Polycystin, the polycystic kidney disease 1 protein, is expressed by epithelial cells in fetal, adult, and polycystic kidney

(autosomal dominant polycystic kidney disease/antibody)

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ABSTRACT Polycystic kidney disease 1 (PKD1) is the major locus of the common genetic disorder autosomal dominant polycystic kidney disease. We have studied PKD1 mRNA, with an RNase protection assay, and found widespread expression in adult tissue, with high levels in brain and moderate signal in kidney. Expression of the PKD1 protein, polycystin, was assessed in kidney using monoclonal antibodies to a monoclonal antibody specific containing the C terminus of the molecule. In fetal and adult kidney, staining is restricted to epithelial cells. Expression in the developing nephron is most prominent in mature tubules, with lesser staining in Bowman's capsule and the proximal ureteric bud. In the nephrogenic zone, detectable signal was observed in comma- and S-shaped bodies as well as the distal branches of the ureteric bud. By contrast, unduced mesenchyme and glomerular tufts showed no staining. In later fetal (>20 weeks) and adult kidney, strong staining persists in cortical tubules with moderate staining detected in the loops of Henle and collecting ducts. These results suggest that polycystin's major role is in the maintenance of renal epithelial differentiation and organization from early fetal life. Interestingly, polycystin expression, monitored at the mRNA level and by immunohistochemistry, appears higher in cystic epithelia, indicating that the disease does not result from complete loss of the protein.

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder (incidence 1/1000), which accounts for ~8% of patients with end-stage renal disease (ESRD). ADPKD is characterized by progressive development and enlargement of renal cysts, typically leading to ESRD by late middle age (1). Cysts are also found in other organs, most notably the liver, which, combined with evidence of an increased prevalence of intracranial aneurysms, heart valve defects, and inguinal herniae, shows ADPKD to be a systemic disorder (2). ADPKD is genetically heterogeneous (3, 4), but ~85% of cases are due to the polycystic kidney disease 1 (PKD1) locus (5) located in 16p13.3.

The complexity of changes in the polycystic kidney disease have made identification of the primary defect by biochemical methods difficult. Nevertheless, it has been noted that the early stages of cyst development are characterized by dedifferentiation of cystic epithelia accompanied by abnormalities of the associated basement membrane (6–8). These observations led to the proposal that disruption of normal cell–basement membrane interaction is the primary defect in this disorder (9). The recent identification (10) and characterization of the PKD1 gene (11, 12) now allows the primary defect to be examined directly. The PKD1 gene encodes a large protein (4302 aa), polycystin, which is likely to be a membrane-associated glycoprotein involved in cell–cell and/or cell–matrix interactions (12). Polycystin may thus play an important role in the interaction of tubular epithelia and the basement membrane, with disease resulting from disruption of this contact by reduced or aberrant polycystin expression.

Characterization of the PKD1 gene has been complicated because all but the terminal 3.8 kb of transcript (~14 kb in total) is encoded by a genomic region reiterated several times at another site on the same chromosome (in 16p13.1). This area contains three genes (HG-A, -B, and -C) with substantial homology to PKD1 (~97%), which produce large polyadenylated transcripts (10); it is not known whether they encode functional proteins. Consequently, to avoid confusion with these products, studies of PKD1 mRNA and protein expression need to assay regions encoded by the single-copy 3′ area. A recent study utilizing polyclonal antibodies to a decapetide at the extreme C terminus of the originally described sequence of the PKD1 protein (10) demonstrated immunoreactivity localized to the renal interstitium and vasculature (13). However, because of a frame-shifting error in that sequence (predicting a longer open reading frame) (11, 12, 14), the epitope to these antibodies does not appear to form part of polycystin and the significance of these results is unclear.

To characterize PKD1 expression we have assayed mRNA levels with an RNase protection assay and generated monoclonal antibodies to a fusion protein containing part of polycystin encoded by single-copy DNA. These results show that polycystin expression in the human kidney is restricted to epithelial cells in fetal, adult, and polycystic tissue.

METHODS

RNase Protection Assay. Normal human tissue, obtained at autopsy or surgery, was snap-frozen in liquid nitrogen and stored at −70°C prior to extraction by established methods (15). Extracted RNA was dissolved in hybridization buffer [80% formamide/40 mM piperazine-N,N′-bis(2-ethanesulfonic acid)/400 mM NaCl/1 mM EDTA, pH 8] for further analysis. 32P-labeled riboprobes to the single-copy area of PKD1 (12, 12–12, 381 nt) (10) and to U6 small nuclear RNA (snRNA) were generated by SP6 (PKD1) or T7 (snRNA) polymerase, using appropriate templates. Hybridization to total RNA (25 μg), PKD1, or snRNA (0.1 μg) (as an internal control) was performed as described (16). Protected bands of the predicted size—PKD1 (260 bp) and snRNA (107 bp)—were cut from the gel and radioactivity was assayed in a flat-bed liquid scintillation counter (1205 Betaplate; Pharmacia-Wallac OY). A linear relationship between RNA quantity and signal was demonstrated within the range assayed. To control for sample losses during processing, counts for PKD1 were expressed as a ratio of that for snRNA.

Abbreviations: PKD1, polycystic kidney disease 1; ADPKD, autosomal dominant polycystic kidney disease; snRNA, small nuclear RNA. §To whom reprint requests should be addressed.

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Generation of PKD1-Specific Monoclonal Antibodies. A 944-bp fragment [12,419–13,362 nt of the PKD1 transcript, including the natural stop codon (12)] was amplified from the PKD1 cDNA AH6 (10) with the proofreading polymerase pfu-1 using the primers C 73892 (5'-CGGAATTCTCTCTCTACCTGTCTGC-3'), and T 130122 (5'-CGGGATCCACTAGAAAACCGTCATTAGCT-3'). This generated a product of 233 aa from the extreme C terminus of polycystin (4070–4302 aa). The PCR product was cloned in frame with the maleI gene in the expression vector pMAL-c2 (NEB), transformed into the Escherichia coli strain XL-1 blue, and verified by sequencing. The maltose binding polycystin fusion protein was expressed and purified by amylose affinity chromatography.

BALB/c mice were immunized with the whole fusion protein and monoclonal antibodies were produced as described (17). The resultant hybridomas were screened on COS-1 cells transfected with a construct pCDNA-3/AH8, which contains the PKD1 cDNA AH8 (9439–14,067 nt of PKD1) and expresses the C-terminal 1225 aa of polycystin (3078–4302 aa) under the control of the cytomegalovirus major intermediate early promoter. The calculated molecular mass of the resultant polypeptide is 134 kDa. Two positive clones were isolated after a further round of subcloning and screening. These were isotypes and found to be PKS-A, IgG1κ and PKS-B, IgAκ.

Immunohistochemistry. A standard two-stage indirect immunoperoxidase protocol utilizing a goat anti-mouse immunoglobulin horseradish peroxidase (HRP) conjugate (Dako P04470) secondary antibody was employed for immunostaining of transfected and tissue samples. Renal tissue was obtained at nephrectomy or autopsy and snap-frozen or fixed in formalin. The optimal conditions for tissue staining were assessed for each antibody on frozen, formalin-fixed, and paraformaldehyde-fixed tissue. Staining on formalin-fixed tissue was also assessed after protease (protease type XXIV subtilisin, Carlsberg; Sigma P8038) treatment (25 min), microwave heating (2 × 4 min), or pressure cooking (90 s). Most consistent staining was, however, found with no pretreatment and this method was used for further detailed analysis.

Western Blotting. Crude protein extracts from transfected COS-1 cells (10^5 per lane) were electrophoresed in a 7.5% SDS/polyacrylamide gel with a 5% stacking layer (18). After electrophoresis the proteins were transferred to an Immobilon-P membrane by electroblotting. Polycystin monoclonal antibodies were used as primary antibodies. Peroxidase activity resulting from binding the secondary HRP antibody conjugate was detected using enhanced chemiluminescence (Amersham).

RESULTS

The PKD1 mRNA Is Widely Expressed. Preliminary analysis of PKD1 by Northern blotting, using tissue-specific cell lines, showed mRNA expression in all cells assayed (10). However, Northern analysis is an unsuitable means to quantify PKD1 expression in tissues because of the large size of the transcript. We have therefore employed an RNase protection assay to a small fragment within the single-copy area. The results of these assays are consistent with the earlier results with expression detected in all tissues surveyed (Fig. 1). Levels were highest in the brain (cerebral cortex) and lowest in the thymus (6- to 7-fold difference); kidney samples (n = 2) showed intermediate levels of expression. When compared to the level in normal kidney, mean expression in the brain (n = 3) was ~3 times greater. Of particular interest, assays showed that expression levels, relative to total RNA, were nearly 2-fold higher in three end-stage ADPKD kidneys and one polycystic ADPKD liver when compared to normal kidney and liver, respectively (Fig. 1).

Specificity of Monoclonal Antibodies to Polycystin. A fusion protein containing 233 aa of polycystin was employed to generate antibodies. To test their specificity, COS-1 cells were transfected with a second construct expressing the terminal 1225 aa of polycystin. Polyclonal sera stained transfected COS-1 cells, showing its specificity to polycystin, and consequently fusions to generate monoclonal antibodies were initiated. Several clones which immunostained the transfected COS-1 cells strongly were detected, and two of these, PKS-B and PKS-A, were characterized in detail. Both antibodies detected a polypeptide of the predicted size (~130 kDa) on Western blots of transfected COS-1 cells (Fig. 2). These antibodies were tested on tissue and PKS-B was found to stain frozen tissue effectively, but not formalin-fixed material, while PKS-A only stained the formalin-fixed tissue. Control experiments of staining tissue without inclusion of the primary antibody showed no signal.

Polycystin Expression in the Fetal Kidney. Although PKD1 mRNA is widely expressed, we have focused our initial immunohistochemical studies on the kidney. The antibody PKS-A was used to assess the pattern of polycystin expression.

![Fig. 1. PKD1 gene expression in normal human tissues. A PKD1 protected band of predicted size (260 bp) was detected in all tissues surveyed. OX18, OX40, and OX1001 are polycystic kidney RNAs from three patients with ADPKD; OX876 is polycystic liver RNA from an ADPKD patient (only the liver sample was proven to be from a PKD1 pedigree). snRNA (107 bp) was used as an internal control for each sample; a single base mismatch between the human sequence and that for Xenopus tropicalis permitted partial cleavage of the main protected band, resulting in two additional bands (16). The "No RNA" lane represents a control lane in which probes were hybridized to tRNA only. The left-hand lane markers are from a pBR322 DNA Msp I digest.](image-url)
in the developing kidney (gestational age, 13–26 weeks). Fig. 3a shows the overall pattern of expression in the 16-week kidney, with strong tubular staining. Closer examination (Fig. 3b) shows that these mature tubules lie proximal to the nephrogenic zone where weaker staining of the earliest nephron precursors—comma- and S-shaped bodies—and the distal tips of the inducing ureteric bud is seen. Significant staining of the proximal part of the ureteric bud and regions of Bowman’s capsule in mature glomeruli was also noted. Thus, all polycystin expression in cells from the earliest stages of nephron formation in the developing kidney is epithelial, derived from either the nephrogenic mesenchyme or the ureteric bud. Uninduced mesenchyme, glomerular tufts, capillaries, venules, arterioles, and the capsule of the developing kidney did not express polycystin at any stage. At a later stage of renal development (20 weeks; Fig. 3c), when the cortico-medullary junction becomes more evident, it is clear that staining of cortical tubules is stronger than that in the loops of Henle and collecting ducts. Significant immunoreactivity persists in Bowman’s capsule and the glomerular tuft remains unstained.

Polycystin Expression in the Adult Kidney. Both formalin-fixed and frozen adult renal tissue were stained with the antibodies PKS-A and PKS-B, respectively. Both showed a pattern of staining similar to that in later fetal samples. Fig. 3d shows strong staining of many cortical tubules in a frozen section. However, as the morphology of formalin-fixed samples was better preserved, further detailed analysis was conducted on these sections. Fig. 3e and f show adult tissue with strong staining of cortical tubules around a glomerulus. In some glomeruli, Bowman’s capsular staining highlighted a region of strong expression in parietal epithelia, possibly where the proximal tubule arises from the capsule. Medullary tubules—the loops of Henle and collecting ducts—also show significant staining, but the capsule of the kidney, stromal cells, blood vessels, and glomerular tufts were consistently negative.

Polycystin Expression in End-Stage ADPKD Kidney. Four formalin-fixed and three frozen sections of end-stage ADPKD kidney, plus one ADPKD cistic liver sample, were stained with PKS-A or PKS-B. Two kidneys and the liver were known to be from PKD1 pedigrees by linkage or mutation analysis (OX3, OX876, and OX1330) and, although it was not possible to determine the status of others (because of a lack of samples or family history), the majority is likely to be PKD1 as this disease accounts for 85% of all ADPKD. Consistent results were obtained in each sample with cistic epithelia staining intensely (Fig. 3g–i) in all cysts, including in the polycystic liver (data not shown), except where the cyst lining is attenuated or denuded. The intense staining is seen in both tubules and Bowman’s capsule showing early cystic dilation (Fig. 3g) as well as in larger well-developed cysts (Fig. 3h). The pattern of expression is similar to that seen in normal adult kidney, with staining restricted to tubular epithelia, although the staining appears more intense. Marked fibrosis and expansion of the renal interstitium were evident in the end-stage ADPKD kidneys, but the interstitial cells, capillaries, and matrix did not stain. Fig. 3i shows a polycystic kidney from a patient (OX1330) with a known deletion mutation [same as OX875 (10)], which removes ~3 kb of the transcript from exon 35 to within exon 46, including the area recognized by the antibodies (and the RNase protection assay). In this case, only protein produced by the normal allele would be detected. A pattern of intense staining, similar to that in the other PKD1 kidneys, was observed. Analysis of mRNA expression in this patient shows a level similar to that in normal kidney (data not shown). However, since only the normal allele is being assayed here, expression from this allele must be increased ~2-fold—a result similar to that seen in the other polycystic tissue assayed (Fig. 1). This indicates that the increase in mRNA and protein expression seen in cistic epithelia results from overexpression of both normal and PKD1 alleles.

DISCUSSION

Previous studies of cistic tissue from ADPKD kidney, especially of cultured cistic epithelia, have shown multiple changes to its growth properties (19) and pattern of protein expression (20, 21). Interpretation of these results has, however, been difficult as it is not clear which changes represent primary, cyst-causing abnormalities and which are secondary to cyst formation. Identification of the PKD1 gene and consequently of the PKD1 protein, polycystin, now provide a means to study directly the expression and distribution of the disease-causing protein in normal and cistic tissue.

Our studies of mRNA show a wide range of tissue expression consistent with the systemic nature of ADPKD. A full survey of fetal and adult tissue by in situ hybridization and immunohistochemistry will, however, be required to determine precisely the cellular localization of PKD1 mRNA and protein in each tissue. Preliminary results from one polycystic liver (OX876) show significant expression of polycystin in the epithelia of liver cysts (data not shown). However, our major immunohistochemical studies have concentrated on the kidney since this is the main site of disease in PKD1.

To localize polycystin expression, we have generated monoclonal antibodies to the single-copy encoded region of polycystin and have shown their specificity to this molecule. In normal fetal and adult renal tissue, polycystin is localized to the cytoplasm of epithelial cells in cortical tubules and, to a lesser extent, to epithelial cells in the loops of Henle, collecting ducts, and regions of the Bowman’s capsule. It is known from careful microdissection studies of ADPKD kidneys that cysts can develop from any of these parts of the nephron (22). It is not known at what time the majority of cysts form, but rare analyses of renal tissue from PKD1 affected fetuses have shown tubular and glomerular microcysts as early as 12 weeks of gestation (23, 24), although there is evidence that cysts continue to form in adult life (22). Our studies show that polycystin is expressed at 13 weeks, the earliest stage available for study, and continues to be expressed in the adult (and cystic) kidney. Hence, polycystin expression is temporally and spatially coincident with cyst development in the ADPKD kidney.

The ADPKD kidney appears to form “normally” (it is not dysplastic) with cysts subsequently developing from just a small
FIG. 3. Immunohistochemical staining of fetal (a–c), adult (d–f), and polycystic (g–i) kidney with the polycystin antibodies PKS-B (d) and PKS-A (all other plates). All sections were formalin-fixed, apart from d, which was frozen. (a and b) Sixteen-week fetal kidney showing strong staining of tubules (T) and fainter signal in S-shaped bodies (S) and the distal ends of the ureteric bud (U). Glomerular tufts (G) and undifferentiated mesenchyme are negative. (c) Twenty-week fetal kidney showing strong tubular staining (T) and a negative glomerular tuft (G). (d and f) Staining of tubules (T) in adult tissue is observed in frozen (PKS-B; d) and formalin-fixed (PKS-A; e and f) tissue. The glomerular tuft (G) is negative but a region of strong staining can be seen in Bowman’s capsule (B). (g–i) Sections of end-stage polycystic kidney from ADPKD patients: OX40 (g), OX3 (h), and OX1330 (i). The cystic epithelia (CE) are intensely stained in tubules and Bowman’s capsule (B) showing early stages of cystic dilation (g) and larger well-developed cysts (h and i). The expanded interstitium (I) is negative. The region recognized by the PKS-A antibody is deleted due to PKD1 mutation in OX1330, and thus this staining must be due to the product of the normal allele. (a, ×16; b, d, e, and g–i, ×100; and c and f, ×200.)

A proportion of otherwise normal nephrons (22). This suggests that the defect in ADPKD is not in epithelial formation but rather, in the maintenance of epithelial differentiation and organization (25). This hypothesis is supported by the expres-
sion pattern of polycystin; the highest levels are in the epithelial cells of mature tubules in the fetal kidney, a pattern that persists in the adult. Nevertheless, weak expression in comma-, S-shaped bodies and distal branches of the ureteric bud during epithelial induction was noted (whereas no expression is seen in uninduced mesenchyme), so that a role for polycystin in the process of induction cannot be ruled out.

It has been suggested that polycystin is a membrane-bound protein which mediates epithelial cell–basement membrane interactions (12). Such a model is consistent with the expression pattern detected here. This model would also imply predominant localization of the molecule to the baso-lateral epithelial cell surface. However, the level of resolution seen here is insufficient to confirm this. Nevertheless, the cytoplasmic location detected here supports the view that the C terminus of polycystin (the region identified by the antibodies) is cytoplasmic.

One consistent observation from our studies of mRNA and protein immunolocalization is the overexpression of polycystin in end-stage ADPKD kidney. PKD1 mRNA levels are 2-fold higher and more intense antibody staining with polycystin were noted in all cysts of the diseased organs. It has previously been suggested that the mechanism of disease in PKD1 may be complete removal of the PKD1 protein from diseased tissue due to the primary constitutive mutation, plus a second somatic event (26), as occurs in several inherited cancer predisposing systems (27, 28). However, these results, which show increased polycystin expression (including evidence of increased expression from the normal allele) in cystic epithelia, make this theory unlikely.

It is tempting to suggest from these results that overproduction of polycystin is the primary defect. However, if this were the case the disease allele should be the only one overexpressed (not the normal allele). Our preliminary analysis of a PKD1 patient with a large deletion in the 3’ end of gene (removing the areas monitored by the mRNA assay and antibodies) show that the normal allele is also overexpressed in cystic tissue. These results suggest that up-regulation of polycystin in end-stage cystic tissue is a secondary phenomenon, possibly reflecting the differentiation state of cystic epithelia. It is known that these cells have a very different pattern of protein expression than normal adult epithelia (20, 21) and it is worth remembering that we are assaying the cystic tissue some period after the initial event(s) which triggered the development of cysts.

One mechanism which might explain overproduction of polycystin is that the PKD1 gene controls its own expression level via a negative feedback loop. Hence, overproduction may, paradoxically, reflect the lack of functional protein, perhaps at the initial stage of cystic transformation. If polycystin molecules normally interact, this may occur if PKD1 mutations generate an aberrant protein which binds the normal product and creates a nonfunctional complex. Analyses of the limited number of mutations described so far do show examples of changes in the 3’ end of PKD1 (10, 29, 30) which may make an abnormal product with a defective C-terminal region. However, there is also evidence that null mutations (but which also disrupt the adjacent TSC2 gene) cause polycystic kidney disease (31), suggesting that a shortage of polycystin may be enough to trigger the cascade leading to cyst development. Clearly, analysis of polycystin expression in early ADPKD kidney and in renal tissue of PKD1/TSC2 deletion patients, the study of more PKD1 mutations, and further elucidation of the normal role of polycystin would help us to better understand the disease process.

In summary, we have described the pattern of renal polycystin expression, localizing it to tubular epithelial cells in fetal, adult, and polycystic kidney. These results suggest that polycystin has a role in maintaining epithelial differentiation and organization and that it is disruption of this which results in the initiation of cyst formation in PKD1.