been recognized that patients with large deletions, overlapping the neighbouring TSC gene, manifest very severe early onset disease, in comparison to patients with smaller mutations.

Additional clinical variatiability may, in theory, result from somatic mutations of the cystic genes. If, as has been suggested, somatic 'second hit' mutations of the wild type allele are either required or contributive to the formation of ADPKD cysts, such mutability is likely to be variable among individual patients and may further explain the variability of disease expression. It will be clear that further analysis of ADPKD mutations and of the corresponding protein defects will be needed to understand these genotype-phenotype relations in more detail.

Most gender studies demonstrate that ADPKD is a more severe disease in men than in women. One of the factors predisposing males to an adverse renal prognosis might be the higher prevalence and severity of hypertension in males. Theoretically, increased transcription of renin under the influence of androgens could also play a role (Wagner '90). It is of interest that a worse prognosis for males has also been noted in the *cy* rat model of ADPKD and it was found that orchidectomy reduced the cystic damage in affected animals. Testosterone abrogated this effect in male rats and aggravated the disease course in female rats (Cowley '97). Better understanding of the endocrine and cellular mechanisms underlying this gender effect may reveal targets for therapeutic intervention.

Ethnicity has also been associated with renal outcome (Yium '94). Black American patients have earlier onset of ESRF than do white patients, indicating the presence of yet unidentified genetic, environmental and/or socio-economic factors that need to be explored.

Hypertension is present in the majority of patients with either ADPKD or ARPKD and multivariate analysis of ADPKD data indicated that hypertension is an independant risk factor for the development of renal failure. In addition, Ritz et al. observed that the age at onset of ESRF in ADPKD was lower in the offspring of families with primary hypertension (Geberth '95). It is now generally assumed that hypertension contributes to the development of renal failure. This may be further supported by histological data from ADPKD tissues, suggesting particular susceptibility of renal vessels to blood pressure induced injury, as compared to arterioles in other viscera (Zeier '91). It has been hypothesized that antihypertensive treatment, especially with ace-inhibitors, may delay the development of renal failure. Such an effect has indeed been demonstrated in the *cy* rat model of PKD, using ace-inhibitors (Ogborn '95[c]). However, direct evidence of a renal protection by treatment with ace-inhibitors in either hypertensive or normotensive human PKD patients, has not yet been reported.

The cumulative data strongly suggest that hypertension is deleterious for renal prognosis in PKD, as it is in other renal disease, for example in glomerulonephritis or diabetic nephropathy. A lot more work is clearly needed to understand the mechanisms and therapeutic options of hypertension and of its damaging effect on cystic kidneys in both ADPKD and ARPKD. These investigations will include clinical trials in patients and/or

animal models as well as experimental studies on haemodynamic, endocrine and cellular factors involved in the pathophysiology of hypertension-induced renal damage in PKD.

Treatment of PKD in experimental models

Several therapeutic options have recently been tested in experimental PKD models. Some of these yielded a significant reduction of the cystic lesions and / or a delay of the development of renal failure. Although most are not (yet) ready for clinical use, these observations will undoubtly direct further investigations.

A number of different genetic manipulations were succesfully applied to ameliorate the manifestations of murine pkd: 1). Supplementation of the pkd inducing mutation with wild type gene can significantly reduce the expression of disease in the *orpk* mouse model (Yoder '96); 2). A change of the overall genetic background can drastically reduce the cystic phenotype in a number of animal models (Fry '85, Nagao '91, Moyer '94, Upadhya '99); 3). Genetic inhibition of the EGFR has been associated with reduced disease manifestations in murine PKD (Richards '98).

Promising results have also been obtained using specific inhibitors of biological processes thought to be part of the pathophysiology of cyst formation: 1). Inhibition of the renin-angiotensin axis (thought to be involved in disease progression) reduces cystic damage and prolongs renal survival in pkd of the *cy* rat model (Ogborn '95[c]). This protective effect could not (yet) be established in human ADPKD patients with renal failure (Ecder '99, Wang '97). However, systematic studies in early ADPKD and in patients with ARPKD have not yet been reported. 2). Inhibition of the vasopressin V-2 receptor (thought to play a role in collecting tubular cyst progression) was found to reduce cyst enlargement in arpkd of the *cpk* mouse model (Gattone '99, Martin '99). 3). Inhibition of the EGFR signaling pathway or of the tubular sodium pump (Na-K ATPase), can block experimental cyst induction in metanephric organ cultures (Avner '85, '87b, Pugh '95).

An interesting observation by Woo et al. indicated that paclitaxel (Taxol) can dramatically reduce the cystic damage to the kidneys and prolong lifespan in affected *cpk* mice (Woo '94). This observation was later suggested to result from the ability of paclitaxel and related taxanes to stabilize microtubules and promote microtubule assembly (Woo '97[a]). These promising results were later confirmed by others (Martinez '97). However, while paclitaxel had a positive effect in *cpk* mutant mice, this effect was not applicable to three other murine models of PKD (Martinez '97, Sommerdahl '97). Moreover, paclitaxel exhibits considerable toxicity. More experimental studies of both efficacy and toxicity would be needed to further explore the therapeutic potential of microtubule specific agents.

Several dietary interventions were reported to have a renal protective effect in murine pkd models. These include protein restriction (Cowley '96, Ogborn '95a, Aukema '92c), soy as a protein source (Aukema '99, Ogborn '98) and enrichment of standard chow with 10% flaxseed (Ogborn '99). Recent clinical evidence indicates that patients with advanced ADPKD may benefit from dietary protein restriction (Klahr '97). However, more and longer systematic prospective studies, also involving early cases of ADPKD and patients with ARPKD, are clearly needed to resolve these issues. If similar protective effects can also be demonstrated in humans with PKD, dietary measures will be a relatively simple way to reduce morbidity and delay renal failure in these patients.

The cumulative data suggest that PKD may be ameliorated also in human patients and provide a basis for further exploration of the therapeutic options in this severe disorder.

Conclusions

PKD research made remarkable progress during the last two decades. New insights were obtained from different research fields. Genes hosting the primary mutations have been localized (ARPKD) and characterized (ADPKD). In addition, several murine genes that either host a primary PKD mutation or encode disease modifying factors, have been identified and localized. At the cellular level, a multitude of structural and functional phenomena were specifically associated with the cystic phenotype. Taken together, these phenomena indicate that abnormal epithelial cell proliferation, altered fluid transport and abnormal regulation of extracellular matrix constituents are all part of the cellular pathophysiology of PKD. Clinical and epidemiological studies demonstrate that disease manifestations of ADPKD and ARPKD are highly variable, both between and within affected families. This variability indicates that genetic and environmental factors, other than the primary mutation, are critical determinants of disease severity in PKD.

Direct treatment is not available to PKD patients. However, a number of promising treatment options have been succesfully applied in animal models of PKD. More fundamental as well as treatment oriented research is clearly needed to further explore these options. A particular challenge to future researchers is to understand the functional relationship between the multitude of PKD abnormalities observed in different fields of research: genetics, cellular pathophysiology and clinical observations. Especially fruitful may also be the exploration of factors that determine the highly variable expression of disease manifestations in PKD.

As in the past, experimental models of cyst formation provide helpful tools for such investigations. Genetically determined animal models can be used to investigate, in a systematic way and at different stages of disease, the genetical, cell biological and clinical aspects of renal cyst formation. The models described in this thesis provide unique opportunities for such studies. The *bpk* mutation expressed in Balb/c Mice is a good

model of ARPKD (Chapter 2.1). Although genetically distinct from its human counterpart, affected *bpk* mice express a clinical and histological phenotype much like human ARPKD patients, including hyperplastic lesions of the intra- and extrahepatic biliary tract. The succesful isolation and culture of the biliary epithelial cells from affected *bpk* mice, enables the investigation of key pathophysiologic features of ARPKD epithelia (Chapter 2.2). The Han:SPRD-*cy* model is not a genetic model of PKD1 (Chapter 2.3). Nevertheless, we consider Han:SPRD-*cy* a valuable model of PKD. Its phenotypic similarities with human ADPKD makes *cy* more appropriate as a model to study specific aspects of the PKD1 phenotype, when compared to the *pkd1* knockout mouse, which expresses only mild cystic lesions and at old age only. The *wpk* rat model, described in Chapter 2.4, manifests a renal phenotype resembling human ARPKD, including a collecting tubular nature of the cysts, proteinuria and severe systemic hypertension. Although genetically distinct from human ARPKD, we consider *wpk* of particular value to study the cellular pathophysiology and therapeutic options of collecting tubular cyst formation. *Wpk* may additionally be used to explore the cause and potential treatment of systemic hypertension in ARPKD, as well as to study the contribution of hypertension to the development of renal failure in PKD. The long history of hemodynamic and physiologic studies in rats makes *wpk* more suitable for such studies than the existing mouse models of ARPKD.

We found that 'programmed cell death' (apoptosis) occurs during normal human kidney development and continues at a very low level after birth. More importantly, apoptosis occurs at an increased level in human ARPKD and dysplastic kidneys as well as in mouse ARPKD (Chapter 3.1). These data parallel the finding of increased apoptosis in human ADPKD by Woo et al. (Woo '95), suggesting that apoptosis is part of a common cystogenic pathway. Whether apoptosis is either the cause or the consequence of cyst formation remains to be determined. In vitro studies suggest that apoptosis may be an essential part of the formation of cysts. Lin et al. demonstrated that the formation of cysts by canine kidney cells (MDCK) in three dimensional cultures can be prevented by inhibiting apoptosis (Lin '99). Apoptosis was inhibited in this system using overexpression of the anti-apoptotic proto-oncogene bcl-2. The fact that apoptosis can be reduced by various molecular means both in vivo and in vitro suggests potential for novel therapeutic strategies (Coles '92, Kennedy '97, Lin '99). However, such therapies may have unintended side effects because apoptosis can also be viewed as a last line of defense against neoplastic transformation of the hyperproliferative epithelia in PKD. Elucidation of the mechanisms responsible for the coupled stimultion of apoptosis and proliferation in PKD may provide opportunities for interventions that avoid the potential risk of selectively inhibiting apoptosis alone.

In Chapter 3.2 we report our efforts in vain to identify polycystin-1 in human renal tissues, using immunological detection methods. This is in contrast to reports by other

investigators. We conclude that the renal localization of polycystin-1 is not yet certain. The issue is clearly controversial and may be elucidated when reagents are exchanged between independant research groups and when adequate control tissue, lacking polycystin-1 expression, becomes available to test the available antisera.

PKD is an inevitable disorder in those who inherit the genetic defect. The main objective of further research will be to reduce the progression of the cystic lesions and to delay the development of renal failure. The experimental models presented in this thesis provide new opportunities to study the pathophysiology underlying these processes.

References

Alvarez F, Bernard O, Brunelle F, et al.: Congenital hepatic fibrosis in children. J Pediatr 99:370-375, 1981

Arnould T, Sellin L, Benzing T, Tsiokas L, Cohen HT, Kim E, Walz G: Cellular activation triggered by the autosomal dominant polycystic kidney disease gene product PKD2. Mol Cell Biol 19:3423-34, 1999

Atala A, Freeman MR, Mandell J, Beier DR: Juvenile cystic kidneys (jck): a new mouse mutation which causes polycystic kidneys. Kidney Int 43:1081-5, 1993

Aukema HM, Chapkin RS, Tomobe K, Takahashi H, Holub BJ: In vivo formation of polyphosphoinositide isomers and association with progression of murine polycystic kidney disease. Exp and Mol Pathol 57:39-46, 1992a

Aukema HM, Yamaguchi T. Takahashi H, Celi B, Holub BJ: Abnormal lipid and fatty acid compositions of kidneys from mice with polycystic kidney disease. Lipids 27:429-35, 1992b

Aukema HM, Ogborn MR, Tomobe K, Takahashi H, Hibino T, Holub BJ: Effects of dietary protein restriction and oil type on the early progression of murine polycystic kidney disease. Kidney Int 42:837-42, 1992c

Aukema HM, Housini I, Rawling JM: Dietary soy protein effects on inherited polycystic kidney disease are influenced by gender and protein level. J Am Soc Nephrol 10:300-8, 1999

Avner ED, Piesco NP, Sweeney WE Jr, Studnicki FM, Fetterman GH, Ellis D: Hydrocortisone-induced cystic metanephric maldevelopment in serum-free organ culture. Lab Invest 50:208-18, 1984

Avner ED, Sweeney WE Jr, Finegold DN, Piesco NP, Ellis D: Sodium-potassium ATPase activity mediates cyst formation in metanephric organ culture. Kidney Int 28:447-55, 1985

Avner ED, Studnicki FE, Young MC, Sweeney WE Jr, Piesco NP, Ellis D, Fetterman GH: Congenital murine polycystic kidney disease. I. The ontogeny of tubular cyst formation. Ped Nephrol 1:587-96, 1987a

Avner ED, Sweeney WE Jr, Piesco NP, Ellis D: Triiodothyronine-induced cyst formation in metanephric organ culture: the role of increased Na-K-adenosine triphosphatase activity. J Lab Clin Med 109:441-53, 1987b

Avner ED, Weeney WE, Young MC, Ellis D: Congenital murine polycystic kidney disease. II. Pathogenesis of tubular cyst formation. Ped Nephrol 2: 210-18, 1988a

Avner ED, Piesco NP, Sweeney WE Jr, Ellis D: Renal epithelial development in organotypic culture. Pediatr Nephrol 2:92-9, 1988b

Avner ED, Sweeney WE, Ellid S: Isolated proximal tubules and proximal tubular cyst of CPK mice have increased Na-K ATPase activity. Kidney Int 36:960-8, 1989

Avner ED: Polypeptide growth factors and the kidney: a developmental perspective. Pediatr Nephrol 4:345-53, 1990

Avner ED, Sweeney WE, Nelson WJ: Abnormal sodium pump distribution during renal tubulogenesis in congenital murine polycystic kidney. PNAS 89:7447-52, 1992

Aziz N, Maxwell MM, St-Jacques B, Brenner BM: Downregulation of Ke 6, a novel gene encoded within the major histocompatibility complex, in murine polycystic kidney disease. Mol and Cell Biol 13:1847-53, 1993

Bear JC, McManamon Polycystin, Morgan J et al.: Age at clinical onset and at ultrasonographic detection of adult polycystic disease: Dtata for genetic counseling. An J Med Genet 18:45-54,1984

Bernstein J: Hepatic involvement in heriditary renal syndromes. Birth Defects 23:115-130, 1987

Bihoreau MT, Ceccherini I, Browne J, Kranzlin B, Romeo G, Lathrop GM, James MR, Gretz N: Location of the first genetic locus, PKDr1, controlling autosomal dominant polycystic kidney disease in Han:SPRD cy/+ rat. Hum Mol Genet 6:609-13, 1997

Boulter CA, Aguzzi A, Evans MJ, Affara N: A chimeric model for ADPKD. In: Breuning MH, Devoto M, Romeo G eds. Polycystic kidney disease. Karger, Basel, Contrib Nephrol 97:60-70, 1992

Brasier JL, Henske EP: Loss of polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss of function model for cyst pathogenesis. J Clin Invest 99:194-199, 1997
Braun C, Ludicke C, Rebsch W, Gretz N, van der Woude FJ, Rohmeiss Polycystin: Autoregulation of renal blood flow and pressure-dependent renin release in autosomal dominant polycystic kidney disease of rats. Nephrol Dial Transplant 11,Suppl 6:52-7, 1996
Breyer MD, Jacobson HR, Breyer J: Epidermal growth factor inhibits the hydroosmotic effect of vasopressin in the isolated perfused rabbit cortical collecting tubule. J Clin Invest 82:1313-20, 1988
Calvet JP: Polycystic kidney disease, primary extracellular matrix abnormality or defective cellular differentiation. Kidney Int 43: 101-8, 1993
Carone FA, Rowland RG, Perlman SG, Ganote CE: The pathogenesis of drug induced renal cystic disease. Kidney Int 5:411-21, 1974
Carone FA, Jin H, Nakamura S, Kanwar YS: Decreased synthesis and delayed processing of sulfated glycoproteins by cwells from human polycystic kidneys. Lab Invest 68:413-8, 1993
Chapman AB, Rubinstein D, Hughes R et al.: Intracranial aneurysms in autosomal dominant polycystic kidney disease. N Eng J Med 327:916-920, 1992
Chapman A, Gabow P: Hypertension in autosomal dominant polycystic kidney disease. Kidney Int 52 (Suppl 61):S71-S73, 1997
Churchill DN, Bear JC, Morgan J, Payne RH, McManamon, Gault MH: Prognosis of adult onset polycystic kidney disease re-evaluated. Kidney Int 26:190-193, 1984
Cole BR: Autosomal reccessive polycystic kidney disease. In:Gardner KD, ed. The cystic kidney. Dordrecht, Netherlands, Kluwer pp327-350, 1990
Coles HSR, Burne JF, Raff MC: Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development 118:777-784, 1993
Cowley BD, Smardo FL, Granthamm JJ, Calvet JP: Elevated c-myc protooncogene expression in autosomal recessive polycystic kidney disease. PNAS 84:8394-8, 1987
Cowley BD, Chadwick LJ, Grantham JJ, Calvet JP: Elevated proto-oncogene expression in polycystic kidneys of the C57BL/6J (cpk) mouse. J Am Soc Nephrol 1:1048-53, 1991
Cowley BD: Autosomal dominant polycystic kidney disease in the rat. Kidney Int 43:522-34, 1993
Cowley BD Jr, Grantham JJ, Muessel MJ, Kraybill AL, Gattone VH 2nd: Modification of disease progression in rats with inherited polycystic kidney disease.Am J Kidney Dis 27:865-79, 1996
Cowley BD Jr, Rupp JC, Muessel MJ, Gattone VH 2nd: Gender and the effect of gonadal hormones on the progression of inherited polycystic kidney disease in rats. Am J Kidney Dis 29:265-72, 1997
Crocker JF, Blecher SR, Givner ML, McCarthy SC: Polycystic kidney and liver disease and corticosterone changes in the cpk mouse. Kidney Int 31:1088-91, 1987
Crowell WA, Hubbell JJ, Riley JC: Polycystic renal disease inrelated cats. J Am Vet Assoc: 175:286-8, 1979
Cuppage FE, Huseman RA, Chapman A, Grantham JJ: Ultrastrucure and function of cysts from human adult polycystic kidneys. Kidney Int 17:372-81, 1980
Dalgaard OZ: Bilateral polycystic disease of the kidneys. A follow-up of two hundred and eighty four patients and their families. Acta Med Scand Suppl 328: 1-255, 1957
D'Angelo A, Mioni G, Ossi E, Lupo A, Valvo E, Maschio G: Alterations in renal tubular sodium and water transport in polycystic kidney disease. Clin Nephrol 3:78-81, 1975
Daoust MC, Reynolds DM, Bichet DG et al. Evidence for a third genetic locus for autosomal dominant polycystic kidney disease. Genomics 25:733-736, 1995
Davisson MT, Guay-Woodford LM, Harris HW, D'Eustachio Polycystin: The mouse polycystic kidney disease mutation (cpk) is located on proximal chromosome 12. Genetics 9:778-81, 1991
Doi T, Striker LJ, Quaife C, Conti FG, Palmiter R, Behringer R, Brinster R, Striker GE: Progressive glomerulosclerosis develops in transgenic mice chronically expressing growth hormone and growth hormone releasing factor but not in those expressing insulinlike growth factor-I. Am J Pathol 131:398-403, 1988

Du J, Wilson PD: Abnormal polarized location of EGF receptors and autocrine stimulation of cyst epithelial growth in human ADPKD. Am J Physiol 269:C487-95, 1995
Eaton KA, Biller DS, DiBartola SP, Radin MJ, Wellman ML: Autosomal dominant polycystic kidney disease in Persian and Persian-cross cats. Vet Pathol 34:117-126, 1997
Ebihara I, Killen PD, Laurie GW, Huang T, Yamada Y, Martin GR, Brown KS: Altered mRNA expression of basement membrane components in a murine model of polycystic kidney disease. Lab Invest 58:262-9, 1988
Eccles MR: The role of PAX2 in normal and abnormal development of the urinary tract. Pediatr Nephrol 12:712-20, 1998
Ecder T, Edelstein CL, Chapman AD, Johnson AM, Tison L, Gill EA, Brosnahan GM, Schrier RW: Reversal of left vebtricular hypertrophy with angiotensin converting enzyme inhibition in hypertensive patients with autosomal dominant polycystic kidney disease. Nephrol Dial Transpl 14: 1113-1116, 1999
Ehara T, Carone FA, McCarthy KJ, Couchman JR: Basement membrane chondroitin sulfate proteoglycan alterations in a rat model of polycystic kidney disease. Am J Pathol 144:612-21, 1994
Elzinga LW, Barry JM, Torres VE, et al. : Cyst decompression for autosomal dominant polycystic kidney diseae. J Am Soc Nephrol 2:1219-1226., 1992
Evan AP, Gardner KD: Comparison of human polycystic and medullary cystic disease with diphenylamine-induced cystic disease. Lab Invest 35: 93-101, 1976
Falkenstein D, Burrow CR, Norman JT, Hartz PA, Wilson PD. Abnormalities in membrane polarity, enzyme and ion secretion in human autosomal recessive polycystic kidney disease. J Am Soc Nephrol 5:621, 1994
Faraggiana T, Bernstein J, Strauss L, et al.: Use of lectins in the study of histogenesis of renal cysts. Lab Invest 53:575-579, 1985
Feather SA, Woolf AS, Donnai D, Malcolm S, Winter RM: The oral-facial-digital syndrome type 1 (OFD1), a cause of polycystic kidney disease and associated malformations, maps to Xp22.2-Xp22.3. Hum Mol Genet 6:1163-7, 1997
Flaherty L, Bryda EC, Collins D, Rudofsky U, Montogomery JC: New mouse model for polycystic kidney disease with both recessive and dominant gene effects. Kidney Int 47:552-8, 1995
Fomitcheva J, Baker ME, Anderson E, Lee GY, Aziz N: Characteriztion of Ke 6, a new 17 β-hydroxysteroid dehydrogenase and its expression in gonadal tissues. J Biol Chem 273:22664-71, 1998
Fox RR, Krinsky WL, Crary DD: Heriditary cortical renal cysts in the rabbit. J Heredity 62:105-9, 1971
Fox S, Eicher EM: Juvenile polycystic kidneys (jpk). Mouse Newsletter 58:47-51, 1970
Fry JL, Koch WE, Jennette JC, McFarland E, Fried FA, Mandell J: A genetically determined murine model of infantile polycystic kidney disease. J Urol 134: 828-33, 1985
Gabow PA: Polycystic kidney disease: Clues to pathogenesis. Kidney Int 40:989-996, 1991
Gabow PA, Johnson AM, Kaehny WD: Factors affecting the progression of renal disease in autosomal dominant polycystic kidney disease. Kidney Int 41:1311-19, 1992
Gabow PA Definition and natural history of aotosomal dominant polycystic kidney disease. In: Polycystic kidney disease. Watson ML and Torres VE (eds), Oxford Universaity Press, Oxford pp333-355, 1996
Gabow P, Grantham J: Polycystic kidney disease. In: Diseases of the kidney, edited by R. Schrier and C. Gotschalk. Boston, Little, Brown, 1997, pp 521-560
Gagnadoux M, Habib R, Levy M:Cystic renal diseases in children. Adv Nephrol 18:33-58, 1989
Gardner KD Jr, Evan AP, Reed WP: Accelerated renal cyst development in deconditioned germ-free rats. Kidney Int 29:1116-23, 1986
Gardner KD, Burnside JS, Elzinga LW, Locksley RM: Cytokines in fluids from polycystic kidneys. Kidney Int 39:718-24, 1991
Gattone VH, Calvet JP, Cowley BD Jr, Evan AP, Shaver TS, Helmstadter K, Grantham JJ: Autosomal recessive polycystic kidney disease in a murine model: a gross and microscopic description. Lab Invest 59:321-8, 1988

Gattone VH, Andrews GK, Nie FW, Chadwich LJ, Klein RM, Calvet JP: Defective epidermal groweth factor gene expression in mice with polycystic kidney disease. Dev Biol 138:225-230, 1990
Gattone VH, Cowley BD, Barash BD, Nagao S, Takahashi H, Yamaguchi T, Grantham JJ: Methylprednisolone retards the progression of inherited polycystic kidney disease in rodents. Am J Kidn Dis 25:302-13, 1995a
Gattone VH, Lowden WA, Cowley BD: Epidermal growth factor ameliorates autosomal recessive polycystic kidney disease in mice. Dev Biol 169:504-10, 1995b
Gattone VH, MacNaughton KA,Kraybill AL: Murine autosomal recessive polycystic kidney disease with multiorgan involvement induced by the cpk gene. Anat Rec 245:488-99, 1996
Gattone VH, Maser RL, Tian C, Rosenberg JM, Branden MG: Developmental expression of urine concentration-associated genes and their altered expression in murine infantile-type polycystic kidney disease. Dev Biol 24:309-18, 1999
Geberth S, Stier E, Zeier M, Mayer G, Rambausek M, Ritz E: More adverse renal prognosis of autosomal dominant polycystic kidney disease in families with primary hypertension. J Am Soc Nephrol 6:1643-1648, 1995
Gilbert E, Morel A, Tulliez M, Maunoury R, Terzi F, Miquerol L, Kahn A: In vivo effects of activated H-ras oncogene expressed in the liver and in urogenital tissues. Int J Cancer 73:749-56, 1997
Goodman T, Grice HC, Becking GC, Salem FA: A cystic nephropathy induced by nordihydroguaiaretic acid in the rat. Light and electron microscopic investigations. Lab Invest 23:93-107, 1970
Gorer PA: Renal lesions found in pure lines of mice. J Path and Bact 50:25-30, 1940
Granot Y, Van Putten V, Przekwas J, Gabow PA, Schrier RW: Intra- and extracellular proteins in human normal and polycystic kidney epithelial cells. Kidney Int 37:1301-9, 1990
Grantham JJ, Donoso VS, Evan AP, Carone FA, Gardner KD: Viscoelastic properties of tubule basement mambranes in experimental renal cystic disease. Kidney Int 32:189-97, 1987a
Grantham JJ, Geiser JL, Evan AP: Cyst formation and growth in autosomal dominant polycystic kidney disease. Kidney Int 31:1145-1152, 1987b
Grantham JJ, Uchic M, Cragoe EJ Jr, Kornhaus J, Grantham JA, Donoso V, Mangoo-Karim R, Evan A, McAteer J: Chemical modification of cell proliferation and fluid secretion in renal cysts. Kidney Int 35:1379-89, 1989
Grantham JJ: Fluid secretion, cellular proliferation and the pathogenesis of renal epithelial cysts. J Am Soc Nephr 3:1843-57, 1993
Grantham JJ, Schreiner GF, Rome L, Grenz L, Joly A: Evidence for inflammatory and secretagogue lipids in cyst fluids from patients with autosomal dominant polycystic kidney disease. Proc Assoc Am Physicians 109:397-408, 1997
Gregoire JR, Torres VE, Holley KE, Farrow GM:Renal epithelial hyperplastic and neoplastic proliferation in autosomal dominant polycystic kidney disease (ADPKD) Am J Kidney Dis 9:27-38, 1987
Gretz N, Ceccherini I, Kranzlin B, Kloting I, Devoto M, Rohmeiss P, Hocher B, Waldherr R, Romeo G: Gender-dependent disease severity in autosomal polycystic kidney disease of rats. J Am Soc Nephrol 4:1925-6, 1994
Gretz N: Accelerated renal death after unilateral nephrectomy in a rat strain suffering from autosomal dominant polycystic kidney disease. Kidney Int 48:496-500, 1995
Gretz N, Kranzlin B, Pey R, Schieren G, Bach J, Obermuller N, Ceccherinin I, Klotong I, Rohmeiss P, Bachmann S et al.: Rat models of autosomal dominant polycystic kidney disease. Nephrol Dial Transplant 11:46-51, 1996
Grunfeld JP, Albouze G, Jungers Polycystin et al. : Liver changes and complications in adult polycystic kidney disease. Adv Nephrol 14:1-20, 1984
Guay-Woodford LM, D'Eustachio Polycystin, Bruns GAP: Identification of the syntenic human linkage for the mousecongenital polycystic kidney (cpk) locus. J Am Soc Nephrol 4:814-, 1993

Guay Woodford L, Muecher G, Hopkins S, Avner E, Germino G, Guillot A, Herrin J, Holleman R, Irons D, Primack W, et al.: The severe perinatal form of autosomal recessive polycystic kidney disease (ARPKD) maps to chromosome 6p21.1-p12: Implications for genetic counseling. Am J Hum Genet 56:1101-1107, 1995

Guay-Woodford LM, Bryda EC, Christine B, Lindsey JR, Collier WR, Avner ED, D'Eustachio Polycystin, Flaherty L: Evidence that two phenotypically distinct mouse PKD mutations, bpk and jcpk are allelic. Kidney Int 50:1158-65, 1996

Hamir AN, Klein L: Polycystic kidney disease in a racoon. J Wildl Dis 32:674-77, 1996

Harding MA, Chadwick LJ, Gattone VH 2d, Calvet JP: The SGP-2 gene is developmentally regulated in the mouse kidney and abnormally expressed in collecting duct cysts in polycystic kidney disease. Dev Biol 146:483-90, 1991

Harding MA, Gattone VH 2d, Grantham JJ, Calvet JP: Localization of overexpressed c-myc mRNA in polycystic kidneys of the cpk mouse. Kidney Int 41:317-25, 1992

Harrap SB, Davies DL, MacNicol AM et al.:Renal, cardiovascular and hormonal characteristics of young adults with autosomal dominant polycystic kidney disease. Kidney Int 49:501-508, 1991

Hocher B, Thone-Reineke C, Rohmeiss P, Schmager F, Slowinski T, Burst V, Siegmund F, Quertermous T, Bauer C, Neumayer HH, Schleuning WD, Theuring F J: Endothelin-1 transgenic mice develop glomerulosclerosis, interstitial fibrosis, and renal cysts but not hypertension. Clin Invest 99:1380-9, 1997

Hocher B, Zart R, Schwarz A, Vogt V, Braun C, Thone-Reineke C, Braun N, Neumayer HH Koppenhagen K, Bauer C, Rohmeiss Polycystin: Renal endothelin system in polycystic kidney disease. J Am Soc Nephrol 9:1169-77, 1998

Hodgkinson KA, Kerzin-Storrar L, Watters EA et al. Adult polycystic kidney disease: Knowledge, experience and attitudes to prenatal diagnosis. J Med Genet27:552-558, 1990

Horikoshi S, Kubota S, Martin GR, Yamada Y, Klotman PE: Epidermal growth factor expression in the congenital polycystic mouse kidney. Kidney Int 339:57-62, 1991

Hossack KF, Leddy CL, Schrier LW, Gabow PA: Incidence of cardiac abnormalties associated with autosomal dominant polycystic kidney diseae. Kidney Int 31:203, 1987

Hossack KF, Leddy CL, Johnson AM, Schrier RW, Gabow PA: Echocardiographic findings in autosomal dominant polycystic kidney disease. N Eng J Med 319:907-912, 1988

Hughes J, Ward CJ, Peral B, Aspinwall R, Clark K, San Millán JL, Gamble V, Harris PC: The ploycystic kidney disease 1 (PKD1) gene encodes a novel protein with multiple cell recognition domains. Nature Genetics 10: 151-160, 1995

Huseman R, Grady A, Welling D, Grantham J: Macropuncture study of polycystic disease in human adult kidneys. Kidney Int 18:375-385, 1980

Iakoubova OA, Dushkin H, Beier DR: Localisation of a murine recessive polycystic kidney disease mutation and modifying loci that affect disease severity. Genomics 26:107-14, 1997

Ibraghimov-Beskrovnaya O, Dackowski WR, Foggensteiner L, Coleman N, Thiru S, Petry LR, Burn TC, Connors TD, Van Raay T, Bradley J,Qian F, Onuchic LF, Watnick TJ, Piontek K, Hakim RM, Landes GM, Germino GG, Sandford R, Klinger KW: Polycystin: In vitro synthesis, in vivo tissue expression, and subcellular localization identifies a large membrane-associated protein. PNAS 94: 6397-6402, 1997

Igarashi Y, Aperia A, Larsson L, Zetterstrom R: Effect of betamethasone on Na-K-ATPase activity and basal and lateral cell membranes in proximal tubular cells during early development. Am J Physiol 245:F232-7, 1983

Iglesias CG, Torres VE, Offord KP et al. : Epidemiology of adult polycystic kidney disease. Olmsted Country, Minn: 1935-1980. Am J Kidney Dis 2:630-639, 1983

Inage Z, Kikkawa Y, Minato M, Owada M, Kitagawa T, Ohno K, Kondo K, Ueda Y, Iidaka K: Autosomal recesive polycystic kidney disease in rats. *Nephron* 59: 637-640, 1991

Inage Z, Kikkawa Y, Minato M, Owada M, Kitagawa T, Ohno K, Kondo K, Ueda Y, Iidaka K: Cystic basement membrane with increased negative charge in rat autosomal recessive polycystic kidney. Nephron 63:107-8, 1993

Iverson WO, Fetterman GH, Jacobson ER, Olsen JH, Senior DF, Schobert EE: Polycystic kidney and liver disease in springbok: Morfology of the lesions. Kidney Int 22:146-55, 1982
Genomics 1997 Feb 15;40(1):101-7
Janaswami PM, Birkenmeier EH, Cook SA, Rowe LB, Bronson RT, Davisson MT: Identification and genetic mapping of a new polycystic kidney disease on mouse chromosome 8. Genomics 40:101-7, 1997
Jones TO, Clegg FG, Morgan G, Wijeratne WV: A vertically transmitted cystic renal dysplasia of lambs. Vet Record: 127:421-4, 1990
Kääriääinen H, Koskimies O, Norio R: Dominant and recessive polycystic kidney disease in children: Evaluation of clinical features and laboratory data. Pediatr Nephrol 2:296-302, 1988
Kaplan BS, Fay J, Shah V, et al.: Autosomal recessive polycystic kidney disease. Pediatr Nehrol 3:43-49, 1989
Kaplan BS, Kaplan Polycystin:Autosomal recessive polycystic kidney disease in : Spitzer A, Avner ED, eds. Inheritance of kidney and urinary tract diseases. Dordrecht, Netherlands: Kluwer pp 165-176, 1990
Kaspareit-Rittinghausen J, Deerberg F, Wcislo A: Heriditary polycystic kidney disease: adult polycystic kidney disease associated with renal hypertension, renal osteodystrophy and uremic enteritis in SPRD rats. Am J Pathol 139:693-6, 1991
Kawa G, Nagao S, Omori K, Komatz Y, Takahashi H, Tashiro Y: Sodium pump distribution is not reversed in the DBA/2FG-pcy polycystic kidney disease mouse model. J Am Soc Nephr 4:2040-9, 1994
Kelley KA, Agarwal, Reeders S, Herrup K: Renal cyst formation and multifocal neoplasia in transgenic mice carrying the simian virus 40 early region. J Am Soc Nephr 2:84-97, 1991
Kelly CJ, Neilson EG: Medullary cystic disease: an inherited form of autoimmune interstitial nephritis? Am J Kidn Dis 10:389-95, 1987
Kaehny , Bell Polycystin, Earnest M et al.:Family clustering of intracranial aneurysms in autosomal polycystic kidney disease. Kidney Int 31:205, 1987
Kennedy WA II, Butyan R, Swwczuk IS: Epidermal growth factor suppresses renal tubular apoptosis following ureteral obstruction. Urology 19:973-980, 1997
Kim E, Arnould T, Sellin L, Benzing T, Comella N, Kocher O, Tsiokas L, Sukhatme VP, Walz G: Interaction between RGS7 and polycystin. Proc Natl Acad Sci U S A 96:6371-6, 1999
Kissane JM:Renal cysts in pediatric patients: a classification and overview. Pediatr Nephrol 4:68-77, 1990
Klahr S, Levey As, Caggiula, Hunsicker L, Kusek JW, Striker G: The effects of dietary protein restriction and blood pressure control on the progression of chronic renal disease. New Engl J Med 330:877-84, 1994
Klahr S, Breyer JA, Beck GJ: Dietary protein restriction, blood pressure control and the progression of polycystic kidney disease. J Am, Soc Nephrol 5:2037-2047, 1995
Klahr S: Prevention of progression of nephropathy. Nephrol Dial Transplant 12 Suppl 2: 63-66, 1997
Klingel R, Dippold W, Storkel S, Meyer K, Kohler H: Expression of differentiation antigens and growth related genes in normal kidney, autosomal dominant polycystic kidney disease and renal cell carcinoma. Am J Kidn Dis 19:22-30, 1992
Klingel R, Ramadori G, Schuppan D, Knittel T, Meyer KH, Kohler H: Coexpression of extracellular matrix glycoproteins undulin and tenascin in human ADPKD. Nephron 65:111-8, 1993
Koptides M, Hadjimichael C, Koupepidou P, Pierides A, Constantinou Deltas C: Germinal and somatic mutations in the pkd2 gene of renal cysts in autosomal dominant polycystic kidney disease. Hum Mol Genet 8:509-513, 1999
Kranzlin B, Schieren G, Gretz N: Azotemia and extrarenal manifestations in old female Han:SPRD (cy/+) rats. Kidney Int51:1160-9, 1997
Lai LW, Chan DM, Erickson RP, Hsu SJ, Lien YH: Correction of renal tubular acidosis in carbonic anhydrase II-deficient mice with gene therapiy. J Clin Invest 101:1320-1325, 1998

Lakshmanan J, Fisher DA: An inborn error in epidermal growth factor prohormone metabolism in a mouse model of autosomal recessive polycystic kidney disease. Bioch Bioph Res Commun 196:892-901, 1993
Lanoix J, D'Agati V, Szabolcs, Trudel M:Dysregulation of cellular proliferation and apoptosis mediates human autosomal recessive polycystic kidney disease (ADPKD). Oncogene 13: 1153-60, 1996
Lee DCW, Chan KW, Chan CY: Expression of transforming growth factor α and epidermal growth factor receptor in adult polycystic kidney disease. J Urol 159:291-96, 1998
Lin H-H, Yang T-P, Jiang S-T, Yang H-Y, Tang M-J: Bcl-2 overexpression prevents apoptosis-induced Madin- Darby canine kidney simple epithelial cyst formation. Kidney Int 55:168-178, 1999
Lo SH, Yu QC, Degenstein L, Chen LB, Fuchs E: Progressive kidney degeneration in mice lacking tensin. J Cell Biol 136:1349-61, 1997
Lowden DA, Lindemann GW, Merlino G, Barash BD, Calvet JP, Gattone VH: Renal cysts in transgenic mice expressing transforming growth factor-α. J Lab Clin Med 124:386-94, 1994
Lozzio BB, Chernoff AI, Machado ER, LozzioCB: Heriditary brenal diseae in a mutant strain of rats. Science 156:1742-4, 1967
Lu W, Peissel B, Babakhanlou H, Pavlova A, Geng L, Fan X, Larson C, Brent G, Zhou J: Perinatal lethality with kidney and pancreas defects in mice with a targetted PKD1 mutation. *Nature Gen* 17: 179-181, 1997
Lu W, Fan X, Basora N, Babakhanlou H, Law T, Rifai N, Harris PC, Perez-Atayde AR, Rennke HG, Zhou J: Late onset of renal and hepatic cysts in Pkd-1 targeted heterozygotes. *Nat genet* 21: 160-161, 1999
Lyon MF, Hulse EV:An inherited kidney disease of mice resembling human nephronophthisis. J Med Genetics 8:41-8, 1971
MacKay K, Striker LJ, Pinkert CA, Brinster RL, Striker GE: Glomerulosclerosis and renal cysts in mice transgenic for the early region of SV40. Kidney Int 32:827-37, 1987
Mainzer F, Saldino RM, Ozonoff MB, Minagi H: Familial nephropathy associated with retinitis pigmentosa, cerebellar ataxia and skeletal abnormalities. Am J Med 49:556-562, 1970
Mangoo-Karim R, Uchic ME, Grant M, Shumate WA, Calvet JP, Park CH, Grantham JJ: Renal epithelial fluid secretion and cyst growth: the role of cyclic AMP. FASEB 3:2629-2632, 1989
Martin PY, Abraham WT, Lieming X,Olson BR, Oren MR, Ohara M, Schrier RW: Selective V2-receptor vasopressin antagonism decreases urinary aquaporin-2 excretion in patients with chronic heart failure. J Am Soc Nephrol 10:2165-70, 1999
Martinez JR, Cowley BD, Gattone VH 2nd, Nagao S, Yamaguchi T, Kaneta S, Takahashi H, Grantham JJ: The effect of paclitaxel on the progression of polycystic kidney disease in rodents. Am J Kidney Dis29:435-44, 1997
McAloose D, Casal Polycystin, Patterson DF, Dambach DM: Polycystic kidney disease in two related West Highland White Terrier litters. Vet Pathol 35:77-81, 1998
McAteer JA, Evan AP, Gardner KD. Morphogenetic clonal growth of kidney epithelial cell line MDCK. Anat Record 217:229-39, 1987
McDonald AT, Crocker JF, Digout SC, McCarthy SC, Blecher SR, Cole DE: Glucocorticoid induced polycystic kidney disease – a threshold trait. Kidney Int 37:901-8, 1990
McDonald RA, Avner ED: Inherited polycystic kidney disease in children. Seminars in Nephrology 11:632-642, 1991
McDonald RA, Watkins SL, Avner ED: Polycystic kidney disease in: Barratt TM, Avner ED, Harmon WE: Pediatric Nephrology. Baltimore: Lippincott Williams and Wilkins. pp459-474, 1999
McGeoch JEM, Darmady EM:Polycystic disease of kidney, liver and pancreas; a possible pathogenesis. J Pathol 119:221-8, 1976
McKenna SC, Carpenter JL:Polycystic disease of the kidney and liver in the Cairn terrier. Vet Path 17:436-42, 1980
Mochizuki T, Wu G, Hayashi T, et al. PKD2, a gene for polycystic kidney dsease that encodes an integral membrane protein. Science 272:1339-42, 1996

Montesano R, Matsumoto K, Nakamura T, Orci L: Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 67:901-8, 1991
Montesano R, Soriano JV, Pepper MS, Orci L: Induction of epithelial branching tubulogenesis in vitro. J Cell Physiol 173:152-61, 1997
Moser M, Pscherer A, Roth C, Becker J, Mucher G, Zerres K, Dixkens C, Weis J, Guay-Woodford L, Buettner R, Fassler R: Enhanced apoptotic cell death of renal epithelial cells in mice lacking transcription factor AP-2 β. Genes Dev 11:1938-48, 1997
Moskowitz DW, Bonar SL, Liu W, Sirgi CF, Marcus MD, Clayman RV: Epidermal growth factor precursor is present in a variety of human renal cystfluids. J Urol53:578-83,1995
Moullier P, Freidlander G, Calise D, Ronco P, Perricaudet M, Ferry N: Adenoviral mediated gene transfer to renal tubular cells in vivo. Kidney Int 45:1220-1225, 1994
Moyer JH, Lee-Tischler MJ, Kwon HY, Schrick JJ, Avner ED, Sweeney WE, Godfrey VL, Cacheiro NL, Wilkinson JE, Woychik RP: Candidate gene associated with a mutation causing recessive polycystic kidney disease in mice. Science 264:1329-33, 1994
Mucher G, Becker J, Knapp M, Buttner R, Moser M, Rudnik-Schoneborn S, Somlo S, Germino G, Onuchic L, Avner E, Guay-Woodford L, Zerres K: Fine mapping of the autosomal recessive polycystic kidney disease locus (PKHD1) and the genes MUT, RDS, CSNK2 β, and GSTA1 at 6p21.1-p12. Genomics 48:40-5, 1998
Nadasdy T, Laszik Z, Lajoie G, Blick KE, Wheeler DE, Silva FG: Proliferative activity of cyst epithelium in human renal cystic diseases. J Am Soc Nephrol 5:1462-8, 1995
Nagao S, Hibino T, Koyama Y, Marunouchi T, Konishi H, Takahashi H: Strain differences in expression of the adult type polycystic kidney disease gene, *pcy*, in the mouse. Jikken Dobutsu 40:45-53, 1991
Nagao S, Watanabe T, Ogiso N, Marunouchi T, Takahashi H: Genetic mapping of the polycystic kidney gene, pcy, on mouse chromosome 9. Biochem Genet 33:401-12 , 1995
Nakamura T, Ebihara I, Nagaoka I, Tomino Y, Nagao S, Takahashi H, Koide H: Growth factor gene expression in kidney of murine polycystic kidney disease. J Am Soc Nephrol 3:1378-86, 1993a
Nakamura T, Ebihara I, Fukui M, Osada S, Tomino Y, Masaki T, Goto K, Furuichi Y, Koide H: Increased endothelin and endothelin receptor mRNA expression in polycystic kidneys of cpk mice. J Am Soc Nephrol 1993 4:1064-72, 1993b
Nauta J, Ozawa Y, Sweeney WE, Rutledge JC, Avner ED: Renal and biliary abnormalities in a new murine model of autosomal recessive polycystic kidney disease. Ped Nephrol 7:163-72, 1993
Nauta J, Sweeney WE, Rutledge JC, Avner ED: Biliary epithelial cells from mice with congenital polycystic kidney disease are hyperresponsive to epidermal growth factor. Ped Res 37:755-63, 1995
Nauta J, Goedbloed MA, Luider TM, Hoogeveen Ath, van den Ouweland, Halley DJJ: The Han:SPRD rat is not a genetic model of human autosomal dominant polycystic kidney disease. Lab Animals 31:241-7, 1997
Nauta J, Goedbloed MA, van Herck H, Hesselink DA, Visser P, van Dokkum RPE, Wright CJ, Guay-Woodford LM: A new rat model that phenotypically resembles ARPKD. Submitted nov 99
Neufeld TK, Douglass D, Grant M, Ye M, Silva F, Nadasdy T, Grantham JJ: In vitro formation and expansion of cysts derived from human renal cortex epithelial cells. Kidney Int 41:122-1236, 1992
Nguyen HQ, Danilenko DM, Bucay N, DeRose ML, Van GY, Thomason A, Simonet WS: Expression of keratinocyte growth factor in embryonic liver of transgenic mice causes changes in epithelial growth and differentiation resulting in polycystic kidneys and other organ malformations. Oncogene 12:2109-19, 1996
Nidess R, Koch WE, Fried FA, McFarland E, Mandell J: Development of the embryonic murine kidney in normal and congenital polycystic kidney disease: characterization of a proximal tubular degenerative process as the first observable light microscopical defect. J Urol 131:156-62, 1984
Obermuller N, Gretz N, Kriz W, van der Woude FJ, Reilly RF, Murer H, Biber J, Witzgall R: Differentiation and cell polarity during renal cyst formation in the Han:SPRD (cy/+) rat, a model for ADPKD. Am J Physiol 273:F357-71, 1997

Ogborn MR, Crocker JFS, McCarthy SC: RU 38486 prolongs survival in murine congenital polycystic kidney disease. J Steroid Bioch 28:783-4, 1987
Ogborn MR, Sareen S: Amelioration of polycystic kidney disease by modification of dietary protein intake in the rat. J Am Soc Nephrol 6:1649-54, 1995a
Ogborn MR, Sareen S, Tomobe K, Takahashi H, Crocker JF: Renal tubule Na,K-ATPase polarity in different animal models of polycystic kidney disease. J Histochem Cytochem 43:785-90, 1995b
Ogborn MR, Sareen S, Pinette G: Cilazapril delays progression of hypertension and uremia in rat polycystic kidney disease. Am J Kidney Dis26:942-6, 1995c
Ogborn MR, Bankovic-Calic N, Shoesmith C, Buist R, Peeling J: Soy protein modification of rat polycystic kidney disease. Am J Physiol 274:F541-9, 1998
Ogborn MR, Nitschmann E, Weiler H, Leswick D, Bankovic-Calic N: Flaxseed ameliorates interstitial nephritis in rat polycystic kidney disease. Kidney Int 55:417-23, 1999
Ohno K, Kondo K: A mutant rat with congenital skeletal abnormalities and polycystic kidneys. *Jikken Dobutsu* 38: 139-146, 1989
Orellana SA, Sweeney WE, Neff CD, Avner ED: Epidermal growth factor receptor expression is abnormal in murine polycystic kidney. Kidney Int 47:490-9, 1995
Osathanondh V, Potter EL:Pathogenesis of polycystic kidneys. Type I due to hyperplasia of interstitial portions of collecting tubules. Arch Pathol 77:466-473, 1964
Parfrey PS, Bear JC, Morgan J et al. : The diagnosis and prognosis of autosomal dominant polycystic kidney disease. N Eng J Med 323:1085-1090, 1990
Parnell SC, Magenheimer BS, Maser RL, Rankin CA, Smine A, Okamoto T, Calvet JP: The polycystic kidney disease-1 protein, polycystin-1, binds and activates heterotrimeric G-proteins in vitro. Biochem Biophys Res Commun 251:625-31, 1998
Peral B, San Millan JL, Ong ACM, Gamble V, Ward CJ, Strong C, Harris PC: Screening the C' region of the polycystic kidney disease 1 (PKD1) gene reveals 6 novel mutations. Am J Hum Genet 58:86-96, 1996
Peral B, Gamble B, Strong C, Ong ACM, Sloane-Stanley J, Zerres K, Winearls CG, Harris PC:Identification of mutations in the duplicated region of the polycystic kidney disease 1 (PKD1) gene by a novel approach. Am J Hum Genet 60:1399-1410, 1997
Pey R, Hafner M, Schieren G, Bach J, Gretz N: In vitro formation of cysts derived from a rat model of autosomal dominant polycystic kidney disease. Nephrol Dial Transplant 11 Suppl 6:58-61, 1996
Potter EL: Normal and abnormal development of the kidney. Year Book Medical Publishers, Chicago , 1972
Preminger GM, Koch WE, Fried FA, McFarland E, Murphy ED, Mandell J: Murine congenital polycystic kidney disease. J Urol 127:556-60, 1982
Pretorius DH, Lee ME, Manco-Johnson ML et al. Diagnois of autosomal dominant polycystic kidney disease in utero and in the young infant. J Ultrasound Med 6:249-255, 1987
Pugh JL, Sweeney WE Jr, Avner ED: Tyrosine kinase activity of the EGF receptor in murine metanephric organ culture. Kidney Int 47:774-81, 1995
Qian F, Watnick TJ, Onuchic LF, Germino GG: The molecular basis of focal cyst formation in human autosomal polycystic kidney disease type I. Cell 87:979-987, 1996
Qian F, Germino FJ, Cai Y, Zhang X, Somlo S, Germino GG: PKD1 interacts with PKD2 through a probable coiled-coil domain. Nature Genetics 16:179-183, 1997
Quaife CJ, Mathews LS, Pinkert CA, Hammer RA, Brinster RL, Palmiter RD: Histopathology associated with elevated levels of growth hormone and insuline like growth factor I in transgeneic mice. Endocrinology 124:40-48, 1989
Rahilly MA, Samuel K, Ansell JD, Michelem HS, Fleming S: Polycystic kidney disease in the CBA?M immunodeficient mouse. J Pathol 168:335-42, 1992
Ramasubbu: K, Gretz N, Bachmann S: Increased epithelial cell proliferation and abnormal extracellular matrix in rat polycystic kidney disease. J Am Soc Nephrol 9:937-45, 1998
Ramirez B, Fimitcheva I, Aziz: Abnormal regulation of the Ke 6 gene, a new 17 β-hydroxysteroid dehydrogenase in the cpk mouse kidney. Mol Cell Endocrinol 143:9-22, 1998

Rankin CA, Grantham JJ, Calvet JP: C-fos expression is hypersensitive to serum-stimulation in cultured cystic kidney cells from the C57BL/6J-cpk mouse. J Cell Physiol 152:578-86, 1992
Rankin CA, Itoh Y, Tian C, Ziemer DM, Calvet JP, Gattone VH 2nd: Matrix metalloproteinase-2 in a murine model of infantile-type polycystic kidney disease. J Am Soc Nephrol 10:210-7, 1999
Ravine D, Walker R, Gibson R:Phenotype and genotype heterogenaicty in autosomal dominant polycystic kidney disease. Lancet 340:1330-1333, 1992
Rayer P: Atlas in: Traité des maladies des reins. Paris, 1841
Reeders ST, Breuning MH, Davies KE et al. A highly polymorphic DNA marker linked to adult polycystic kidney disease on chromosome 16. Nature 317:542-544, 1985
Ribacchi R: Riducione progressiva della fosfatasi alcaline e della adenosintri-fosfatasi mg-dipendente nel rene policistico congenito dei topi PM/Se. Lav Anat Pat Perugia 37:9-14, 1977
Richards WG, Sweeney WE, Yoder BK, Wilkinson JE, Woychik RP, Avner ED: Epidermal growth factor receptor activity mediates renal cyst formation in polycystic kidney diseae. J Clin Invest 101:935-939, 1998
Rocco MV, Neilson EG, Hoyer JR, Ziyadeh FN: Attenuated expression of epithelial cell adhesion molecules in murine polycystic kidney disease. Am J Physiol 262:F679-86, 1992
Roelfsema JH, Spruit L, Saris JJ, Chang Polycystin, Pirson Y, van Ommen GJB, Peters PJM, Breuning MH: Mutation detection in the rrepeated part of the PKD1 gene. Am J Hum Genet 61: 1044-1052, 1997
Romeo G, Devoto M, Costa G et al. : A second genetic locus for autosomal dominant polycystic kidney disease. Lancet 2:8-11, 1988
Romero R, Cullen M, Jeanty Polycystin, et al.: The diagnosis of congenital renal anomalies with ultrasound. II. Infantile polycystic kidney disease. Am J Obstet Gynecol 150:259-262, 1984
Rupple BM: Congenital polycystic disease of the kidney occuring in mice. J Nat Cancer Inst 5:1183-87, 1955
Russel ES, McFarland EC: Cystic kidneys. Mouse Newsletter 56:40-3, 1977
Sampson JR, Maneshwar MM, Aspinwall R, Thompson Polycystin, Cheadle JP, Ravine D, Roy S, Haan E, Bernstein J, Harris PC: Renal cystic disease in tuberous sclerosis: The role of the polycytic kidney disease 1 geen. Am J Hum Genet 61:843-851, 1997
Sandford R, Sgotto B, Aparicio S, Brenner S, Vaudin M, Wilson RK, Chissoe S, Pepin K, Bateman A, Chothia C, Hughes J, Harris Polycystin: Comparitive analysis of the polycystic kidney disease 1 (PKD1) gene reveals an integral membrane glycoprotein with multiple evolutionary conserved domains. Hum Mol Genet 6:1483-1489, 1997
Schaefer L, Han X, Gretz N, Hafner C, Meier K, Matzkies F, Schaefer RM: Tubular gelatinase A (MMP-2) and its tissue inhibitors in polycystic kidney disease in the Han:SPRD rat. Kidney Int 49:75-81, 1996
Schäfer K, Bader M, Gretz N, Oberbaumer I, Bachmann S: Focal overexpression of collagen IV characterizes the initiation of epithelial changes in polycystic kidney disease. Exp Nephrol 2:190-5, 1994a
Schäfer K, Gretz N, Bader M, Oberbaumer I, Eckardt KU, Kriz W, Bachmann S: Characterization of the Han:SPRD rat model for hereditary polycystic kidney disease. Kidney Int 46:134-52, 1994b
Schaffner DL, Barrios R, Massey C, Banez EI, Ou CN, Rajagopalan S, Aguilar-Cordova E, Lebovitz RM, Overbeek PA, Lieberman MW: Targeting of the rasT24 oncogene to the proximal convoluted tubules in transgenic mice results in hyperplasia and polycystic kidneys. Am J Pathol 142:1051-60, 1993
Schieren G, Pey R, Bach J, Hafner M, Gretz N: Murine models of polycystic kidney disease. Nephrol Dial Transplant 11 (Suppl6): 38-45, 1996
Schwab SJ, Bander SJ, Klahr S: Renal infection in autosomal dominant polycystic kidney disease. Am J Med 82:714-718, 1987
Scott PC, Vasey J: Progressive polycystic renal disease in an aged horse. Austr Vet J 63:92,xv, 1986
Sedman A, Bell Polycystin, Manco-Johnson M et al.: Autosomal dominant polycystic kidney disease in childhood: A longitudinal study. Kidney Int 31:1000-1005, 1987

Simon EA, Cook S, Davisson MT, D'Eustachio P, Guay-Woodford LM: The mouse congenital polycystic kidney (cpk) locus maps within 1.3 cM of the chromosome 12 marker D12Nyu2. Genomics 21:415-8 , 1994
Slade MJ, Kirby RB, Posci I, Jones JK, Price RG: Presence of laminin fragments in cyst fluid from patients with autosomal dominant polycystic kidney diseae (ADPKD): role in proliferation of tubular eoithelial cells. Biochem Biophys Acta 1401:203-10, 1998
Sommerdahl CS, Woychik RP, Sweeney WE, Avner ED, Wilkinson WE: Efficacy of taxol in th eorpk mouse model of polycystic kidney disease. Pediatr Nephrol 11:728-733, 1997
Stricklett PK, Nelson RD, Kohan DE: Targeting collecting tubules using the aquaporin-2 promotor. Exp Nephrol 7:67-74, 1999
Sullivan LP, Grantham JJ: Mechanisms of fluid secretion by polycystic epithelia. Kidney Int 49:1586-91, 1996
Sweeney WE, Avner ED: BPK cystfluid contains EGF and TGF α like peptides which are mitogenic and phosphorylate apical EGF-R. J Am Soc Nephrol7:1606, 1996
Sweeney WE, Avner ED: Functional activity of epidermal growth factor receptors in autosomal recessive polycystic kidney disease. Am J Physiol 275:F387-94, 1998
Sweeney WE, Futey L, Frost P, Avner ED: In vitro modulation of cyst formation by a novel tyrosine kinase inhibitor. Kidney Int 56:406-413, 1999
Sweet HO, Lane PW: X-linked polydactyly (*Xpl*), a new mutation in the mouse. J Hered. 7:207-209, 1980
Takahashi H, Ueyama Y, Hibino Y, Kuwahara Y, Suzuki S, Hioki K,Tamaoki N: A new mouse model of genetically transmitted kidney disease. J of Urol 135:1280-3, 1986
Takahashi H, Calvet JP, Dittemore-Hoover D, Yoshida K, Grantham JJ, Gattone VH: A hereditary model of slowly progressive polycystic kidney disease in the mouse. J Am Soc Nephr 1:980-9, 1991
Takayama H, LaRochelle WJ, Sabnis SG, Otsuka T, Merlino G: Renal tubular hyperplasia, polycystic disease, and glomerulosclerosis in transgenic mice overexpressing hepatocyte growth factor/scatter factor. Lab Invest 77:131-8, 1997
Tanner GA, Gretz N, Connors BA, Evan AP, Steinhausen M: Role of obstruction in autosomal dominant polycystic kidney disease in rats. Kidney Int 50:873-86, 1996
Tanner GA: Potassium citrate/citric acid intake improves renal function in rats with polycystic kidney disease. J Am Soc Nephrol 9:1242-8, 1998
Taub M, Laurie GW, Martin GR, Kleinman HK: Altered basement membrane protein biosynthesis by primary cultures of cpk/cpk mouse kidney. Kidney Int 37:1090-7, 1990
The European Polycystic Kidney Disease Consortium: The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. Cell 77:881-894, 1994
The International Polycystic Kidney Disease Consortium: Polycystic kidney disease: The complete structure of the PKD1 gene and its protein. Cell 81:289-298, 1995
Tomobe K, Philbrick DJ, Ogborn MR, Takahashi H, Holub BJ: Effect of dietary soy protein and genistein on disease progression in mice with polycystic kidney disease. Am J Kidney Dis 31:55-61, 1998
Torres VE, Wiebers DO, Forbes GS:Cranial computer tomography and magnetic resonance imaging in autosomal dominant polycystic kidney disease. J Am Soc Nephrol 1:84-90, 1990
Torres VE: Apoptosis in cystogenesis: Hands on or hands off? Kidney Int 55:334-335, 1999
Trudel M, D'Agati V, Costantini F: C-myc as an inducer of polycystic kidney disease in transgenic mice. Kidney Int 39:665-71, 1991
Trudel M, Lanoix J, Barisoni L, Blouin MJ, Desforges M, L'Italien C, D'Agati: C-myc-induced apoptosis in polycystic kidney disease is Bcl-2 and p53 independent. J Exp Med 186:1873-84, 1997
Trudel M, Barisoni L, Lanoix J, D'Agati V: Polycystic kidney disease in SBM transgenic mice: role of c-myc in disease induction and progression. Am J Pathol 152:219-29, 1998
Tsiokas L, Kim E, Arnould T, Sukhatme VP, Walz G: Homo- and heterodimeric interactions between the gene products of PKD1 and PKD2. Proc Natl Acad Sci U S A 94:6965-70, 1997

Uchida S, Tsutsumi O, Hise MK, Oka T: Role of epidermal growth facto in compensatory renal hypertrophy in mice. Kidney Int 33: 387, 1988
Uemasu J, Fujiwara M, Munemura C, et al.: Effects of topical installation of minocycline hydrochloride on cyst size and renal function in polycystic kidney disease.Clin Nephrol 39:140-144., 1993
Upadhya P, Churchill G, Birkenmeier EH, Barker JE, Frankel WN: Genetic modifiers of polycystic kidney disease in intersubspecific KAT2J mutants. Genomics 58:129-37, 1999
Van Adelsberg J: Peptides from the PKD repeats of polycystin, the PKD1 gene product, modulate pattern formation in the developing kidney. Dev Genet 24:299-308, 1998
Van Alstine WG, Trampel DW: Polycystic kidneys in a pigeon. Avian Dis 28:758-64, 1984
Vanden Heuvel GB, Bodmer R, McDonnell KR, Nagami GT, Igarashi Polycystin: Expression of a cut-related homeobox genme in developing and polycystic mouse kidney. Kidney Int 50:453-61, 1996
Veis DJ, Sorensen CM, Shutter JR, Korsmeyer SJ: Bcl-2-deficient mice demonstrate fulminant lymfoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75:229-40, 1993
Verani R, Walker Polycystin, Silva FG: Renal cystic disease of infancy: results of histochemical studies. Pediatr Nephrol 3:37-42, 1989
Wagner D, Metzger R, Paul M, Ludwig G, Suzuki F, Takahashi S, Murakami K, Ganten D: Androgen dependence and tissue specificity of renin messenger RNA expression in mice. J Hypertension 8:45-52, 1990
Wang D, Strandgaard S: The pathogenesis of hypertension in autosomal dominant polycystic kidney disease. J Hypertension 15:925-933, 1997
Wanke R, Hermanns W, Folger S, Wolf E, Brem G: Accelerated growth and visceral lesions in transgenic mice expressing foreign genes of the growth hormone family: an overview. Pediatr Nephrol 5:513-21, 1991
Ward CJ, Turley H, Ong ACM, Comley M, Biddolph S, Chetty R, Ratcliffe PJ, Gatter K, Harris PC: Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult, and polycystic kidney. PNAS 93: 1524-1528, 1996
Watnick TJ, Torres VE, Gandolph MA, Qian F, Onuchic LF, Klinger KW, Landes G, Germino GG: Somatic mutation in individual liver cysts supports a two-hit model of cystogenesis in autosomal dominant polycystic kidney disease. Mol Cell 2:247-251, 1998
Watson ML, Macnicol AM, Allan PL, Wright AF: Effects of angiotensin converting enzyme inhibitor in adult polycystic kidney disease. Kidney Int 41:206-210, 1992
Welling LW, Grantham JJ: Cystic and developmental diseases of the kidney. In: Brenner BM (ed.) The Kidney. Saunders, Philadelphia, 1996, pp1828-1863
Werder AA, Amos MA, Nielson AH, Wolfe GH: Comparative effects of germfree and ambient environments on the development of cystic kidney disease in CFWwd mice. J Lab Clin Med 103:399-407, 1984
Wijeratne WV, Wells GA: Inherited renal cysts in pigs: results of breeding experiments. Vet Record 107:484-8, 1980
Wilson PD, Sherwood AC, Palla K, Du J, Watson R, Norman JT: Reversed polarity of Na^+-K^+-ATPase: mislocation to apical plasma membranes in polycystic kidney disease epithelia. Am J Physiol 260:F240-30, 1991
Wilson PD, Hreniuk D, Gabow PA: Abnormal extracellular matrix and excessive growth of human adult polycystic kidney disease epithelia. J Cell Physiol 150:360-9, 1992
Wilson PD, Du J, Norman JT: Autocrine, endocrine and paracrine regulation of growth abnormalities in autosomal dominant polycystic kidney disease. Eur J Cell Biol 61:131-8, 1993
Wilson PD: Pathogenesis of polycystic kidney disease: Altered cellular function; in Watson ML, Torres VE (eds); Polycystic kidney disease. Oxford, Oxford Medical Publications, pp 125-63, 1996
Wilson PD, Hovater JS, Casey CC, Fortenberry JA, Schwiebert EM: ATP release mechanisms in primary cultures of epithelia derived from the cysts of polycystic kidneys. J Am Soc Nephrol 10:218-29, 1999a

Wilson PD, Burrow CR: Cystic diseases of the kidney: Role of adhesion molecules in normal and abnormal tubulogenesis. Exp Nephrol 7:114-24, 1999b
Wilson PD, Geng L, Li X, Burrow CR: The PKD-1 encoded protein 'polycystin-1 co-localizes with α2β1 integrin in focal adhesions and is tyrosine phosphorylated. Lab Invest in press, 1999c
Winyard PJ, Nauta J, Lirenman DS, Hardman P, Sams VR, Risdon RA, Woolf AS: Deregulation of cell survival in cystic and dysplastic renal development. Kidney Int 49:135-46, 1996
Woo DD, MiaoSY, Pelayo JC, Woolf AS: Taxol inhibits progression of congenital polycystic kidney disease. Nature 368:50-53, 1994
Woo D: Apoptosis and loss of renal tissue in polycystic kidney diseases. N Eng J Med 333:18-25, 1995
Woo DD, Tabancay AP Jr, Wang CJ: Microtubule active taxanes inhibit polycystic kidney disease progression in cpk mice. Kidney Int 51:1613-8, 1997a
Woo DD, Nguyen DK, Khatibi N, Olsen Polycystin: Genetic identification of two major modifier loci of polycystic kidney disease progression in pcy mice. J Clin Invest 100:1934-40, 1997b
Woo D: Progression of polycystic kidney disease in pcy mice is a quantitative trait under polygenic control. J Clin Invest 100:1934-1940, 1998
Wright C, Guay-Woodford L: Genetic analysis of loci that modify renal cystic disease in the mouse bpk model. J Am Soc Nephrol 8:1781A, 1997
Wu CH, Wilson JM, Wu GY: Targeting genes: delivery and persistent expression of a foreign gene driven by mammalian regulatory elements in vivo, J Biol Chem 264: 16985-7, 1989
Wu G, D'Agati V, Cai Y, Markowitz G, Park JH, Reynolds DM, Maeda Y, Le TC, Hou H Jr, Kucherlapati R, Edelmann W, Somlo S:Somatic inactivation of Pkd2 results in Polycystic kidney disease. *Cell* 93: 177-188, 1998
Yamaguchi T, Valli VE, Philbrick D, Hlub B, Yoshida K, Takahashi H: Effects of dietary supplementation with n-3 fatty acids on kidney morphology and the fatty acid composition of phospholipids and triglycerides from mice with polycystic kidney disease. Res Commun Chem Pathol Pharmacol 69:335-51, 1990
Yamaguchi T, Nagao S, Takahashi H, Ye M, Grantham JJ: Cystfluid from DBA-2FG PCY mice stimulates fluid secretion and cyclic AMP accumulation by MDCK cells. J Am Soc Nephrol 4:827, 1993
Yamaguchi T, Nagao S, Kasahara M, Takahashi H, Grantham JJ: Renal accumulation and excretion of cyclic adenosine monophosphate in a murine model of slowly progressive polycystic kidney disease. Am J Kidney Dis30:703-9, 1997
Ye M, Grant M, Sharma M, Elzinga L, Swan S, Torres VE, Grantham JJ: Cyst fluid from human autosomal dominant polycystic kidneys promotes cyst formation and expansion by renal epithelial cells in vitro. J Am Soc Nephrol 3:984-94, 1992
Yoder BK, Richards WG, Sweeney WE, Wilkinson JE, Avener ED, Woychik RP: Insertional mutagenesis and molecular analysis of a new gene associated with polycystic kidney disease. Proc Assoc Am Physicians 107:314-23, 1995
Yoder BK, Richards WG, Sommardahl C, Sweeney WE, Michaud EJ, Wilkinson JE, Avner ED, Woychik RP: Functional correction of renal defects in a mouse model for ARPKD through expression of the cloned wild-type Tg737 cDNA. Kidney Int 1996 Oct;50(4):1240-8
Yoder BK, Richards WG, Sommardahl C, Sweeney WE, Michaud EJ, Wilkinson JE, Avner ED, Woychik RP: Differential rescue of the renal and hepatic disease in an autosomal recessive polycystic kidney disease mouse mutant. A new model to study the liver lesion. Am J Pathol 150:2231-41, 1997
Yium J, Gabow P, Johnson A, Kimberling W, Martinez M: Autosomal dominant polycystic kidney disease in blacks: Clinical course and effects of sickle-cell hemoglobin. J Am Soc Nephrol 4:1670-1674, 1994
Zeier M, Geberth S, Gonzalo A, Mohring K, Waldherr R, Ritz E: Renal histology in polycystic kidney disease with incipient and advanced renal failure. Kidney Int 42:1259-65, 1991

Zeier M, Gebrth S, Mandelbaum A, Ritz E: Elevated bloodpressure profile and left ventricular mass in children and young adults with autosomal dominant "polycystic kidney disease. J Am Soc Nephrol 3:1451-1457, 1993

Zerres K, Hansmann M, Mallmann R, et al.:Autosomal recessive polycystic kidney disease: problems of prenatal diagnosis. Prenat Diagn 8:215-229, 1988

Zerres K, Rudnik-Schoneborn S, Deget F: Routine examination of children at risk for autosomal dominant polycystic kidney disease. Lancet 339: 1356-1357, 1992

Zerres K, Mucher G, Bachner L, Deschennes G, Eggermann T, Kaariaainen H, Knapp M, Lenner T, Misselwitz J, Muhlendahl K, et al.: Mapping of the gene for autosomal recessive polycystic kidney disease (ARPKD) to chromosome 6p21-cen. Nat Genet 7:429-432, 1994

Zerres K, Rudnik-Schoneborn S, Deget F, Holtkamp U, Brodehl J, Geisert J, Scharer K: Autosomal recessive polycystic kidney disease in 115 children: Clinical presentation, course and influence of gender. Acta Paediatr 85:437-445, 1996

Zhu G, Nicolson AG, Cowley BD, Rosen S, Sukhatme VP: In vivo adenovirus-mediated gene transfer into normal and cystic rat kidneys. Gene Ther 3:298-304, 1996

Summary

Summary

In this thesis we report experimental data regarding the pathophysiology of polycystic kidney disease, PKD. PKD is the most frequent inherited cause of renal failure in humans and is characterized by the progressive replacement of normal renal tissues by fluid filled cavities (cysts). A direct treatment of the cystic process is not available. The development of such a treatment will require a proper understanding of the biological mechanisms involved in the pathophysiology of cyst formation.

In **chapter 1** we review the PKD literature. This review presents the clinical entities of PKD, as well as a summary of the current understanding of cyst formation. Specifically, we focus on the contribution of experimental models of PKD to current knowledge.

PKD includes autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD). This classification parallels differences in clinical, genetic and histopathological features. ADPKD affects 1 in 1.000 individuals and leads to renal failure in half the patients, usually after the third decade of life. The disease results from the progressive formation of cysts, often complicated by hypertension and sometimes by renal infections. Many patients have additional cysts in the liver. Other manifestations include cerebral aneurysms, cardiac valve abnormalities and cysts in other excretory organs. In contrast, ARPKD affects only 1 in 40.000, but is a more severe disease than ADPKD, leading to renal failure either at birth or during childhood in all patients. The renal lesions are associated with severe hypertension, biliary hyperplasia and hepatic fibrosis.

Genetic analysis has revealed a number of causative gene defects. The gene involved in ARPKD has been localised to chromosome 6p21.1-p12, but has not yet been cloned. ADPKD results from a mutation to any of at least three distinct genes. Two of these genes, PKD1 and PKD2, have been cloned and sequenced. The proteins PKD1 and PKD2 gene products, polycystin-1 and polycystin-2, are predicted to be transmembrane proteins involved in cell-cell and cell-matrix interactions. At present, many different research groups are attempting to define the normal functions of these proteins and to investigate how their absence and/or disfunction leads to renal cyst formation.

Histopathological and cell biological analysis indicates that cysts develop as dilations of histologically normal nephrons. Interestingly, this process does not affect all nephrons, suggesting that factors other than the genetic defect are involved in the initiation of cyst formation. It has been proposed that the formation and subsequent expansion of cysts requires at least three key pathologic features: epithelial cell proliferation, altered transepithelial fluid transport and abnormal regulation of the extracellular matrix. These features have been studied extensively, using both human tissues and experimental models of PKD.

In order to better understand the factors involved in renal cystogenesis and in the progression towards renal failure, many investigators have focussed on either animal models, whole organ models or cell models of renal cyst formation. In this first chapter we present a summary of the currently available models and assess their contribution to the understanding of the pathophysiology of renal cystic disease.

In **chapter 2** we report the exploration of a number of experimental PKD models.

In **chapter 2.1** we describe the characteristics of a new spontaneous mouse model of ARPKD, *bpk*. Affected *bpk* mice manifest severe renal cystic disease in association with biliary abnormalities, much like human ARPKD. Homozygotes typically die before reaching maturity, due to renal failure with enlarged kidneys. Renal cysts shift from proximal tubules at birth to collecting tubules 20 days later. The epithelium lining these cysts is clearly over-proliferative, as indicated by the increased amount of mitosis in these cells compared to normal epithelium. In addition, epithelial hyperplasia was found in the intra- and extrahepatic biliary tract of affected mice, including nonobstructive hyperplastic dilatation of the extrahepatic biliary tract. The hyperplastic renal and biliary lesions make the *bpk* mouse strain a good model for the dual organ cellular pathophysiology of PKD. We isolated, cultured and characterized biliary tract epithelium from common bile duct explants of *bpk* mice in order to explore the cellular basis of this hyperplasia (**chapter 2.2**). Primary cultures resulted in dense colonies of contact-inhibited epithelial cells with a homogenous growth pattern. Colony growth in serum-free basal medium of *bpk*-derived cells was not different from controls, as assessed by thymidine uptake and colony size increments. Supplementation of the medium with epidermal growth factor (EGF) however, induced a proliferative response in the *bpk* colonies that was significantly increased over controls, as assessed by thymidine uptake and colony size increments. This increased response was not observed using other growth factors. We concluded that primary cultures of *bpk* biliary epithelium may provide a useful in vitro model for the study of the cellular pathophysiology of ARPKD.

While mouse models are particularly well suited for investigating the molecular pathogenesis of PKD, the rat is well-established as an experimental model of renal physiology. The Han:SPRD-*cy* rat has been proposed as a model for ADPKD. The study described in **chapter 2.3** explores the genetic basis of the Han:SPRD-*cy* rat model of ADPKD. We investigated whether the disease in these rats is linked to the rat homologue of the PKD1 gene. PKD1 carries the disease-causing mutation in most patients with ADPKD. We used the protamine gene (*prm1*) as a polymorphic marker for the PKD1 region in the rat. Mating Han:SPRD-cy rats with the inbred BB strain and backcrossing the offspring with BB rats yielded animals informative for linkage analysis. This analysis indicated that PKD in the Han:SPRD-*cy* rat is not caused by a mutation in the PKD1 gene.

We have now identified a new spontaneous rat mutation, designated *wpk*. In **chapter 2.4** we describe the characterization of the *wpk* phenotype as well as the mapping of the *wpk* locus. The phenotype of homozygous mutants resembles human ARPKD, including nephromegaly, hypertension, proteinuria, uremia and death before reaching maturity. The renal cysts shift from both proximal and distal nephron segments at birth to collecting tubules during neonatal development. Affected rats show no evidence of biliary tract abnormalities. The *wpk* locus was mapped just proximal of *cy*, the locus involved in the Han:SPRD-*cy* rat model, on chromosome 5 and complementation testing has demonstrated that these two loci are not allelic. While homology mapping indicates that rat *wpk* and human ARPKD involve distinct genes, this new mutation provides an excellent experimental model to study the molecular pathogenesis as well as the renal pathophysiology of ARPKD.

Organogenesis involves an increase in cell numbers, cell differentiation and morphogenesis. The increase of cell number results from both proliferation and death. 'Programmed cell death' is a well defined cellular phenomenon and thought to be play a role in normal differentiation and morphogenesis. We report in **chapter 3.1** that apoptosis occurs during normal renal development and continues at a very low level after birth. Moreover, we noted that apoptosis occurs at an increased incidence in pre- and postnatal human ARPKD and dysplastic kidneys, when compared to age matched controls. In ARPKD kidneys we found widespread apoptosis in the interstitium around undilated tubules, in undilated tubules between cysts and in the cyst lining epithelia. We speculate that deregulation of cell survival may reflect incomplete tissue maturation and may contribute to the progressive destruction of functional kidney tissue.

Mutations in the PKD1 gene are responsible for most cases of polycystic kidney disease, but little is known about how these mutations lead to the development of cysts. Even less is known about the normal role of polycystin-1, the PKD1 gene product. In **chapter 3.2** we describe our studies on the cellular localization of polycystin-1. We generated 14 different polyclonal antisera against fragments of the predicted 462 kD polycystin-1 protein to enable us to investigate the expression of polycystin-1 in cells and tissues by immunocytochemistry, Western blotting and immunoprecipitation. These antibodies readily recognized a 134 kD polycystin-1 fragment overexpressed in COS cells and stained the epithelial cells of fetal, adult and cystic kidney sections with the same pattern as reported by others. However, further investigations revealed that this pattern was not specific for polycystin-1. We could not unequivocally detect polycystin-1 in vivo, either by immunocytochemistry or immunoblotting. Our studies do not support the reported pattern of polycystin-1 expression in the kidney and we therefore conclude that the renal localization of polycystin-1 remains uncertain.

In **chapter 4** we discuss the future perspectives of PKD research. PKD is an inevitable disease for those inheriting the genetic defect. The ultimate goals of biomedical research into PKD are to reduce the cystic process and to delay the development of renal failure. The current understanding of these processes comes from clinical, genetical and cell biological observations. However, little is known about the relationship between these three different sets of observations. A major challenge of future PKD research is to integrate these three different fields of research in order to understand how gene defects disrupt the normal functions of cells, leading to the formation of cysts and ultimately to renal failure.

Particularly fruitful may be to elucidate the heterogeneity of the PKD phenotype. Remarkable heterogeneity is present at the cellular level, where the cystic process affects only a minority of the nephrons. Heterogeneity at the clinical level has been well documented, both between and within PKD families. Understanding the mechanisms that determine whether a particular nephron and a particular patient will be more affected than others, may provide important insights for the development of effective therapeutic strategies.

Experimental models of PKD have contributed significantly to the current understanding of PKD. Genetic studies have revealed a number of genes and gene products that are involved in the formation of renal cysts and/or in subsequent renal failure. In addition, experimental models allow the systematic study of specific cellular processes involved in PKD, during different stages of development of normal and cystic kidneys. Moreover, animal models have been succesfully used to explore a large number of potential treatment strategies. It is therefore to be expected that these experimental models may also guide investigations in the future and contribute to the understanding of the pathophysiology and therapeutic options of human PKD.

Samenvatting

Samenvatting

In dit proefschrift beschrijven we resultaten van experimenteel onderzoek van polycysteuze nierziekten (polycystic kidney disease, PKD). Deze groep ziekten wordt gekenmerkt door de vorming van cysten in beide nieren en door een hoge mate van erfelijkheid. Cysten zijn met vocht gevulde blaasjes die, behalve in de nieren, ook in andere organen kunnen voorkomen. Naar het erfelijkheidspatroon wordt onderscheid gemaakt tussen autosomaal recessieve PKD (ARPKD) en autosomal dominante PKD (ADPKD). Naast PKD bestaan er nog andere vormen van cystenieren, die in dit proefschrift buiten beschouwing blijven.

Ondanks intensief onderzoek bestaat er nog geen gerichte behandeling voor patiënten met PKD. Voor het ontwikkelen van een dergelijke behandeling is meer begrip nodig van het biologisch mechanisme van cystevorming. Naar dit mechanisme is in de laatste twee decennia veel onderzoek gedaan, waarbij enerzijds gebruik is gemaakt van materiaal afkomstig van patiënten met PKD en anderzijds van verschillende diermodellen van PKD.

Het huidige inzicht is vooral afkomstig uit drie verschillende onderzoeksgebieden: Klinische observaties van de ziekteverschijnselen, celbiologische observaties betreffende abnormale eigenschappen van de cellen die betrokken zijn bij het cysteuze proces en genetische observaties betreffende de defecten in het erfelijk materiaal dat ten grondslag ligt aan de verschillende vormen van PKD.

In **hoofdstuk** 1 wordt een overzicht gegeven van de beschikbare literatuur gegevens over PKD. We belichten hierin naast ziekteverschijnselen vooral de huidige inzichten in de ontstaanswijze van cysten. Als inleiding op de eigen onderzoekingen wordt daarbij met name aandacht gegeven aan de bijdrage die de verschillende experimentele modellen van PKD tot op heden hebben geleverd.

PKD is de meest frequente erfelijke oorzaak van nierfunctie verlies bij de mens. De ernstigste vormen van de ziekte worden op de kinderleeftijd gezien, maar de meest frequente vormen komen meestal pas op volwassen leeftijd tot uiting. Op grond van het erfelijkheidspatroon wordt PKD onderscheiden in ADPKD (overerving naar 50% van de nakomelingen) en ARPKD (25% van de nakomelingen). Dit onderscheid is tevens van belang in verband met verschillen in klinisch beloop en prognose. De verschillen in erfelijkheidspatroon weerspiegelen verschillende genetische defecten en gaan gepaard met specifieke veranderingen in de betrokken weefsels en cellen.

ADPKD komt relatief frequent voor (1:1000 mensen) en leidt bij de helft van de patiënten tot nierfalen en dus tot afhankelijkheid van dialyse en /of niertransplantatie. De ziekte leidt in het algemeen pas op middelbare leeftijd tot ernstige problemen. In uitzonderlijke gevallen komt ADPKD op de kinderleeftijd tot uiting en soms al voor de geboorte. De belangrijkste gevolgen van de nieraandoening zijn nierfalen en hoge

bloeddruk. Daarnaast onstaan in de loop van de tijd bij veel patiënten cysten in de lever en soms in andere organen. Een zeldzame en onbegrepen, maar bijzonder ernstige manifestatie van ADPKD is het optreden van cerebrale aneurysmata, die tot hersenbloedingen aanleiding kunnen geven.

ARPKD komt minder frequent voor (1:40.000) en leidt bij vrijwel alle patiënten al op de kinderleeftijd tot nierfalen en dialyse behoefte. Een deel van de patiënten overlijdt al bij de geboorte aan ademhalingsproblemen die indirect het gevolg zijn van onvoldoende nierfunctie tijdens de embryonale ontwikkeling. ARPKD gaat in vrijwel alle gevallen gepaard met afwijkingen aan de galwegen, die kunnen leiden tot leverfalen en portale hypertensie.

Het maken van onderscheid tussen ADPKD en ARPKD kan op de kinderleeftijd moeilijk zijn. In moeilijke gevallen wordt het onderscheid gemaakt op grond van familie geschiedenis, ziekte beloop, beeldvormend onderzoek van nieren en lever en zonodig weefselonderzoek (biopsie). Wellicht zal dit in de toekomst kunnen worden aangevuld met moleculaire analyse van de betrokken genen.

Het huidige begrip van de vorming van cysten in de nier is vooral gebaseerd op genetisch en celbiologisch onderzoek.

Genetisch onderzoek heeft de erfelijke basis van PKD geidentificeerd en specifieke gendefecten (mutaties) aangetoond. ADPKD wordt veroorzaakt door een mutatie in één van tenminste drie verschillende genen. Kennelijk is de (onbekende) functie van ieder van deze genen voor de nier onmisbaar. Twee van de genen (PKD1 en PKD2), die samen verantwoordelijk zijn voor de overgrote meerderheid van de patiënten, werden geisoleerd en ontrafeld. Op grond van de hierbij gevonden genetische code kan de samenstelling worden voorspeld van de eiwitten waar deze genen voor coderen. Voorlopige gegevens suggereren dat deze twee eiwitten, polycystine-1 en polycystine-2, zijn gelocaliseerd in de celwand, waar ze gezamenlijk een functie uitoefenen die te maken heeft met het contact tussen de cel en zijn omgeving. Verder onderzoek is er op gericht deze functie verder in kaart te brengen en te begrijpen hoe een dysfunctioneren van deze eiwitten bij PKD kan leiden tot de vorming van cysten. Van ARPKD werd de genetische basis gelocaliseerd op het zesde chromosoom. Over de aard van het gemuteerde gen is nog niets bekend.

Celbiologisch en histopathologisch onderzoek heeft geleerd dat cysten onstaan als toenemende verwijdingen van histologisch normaal aangelegde nierbuisjes (nefronen). Opvallend is daarbij dat dit proces in slechts een beperkt aantal van de nefronen optreedt, hoewel de genetische mutatie aanwezig is in alle lichaamscellen. Welke factoren bepalen of een bepaald nefron cysteus wordt, is onbekend. Er zijn aanwijzingen dat een (later verkregen) extra beschadiging van het erfelijk materiaal in de betrokken cellen daarbij een rol zou kunnen spelen.

Op theoretische gronden wordt algemeen verondersteld dat voor de vorming van een cyste tenminste drie abnormale biologische processen minimaal benodigd zijn: Vermeerdering van het aantal cellen in de cystewand (proliferatie), secretie van vloeistof naar de cyste holte en een afwijkend gedrag van het omliggend biologisch milieu (extracellulaire matrix). Deze processen werden in de afgelopen jaren uitgebreid bestudeerd, waarbij veelal gebruik werd gemaakt van experimentele modellen van cystevorming.

Hoewel PKD werd vastgesteld in diverse diersoorten, werden voor wetenschappelijk onderzoek vooral muizen en, in mindere mate, ratten gebruikt. Een deel van deze diermodellen berust op 'natuurlijk' voorkomende PKD (spontane mutaties), en een deel is het gevolg van menselijk ingrijpen in het erfelijk materiaal van muizen (transgene muizen). Deze transgene modellen berusten in het algemeen op een mutatie in een gen waarvan de samenstelling en/of functie meer of minder bekend is. De informatie die deze modellen tot op heden hebben opgeleverd is, voor ieder model afzonderlijk, in dit eerste hoofdstuk samengevat. Daarbij presenteren we een arbitraire selectie van beste diermodellen voor wetenschappelijk onderzoek (Tabel 3).

Tot slot wordt in het eerste hoofdstuk een theoretisch raamwerk gepresenteerd waarbinnen de verschillende klinische, genetische en celbiologische observaties geplaatst kunnen worden en waarin mogelijke verbanden tussen deze observaties worden aangegeven (fig 1).

Hoofdstuk 2 bevat een onderzoeksverslag over de bruikbaarheid van verschillende experimentele PKD modellen.

Allereerst wordt in **hoofdstuk 2.1** een nieuw diermodel van ARPKD beschreven. Eerdere diermodellen hebben als belangrijke tekortkoming het ontbreken van afwijkingen aan de galwegen. Dergelijke afwijkingen zijn bij de mens kenmerkend voor ARPKD en komen, in wisselende mate, bij alle patiënten voor. In de door ons onderzochte 'bpk' muizenstam bleken aangedane dieren naast dubbelzijdige cystenieren ook afwijkingen aan het galwegsysteem te hebben, overeenkomstig de afwijkingen bij patiënten met ARPKD.

Gevonden werd dat aangedane muizen op jonge leeftijd overlijden aan nierinsufficientie door cysteuze destructie van de nieren. We vonden dat de nier- en leverafwijkingen op weefsel niveau sterke gelijkenissen vertonen met die van patiënten met ARPKD. Bovendien bleken deze dieren een sterke verwijding te hebben van de centrale afvoerbuis voor gal, de ductus choledochus. Een van de meest opvallende eigenschappen van zowel de nier- als de leverafwijkingen in bpk muizen is een verhoogd aantal epitheliale cellen. Daarbij werd bovendien in de niercysten een verhoogd aantal celdelingen aangetoond. Deze 'hyperplastische' afwijkingen van nier- en gal-epitheel maken de bpk muis een uitstekend model van ARPKD.

In **hoofdstuk 2.2** beschrijven we de ontwikkeling van een experimenteel systeem voor onderzoek van deze hyperplastische nier- en leverafwijkingen in bpk muizen (zie hoofdstuk 2.1). Teneinde de abnormale groeieigenschappen van deze epithelia te kunnen onderzoeken werd gezocht naar methoden om de betrokken cellen te isoleren en te kweken. Zuivere isolatie van dergelijke cellen uit nier- of leverweefsel bleek niet goed mogelijk. Daarentegen bleek de afwijkende ductus choledochus, door haar ligging buiten de lever, zeer geschikt voor de isolatie van epitheel cellen. Vervolgens werd een kweek systeem ontwikkeld waarin de karakteristieken van deze cellen onder gecontroleerde (serum-vrije) omstandigheden kunnen worden onderzocht. Enigszins tegen de verwachting, bleken de cellen afkomstig van zieke bpk muizen niet sneller te delen dan cellen van gezonde muizen. Indien echter aan het kweeksysteem EGF werd toegevoegd, bleken bpk cellen daarvan een sterkere groeiprikkel te ondervinden dan controle cellen. EGF is een groeifactor die onder natuurlijke omstandigheden ondermeer voorkomt in gal. Een dergelijke overgevoeligheid werd niet gevonden voor andere groeifactoren. Deze specifieke overgevoeligheid van bpk cellen voor EGF past in de hypothese dat epitheel proliferatie een onderliggend mechanisme is van de nier- en galweg pathologie in PKD en dat daarbij abnormale regulatie van het EGF syteem betrokken is.

In **hoofdstuk 2.3** wordt onderzoek beschreven naar de genetische achtergrond van het meest gebruikte proefdiermodel van ADPKD, de Han:SPRD rat. Deze rat wordt veelvuldig gebruikt voor onderzoek naar het mechanisme van cystevorming en naar eventuele behandelingsvormen van PKD. In Han:SPRD is, na een spontane mutatie (later *cy* genoemd; zie hoofdstuk 1), een erfelijke vorm van PKD onstaan die gelijkenissen vertoont met ADPKD bij de mens. Deze gelijkenissen betreffen zowel ziekteverschijnselen als verschillende histopathologische afwijkingen. We hebben onderzocht of de nierziekte bij dit diermodel berust op een mutatie van het gen dat gemuteerd is bij de meeste patiënten met ADPKD, het *pkd1* gen. Hiertoe werd een genetische (polymorfe) marker gekozen die op het chromosoom van de rat is gelegen in de regio waarin ook het *pkd1* gen ligt. Polymorfe markers komen in de natuur voor in verschillende 'gedaanten'. De gekozen marker heeft bij alle ratten van het *cy* model dezelfde 'gedaante'. Kruising met een rattenstam waarin dezelfde polymorfe marker een andere 'gedaante' heeft, levert na twee generaties een nageslacht op waarin onderzocht kan worden of de nierziekte gekoppeld overerft met de 'gedaante' van de marker. Uit dergelijk onderzoek kon worden afgeleid dat de ziekte bij de Han:SPRD-*cy* rat niet wordt veroorzaakt door een gendefect dat dicht bij de gekozen marker ligt en dus niet berust op een mutatie in het *pkd1* gen van de rat.

Hoofdstuk 2.4 bevat de beschrijving van een nieuw rattenmodel van ARPKD. De ziekte bleek te berusten op een spontaan onstane recessief overervende mutatie, die we *wpk* noemden. Zieke ratten ontwikkelen nierfalen op basis van massale cysteuze destructie van het normale nierweefsel en overlijden op de leeftijd van 4 tot 6 weken. Het ziekte beloop wordt, evenals bij mensen met PKD, gecompliceerd door eiwit verlies in de urine

en door verhoogde (intra-arteriele) bloeddruk. Immunohistologisch en electronenmicroscopisch onderzoek toonde dat vroege cysten kunnen ontstaan in alle segmenten van het nefron, terwijl in latere ziekte stadia vrijwel uitsluitend cysten worden gezien met eigenschappen van de distaal gelegen verzamelbuizen. Deze klinische en histologische eigenschappen komen goed overeen met die van patiënten met ARPKD. In tegenstelling tot de ziekte bij de mens werden in deze jonge dieren echter geen afwijkingen van de galwegen aangetoond.

Genetisch onderzoek, naar de localisatie van de *wpk* mutatie in de rat, bestond uit het vast stellen of de ziekte in deze dieren wel of niet gekoppeld overerft met speciaal gekozen genetische markers. Hiertoe werd gebruik gemaakt van het DNA van de tweede generatie nageslacht van een kruisfok tussen de Wistar-*wpk* ratten en een andere rattenstam (BBO). Een groot aantal genetische (polymorfe) markers werden gekozen op grond van een goede spreiding over het hele genoom van de rat en mits ze 'informatief' bleken te zijn. Een marker is 'informatief' indien deze voor ieder van de 2 stammen een eigen 'gedaante' heeft. Door dit onderzoek werd een localisatie op chromosoom 5 van de rat zeer waarschijnlijk. Hoewel het *cy* locus van de rat (hoofdstuk 2.3) ook op chromosoom 5 werd gelocaliseerd, toonde kruisfok experimenten dat *wpk* en *cy* niet allelisch zijn en dus zeer waarschijnlijk niet hetzelfde gen betreffen. Meer gedetailleerd koppelingsonderzoek bevestigde de localisatie op chromosoom 5 en localiseerde *wpk* in een 11 centimorgan interval rond de marker *D5Rat73*. Vergelijking met bekende gegevens toont dat het *wpk* gen verschilt van het ARPKD gen bij de mens en ook van de *cpk* en *bpk* genen bij de muis.

Naast de identificatie van een nieuw gen dat betrokken kan zijn bij het ontstaan van cystenieren biedt de wpk mutatie een uitstekend experimenteel model voor verder onderzoek van de ontstaanswijze, de gevolgen en de behandelingsmogelijkheden van nierfalen en hoge bloeddruk bij ARPKD.

Hoofdstuk 3.1 gaat over 'geprogrammeerde cel-dood' (apoptose). Dit is een verschijnsel waarin een cel als het ware zelfmoord pleegt. Apoptose kan worden onderscheiden van 'niet geprogrameerde cel dood' (necrose) op grond van een specifiek patroon van DNA afbraak. Dit verschijnsel doet zich in de nier vooral voor gedurende de embryonale ontwikkeling en in de herstelfase na weefselschade. Gedacht wordt dat apoptose een rol zou kunnen spelen bij de selectie van cellen in het kader van vormgeving aan complexe structuren. Omdat cyste vorming kan worden gezien als abnormale vormgeving, hebben we onderzocht of cyste vorming gepaard gaat met een abnormaal optreden van apoptose. We vonden dat apoptose in de zich ontwikkelende gezonde nier vooral te vinden is op plaatsen waar differentiatie van cellen van belang is en waar structuren vorm krijgen. In cysteus veranderd nierweefsel vonden we een vehoogd voorkomen van apoptose vergeleken met gezonde nieren in dezelfde fase van ontwikkeling. Apoptose werd vooral gezien in de cystewanden en in het steunweefsel tussen de cysten. Omdat apoptose onder bepaalde omstandigheden beïnvloedbaar is

gebleken, zal verder onderzoek wellicht nieuwe behandelings strategiën voor PKD aan het licht kunnen brengen.

In **hoofdstuk 3.2** beschrijven we de ontwikkeling en karakterisering van antilichamen voor het aantonen van polycystine-1. Polycystine-1 wordt gecodeerd door PKD1, het gen dat gemuteerd is in de meeste patiënten met ADPKD. De studie is gericht op het aantonen van dit eiwit in weefselplakjes en in biochemische weefselpreparaten, met als doel meer te weten te komen over haar functie in gezonde en in cysteuze nieren. Hoewel de structuur van polycystine-1 kan worden voorspeld uit de samenstelling van het gen, werd dit eiwit nog niet geïsoleerd en is dus ook niet beschikbaar voor onderzoek. We beschrijven de ontwikkeling van antistoffen tegen polycystine-1. Deze werden opgewekt in 14 geselecteerde konijnen door inenting met verschillende, relatief kleine, fragmenten van het voorspelde polycystine molecuul. Deze fragmenten werden tevoren kunstmatig in het laboratorium geproduceerd. De verkregen antistoffen werden getest op reactiviteit met een groot (135kD) fragment van het polycystine molecuul. Dit fragment (AH8) werd daartoe tot expressie gebracht in gekweekte niercellen. Ons onderzoek toonde een goede reactiviteit van vrijwel alle verkregen antistoffen tegen het AH8 fragment. Deze antistoffen bleken goed bruikbaar voor zowel immunolocalisatie in hele cellen als immunoblotting in biochemische homogenaten van deze cellen. Vervolgens werden de antistoffen getest op plakjes van foetale, volwassen en cysteuze nieren. Daarbij werd een specifiek aankleurings patroon gevonden dat niet of nauwelijks werd gezien met de pre-immuun sera (afgenomen van de zelfde konijnen voorafgaande aan de immunisaties). Dit patroon komt overeen met observaties door andere onderzoeksgroepen.

Verder onderzoek toonde echter aan dat dit aankleuringspatroon niet specifiek is voor polycystine-1 en ondermeer ook kan worden verkregen met sera van sommige niet-geimmuniseerde konijnen. Na zuivering van onze beste antistoffen, door absorptie aan polycystine fragmenten, bleek de geabsorbeerde (en later weer vrij gemaakte) fractie goede reactiviteit tegen polycystine te bezitten, terwijl de reactiviteit tegen nierweefsel grotendeels of geheel verdwenen was. Onze conclusie is daarom dat we polycystine-1 met deze methoden niet goed hebben kunnen aantonen en dat we de gegevens van anderen dan ook niet kunnen bevestigen. Hoewel daaruit niet kan worden geconcludeerd dat de gegevens van anderen onjuist zijn, vinden we op grond van onze bevindingen, dat er ruimte is voor twijfel en dat daarom de precieze localisatie van polycystine-1 in nierweefsel nog niet zeker is. Deze twijfel kan in de toekomst wellicht worden weggenomen indien materialen worden uitgewisseld tussen onafhankelijke onderzoeksgroepen en indien voor het testen van de antisera controle weefsel beschikbaar komt waarin polycystine-1 niet tot expressie komt.

De algemene beschouwing in hoofdstuk 4 is vooral gericht op toekomstig onderzoek. PKD is een onontkoombare aandoening voor hen die het genetisch defect erven. Het uiteindelijk doel van wetenschappelijk onderzoek naar PKD is dan ook de cystevorming te beperken en de ontwikkeling van nierfalen te vertragen. Daarvoor is een beter begrip nodig over het ontstaan van de afwijkingen. Gedurende de afgelopen twee decennia werd hiernaar al veel onderzoek verricht. De huidige inzichten zijn vooral ontstaan uit drie verschillende gezichtshoeken, namelijk genetische, celbiologische en klinische waarnemingen (hoofdstuk 1). Over het onderlinge verband tussen deze drie soorten waarnemingen is nog weinig bekend.

Een bijzondere uitdaging voor toekomstig PKD onderzoek is dan ook vast te stellen hoe gen-defecten, via afwijkingen op cel- en weefselniveau, uiteindelijk leiden tot ziekteverschijnselen. Of een dergelijk geintegreerd inzicht in het ziekteproces ook zal leiden tot behandelingsmogelijkheden, zal moeten worden afgewacht. Wel is wat dat betreft hoop te putten uit de sterk wisselende ziekte-ernst onder patiënten met PKD. Kennelijk spelen hierbij factoren een rol die, naast het primaire gendefect, mede bepalend zijn voor de ernst van de afwijkingen. Het ontrafelen van deze invloeden op het ziekte proces zou kunnen bijdragen aan de ontwikkeling van behandelingen en verdient dan ook extra aandacht in toekomstig onderzoek.

Diermodellen hebben een belangrijke bijdrage geleverd aan de huidige kennis over PKD. Deze modellen zijn zeer bruikbaar gebleken voor zowel fundamenteel als meer klinisch georiënteerd onderzoek. Genetisch onderzoek van diermodellen heeft een groot aantal genen aan het licht gebracht waarvan de eiwitproducten een rol zouden kunnen spelen bij het ontstaan van niercysten. Ook werden genen gelocaliseerd die mede bepalend zijn voor de ernst van ziekteverschijnselen. Daarnaast heeft de beschikbaarheid van weefsels, afkomstig van proefdieren in verschillende stadia van normale en cysteuze nierontwikkeling, systematische studies mogelijk gemaakt van de cellulaire processen die een rol spelen bij PKD. Ook zijn diermodellen uitermate geschikt gebleken om potentiële behandelings strategiën te testen. Verwacht kan dan ook worden dat experimentele modellen van PKD ook in de toekomst in belangrijke mate kunnen bijdragen aan het onderzoek van PKD bij de mens.

Het uiteindelijk doel van dergelijk experimenteel onderzoek is het beperken van de cysteuze destructie van de nieren en het vertragen van nierfunctie verlies bij patiënten met PKD.

Abbreviations

ACE	angiotensin-converting enzyme
ADPKD	autosomal dominant polycystic kidney disease (PKD1 or PKD2)
ARPKD	autosomal recessive polycystic kidney disease
BM	basal medium
BPK	Balb/c polycystic kidneys; ARPKD mouse model reported in Ch.2.1
cAMP	cyclic adenosin monophosphate
cDNA	complementary DNA
CHF	congenital hepatic fibrosis
cM	centi Morgan
DBA	Dolichos biflorus agglutinin
DNA	desoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ESRF	end stage renal failure
a/b-FGF	acidic / basic fibroblast growth factor
GRF	growth hormone releasing factor
HGF	hepatocyte growth factor
hGH	human growth hormone
IGF-I/II	insulin-like growth factor I / II
JN/MCD	juvenile nephronophthisis/medullary cystic disease
kD	kilo Dalton (molecular weight)
KGF	keratinocyte growth factor
LOH	loss of heterozygosity
LTA	Lotus tetragonolobus agglutinin
MDCK	Madin-Darby canine kidney cells
mRNA	messenger ribo-nucleic acid
PCNA	proliferating cell nuclear antigen
PDGF- α /β	platelet derived growth factor – α /β
PKD	polycystic kidney disease (either ADPKD or ARPKD)
PKD1/PKD2	ADPKD caused by a mutation in either the PKD1 or the PKD2 gene
RAAS	renin angiotensin aldosteron system
RFLP	restriction fragment length polymorphism
TGF-α/β	transforming growth factor – α /β
TNF-α	tumor necrosis factor-α
V_2R	vasopressin type-2 receptor
WPK	Wistar polycystic kidneys; ARPKD rat model reported in Ch.2.4

Dankwoord

Vanwege haar grote bijdrage aan de totstandkoming, draag ik dit proefschrift op aan Louisette, mijn vrouw. Haar eindeloze geduld en haar vertrouwen in de goede afloop gaven de ruimte dit onderzoek, dikwijls ook buiten 'kantooruren', uit te broeden en uit te voeren. Bovendien heeft zij me aangezet om na publicatie van de resultaten niet stilletjes 'bij Bartlehiem af te stappen', maar om nog even door te zetten en de verschillende gegevens samen te voegen tot dit proefschrift.

Ook mijn collegae Eric Wolff en Karlien Cransberg ben ik zeer dankbaar voor het volle vertrouwen dat ze me steeds gaven en voor het 'terug op het ijs zetten', wanneer het onderzoek weer eens tegen zat. De scheve balans betreffende de verdeling van patiëntenzorg, hoop ik in de komende tijd te herstellen.

Mijn ouders herinner ik aan de Chinese wijsheid dat eventuele eer niet toekomt aan degene die de prestatie heeft verricht, maar vooral aan zijn voorgeslacht.

De verschillende onderzoeken werden mogelijk gemaakt door de enthousiaste steun van vier afdelingshoofden. Professor Hans Büller heeft de uiteindelijke promotie binnen de Kindergeneeskunde mogelijk gemaakt. Bovendien ben ik hem dankbaar voor de uitermate constructieve en efficiënte bijdrage aan de samenstelling van dit proefschrift. Zijn voorganger en mijn opleider, Professor Henk Visser bood me gelegenheid om twee jaar onderzoek te doen in Seattle en daarna terug te komen binnen zijn afdeling Kindergeneeskunde, waarvoor ik hem zeer erkentelijk ben. Professor Ellis Avner bood me ruimte in zijn laboratorium in Seattle en bracht me in contact met modern laboratoriumonderzoek en met de ontwikkelingsbiologie van de nier. Terug in Nederland, bood Professor Hans Galjaard ons gelegenheid het onderzoek onder te brengen binnen de zeer stimulerende laboratorium omgeving van zijn afdeling Klinische Genetica. Ik heb dat zeer gewaardeerd.

Bij het opzetten en uitvoeren van de onderzoeken ben ik geholpen door een groot aantal mensen. Hiervan wil ik met name Miriam Goedbloed graag bedanken. Zonder haar was het niets geworden. Zij is een uitstekend analiste en een bijzondere persoonlijkheid, waarmee ik de laatste jaren met zeer veel plezier heb samengewerkt. Haar vasthoudendheid en resistentie tegen tegenvallers kwamen goed van pas bij het onderzoek naar polycystine-1. Bij de uitvoering van de experimenten werd zij goed bijgestaan door Anita Korteland-van Malen. Experimenten in eerdere jaren waren mogelijk dankzij de uitstekende hulp van Lisanne Severijnen, Nicolle Meyer-van der Boom en Nicole Cornelissen. Bij de intra-arteriele bloeddruk metingen in wpk ratten heb ik de vakkundige hulp van Ineke Hekking-Weijma, Richard van Dokkum en Fred Bonthuis zeer gewaardeerd.

Voor de wetenschappelijke onderbouwing van het onderzoek binnen de Klinische Genetica heb ik heel veel gehad aan de inspiratie en stimulerende adviezen van Ans van den Ouweland, Dickey Halley, André Hoogeveen, Arnold Reuser, Mark Nellist, Rob Willemsen en Pim Visser. Ik heb me bij hen steeds bijzonder welkom gevoeld en heb genoten van de samenwerking, waarvoor Veel Dank!

In addition I am especially indebted to our collaborators in the United States. In Seattle, Ellis Avner took care of an excellent scientific environment and Bill Sweeney provided lots of 'hands on' skills in the lab, as well as moral support and friendship. Yuko Ozawa taught me some Japanese methods of mouse research. Phil Tarr took me out for many beers and brought me up the slopes of Mount Rainier as a compensation for the 'character building experiences' in the lab. Joe Rutledge was always fun to visit and an excellent help in mouse pathology. Dennis Benjamin took me out for a sail and let me use his Pathology lab. During more recent years, I had the privilege to collaborate on the wpk project with Lisa Guay-Woodford in Birmingham, Alabama. Lisa is a very nice person to work with and an excellent and efficient investigator. Much of the work in her lab was carried out by Chris Wright.

Namens de proefdieren wil ik graag de medewerkers van het E.D.C. bedanken voor de uitstekende verzorging en het toezicht op de ethische aspecten van de experimenten. Met name waren hierbij betrokken Christa Klein, John Mahabier, Ron Briegoos, Ed Lansbergen, Jaap Kasbergen en Wil Kort.

Harry van Herck heeft de cysteuze nierziekte in wpk ratten ontdekt en ons in de gelegenheid gesteld deze te onderzoeken als model voor PKD. Het bleek een vruchtbare samenwerking, waarvoor veel dank.

Graag wil ik Tom de Vriesch Lentsch (foto's in hfdst. 2.4 en 3) en Ria van Dongen (secretariele ondersteuning) hartelijk bedanken voor hulp bij de vormgeving van het proefschrift.

Tenslotte, maar zeker niet in de laatste plaats, dank ik de promotie commissie voor het beoordelen van dit proefschrift.

De onderzoeken werden financieel ondersteund door de Nierstichting en door de Sophia Stichting voor Wetenschappelijk Onderzoek.

Curiculum vitae

De auteur werd geboren op 28 december 1951 te Leiden. In 1969 behaalde hij het HBS-B diploma aan het Rijnlands Lyceum te Leiden. Hierna volgden de studies Biologie gedurende 1 jaar en Medicijnen gedurende 7 jaar aan de Rijks Universitieit van Groningen. Het artsexamen werd behaald in 1978. De militaire dienstplicht werd vervuld als Eerste Luitenant verbonden aan de afdeling Immunohaematologie en Bloedbank van het Academisch Ziekenhuis te Leiden (Professor J. van Rood). Op 1 juli 1980 begon hij zijn opleiding tot kinderarts in het Sophia Kinderziekenhuis te Rotterdam (Professor H.K.A. Visser). Hij werd ingeschreven in het specialisten register op 1 juli 1984. De opleiding tot kindernefroloog vond in hetzelfde ziekenhuis plaats (Dr. E.D. Wolff) en omvatte bovendien een bezoek van drie maanden aan de afdeling voor niertransplantaties bij kinderen in Guy's Hospital, Londen (Prof. C. Chantler). Sindsdien is hij als kindernefroloog verbonden aan het Sophia Kinder Ziekenhuis in Rotterdam. Dit verband werd vanaf 1990 gedurende twee jaar onderbroken voor een onderzoek stage 'renal developmental biology' (Professor E.D.Avner, Seattle, USA).
De onderzoeken die hebben geleid tot dit proefschrift werden verricht binnen de afdelingen Pediatric Nephrology, Childrens Hospital, Seattle (Prof.E.D. Avner), Klinische Genetica, EMCR, Rotterdam (Professor H.Galjaard) en Kindergeneeskunde, EMCR, Rotterdam (Professor H.Büller).
De auteur is getrouwd met Louisette Borgerhoff Mulder en heeft drie fantastische kinderen, Jan, Sacha en Foeke.