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Impaired endocytosis may represent an obstacle to gene therapy in polycystic kidney disease

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Impaired endocytosis may represent an obstacle to gene therapy in polycystic kidney disease.

Background. Autosomal-dominant polycystic kidney disease (ADPKD) is the most common hereditary renal disease and a frequent cause of chronic renal failure. The cloning of the *PKD1* and *PKD2* genes, which are mutated in the great majority of patients with this disease, opens up the opportunity for somatic gene therapy by introduction of the wild-type gene or cDNA. Several publications have provided evidence, that many portions of the nephron and the collecting duct can form cysts, including the proximal tubule. Alterations in the proximal tubule may prevent the efficient endocytosis of filtered proteins and thus contribute to proteinuria, a frequent symptom in patients with polycystic kidney disease. At the same time this may also negatively affect various gene therapy strategies, since endocytosis is important for the uptake of foreign DNA at least under some circumstances. In the (*cy*/+) rat, a widely used animal model for ADPKD, cysts almost exclusively develop from proximal tubules, and we have therefore investigated whether proteinuria and defective endocytosis also occur in this model.

Methods. Proteinuria was demonstrated by direct measurement and by protein gel electrophoresis of urines from 16 week-old (*cy*/+) rats. Endocytosis was investigated by injection of FITC-dextran and immunohistochemical staining with anti-ClC-5 and anti-megalin antibodies.

Results. Similar to the observations made in ADPKD patients, proteinuria also develops in the (*cy*/+) rat. Using FITC-labeled dextran as an in vivo tracer for renal tubular endosomal function, we could show that portions of cyst-lining epithelia from proximal tubules have lost the ability to endocytose, which is necessary for the reabsorption of albumin and lower-molecular-weight proteins. By immunohistochemistry the expression of other proteins implicated in endocytosis, such as the chloride channel ClC-5 and the albumin receptor megalin, correlated well with the presence and absence of FITC-dextran in cyst wall epithelia.

Conclusion. These data indicate that proteinuria and albuminuria in the (*cy*/+) rat model for ADPKD are due to a loss of the endocytic machinery in epithelia of proximal tubular cysts. Such a defect may also reduce the efficacy of certain gene therapy protocols.

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THE PROXIMAL TUBULE REPRESENTS AN IMPORTANT TARGET IN GENE THERAPY OF THE KIDNEY

With a prevalence of ~1:1000 [1, 2], ADPKD is the most frequent hereditary renal disease in man. The course of the disease is slowly progressive, thus leading to chronic renal failure in ~50% of the patients at age 60 [3–5] and contributing to ~5–10% of all cases with end-stage renal disease [6–9]. The cloning of the *PKD1* [10] and *PKD2* [11] genes, which are mutated in far more than 90% of the patients with ADPKD [12, 13], has opened up new treatment opportunities. It is now at least conceivable that a cDNA encoding the wild-type protein can be introduced into the kidneys of ADPKD patients, where it may functionally replace the mutated genes.

Human ADPKD can affect the collecting duct and many portions of the nephron, including the proximal tubule [14–18]. A variety of different approaches, which have already been tried to carry out renal gene therapy, have indeed resulted in the targeting of proximal tubules. In the earliest publication the use of a retroviral vector has been described [19]. This strategy, however, is severely limited because of the fact that retroviruses will only integrate into the host genome of replicating cells. Under normal circumstances most of the cells in the kidney are quiescent, but they can enter the cell cycle after an insult [20]. In the study just mentioned this was achieved by the intraperitoneal injection of folic acid [19], which leads to pronounced cell death in the proximal tubule. The surviving cells then leave the G_0-phase in order to replace the dead cells, thus making them accessible to retroviral gene therapy. It is obvious that retroviruses can only be used under very specific circumstances, which severely limits their applicability in the setting of the kidney.

In addition to retroviruses, other viruses such as adenovirus and adeno-associated virus have attracted a lot of attention. Since adenoviruses can also transfer foreign DNA into non-replicating cells, they are much better suited

Table 1. Total protein and albumin excretion in the urine of 16-week-old male (*cy*/+) and (+/+) rats.

	(cy/+) ($N = 5$)	(+/+) ($N = 5$)	P
Protein excretion [mg/24 h]	50.2 ± 15.9	20.0 ± 5.6	0.004[a]
Albumin excretion [mg/24 h]	13.1 ± 6.3	3.1 ± 1.3	0.022[a]

Data are presented as means ± standard deviation.
[a] Statistically significant at $p \leq 0.05$

for the kidney. In the first study reported, a recombinant adenovirus was administered through the renal artery and the ureter. While the retrograde route resulted in prominent β-galactosidase activity in the papilla, the injection into the renal artery led to the infection of the proximal tubules [21]. Similar results were published, when cadaveric human kidneys were used. Also in this case, reporter protein activity was predominant in the epithelium of proximal tubules [22]. The efficiency of recombinant adenovirus to restore a genetic defect was finally demonstrated in the case of the aquaporin-1 knockout mouse. The water channel aquaporin-1 is normally expressed in proximal tubules, descending thin limbs and vasa recta, where it is important for the transcellular movement of water [23–26]. Its absence in aquaporin-1 knockout mice severely compromises the concentrating ability of the kidney [27, 28]. When an adenovirus encoding aquaporin-1 was injected into the tail vein of aquaporin-1 knockout mice, a strong expression of aquaporin-1 was evident in the liver and in the kidney. In the latter organ staining with an anti-aquaporin-1 antibody only resulted in the detection of aquaporin-1 in proximal tubules. Moreover, the concentrating ability of the aquaporin-1 knockout mice was partially restored [29].

Other widely applicable gene therapy protocols use cationic liposomes and cationic polymers. The retrograde injection of DNA/liposome complexes through the uretero-pelvic junction can result in the expression of a reporter gene in tubular profiles, possibly proximal tubules [30]. Following their promising initial studies, the same group successfully used liposomes to correct renal tubular acidosis in a mouse line, in which the gene coding for carbonic anhydrase II was inactivated [31]. Using different liposomes and an access through the renal artery, however, another group found a rather inefficient expression of the reporter protein β-galactosidase in the kidney [32, 33]. A complex between DNA and the cationic polymer polyethylenimine (PEI), however, yielded a much higher transfection efficiency, and again the reporter protein was predominantly detected in the proximal tubule [32, 33].

The use of viruses, liposomes, and cationic polymers usually is aimed to restore or add function, but the introduction of an exogenous protein may sometimes be impossible or not desirable; rather, the ablation of a certain protein may be preferrable. In such a case, antisense oligonucleotides represent a very valuable option. Systemic administration of oligonucleotides leads to their preferential accumulation in the kidney and the liver. At closer inspection, it turned out that the oligonucleotides were predominantly taken up by proximal tubules [34–36]. Furthermore, an antisense strategy also resulted in the downregulation of NO-synthase type II [36] and of the sodium phosphate cotransporter [37], which demonstrates that the administration of antisense oligonucleotides can indeed have functional consequences.

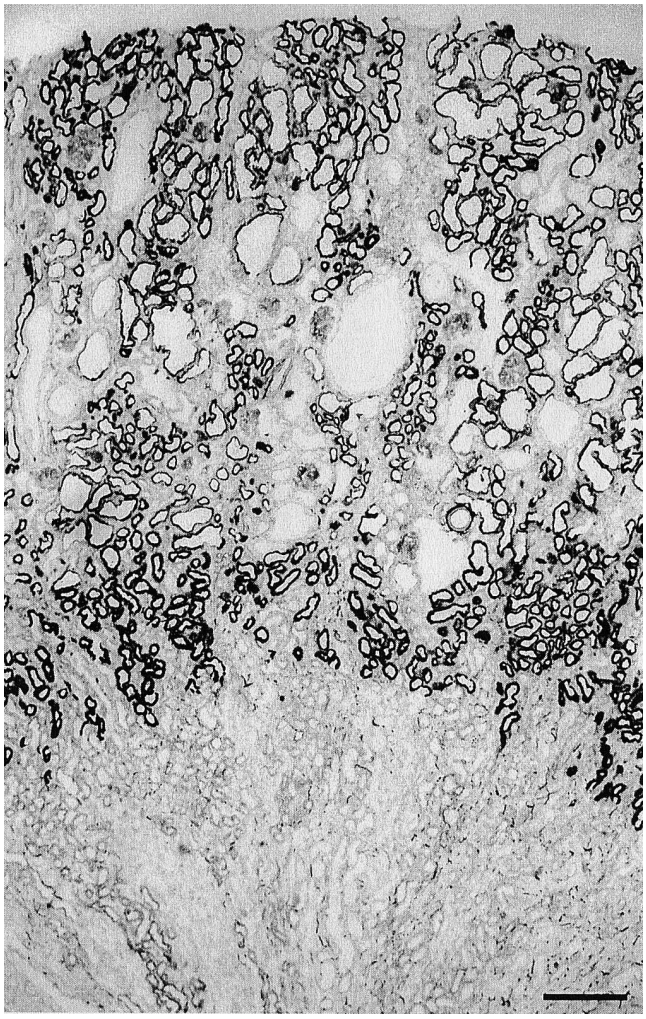

Fig. 1. Location of renal cysts in the (*cy*/+) rat model of ADPKD. Histochemical staining of a kidney section from a (*cy*/+) rat for alkaline phosphatase, a marker enzyme of the proximal tubule, leads to the convenient identification of proximal tubules. On this overview it can be easily seen that most cysts are derived from proximal tubules located in the cortex. It is likely that the unstained cysts nevertheless have originated from proximal tubules, but have lost alkaline phosphatase in the course of cyst formation. Bar, 400 μm.

WILL DEFECTIVE ENDOCYTOSIS IN CYST WALL EPITHELIA HINDER GENE THERAPY OF POLYCYSTIC KIDNEY DISEASE?

For all the approaches described above, i.e., adenovirus [38, 39], adeno-associated virus [40], liposomes [41, 42],

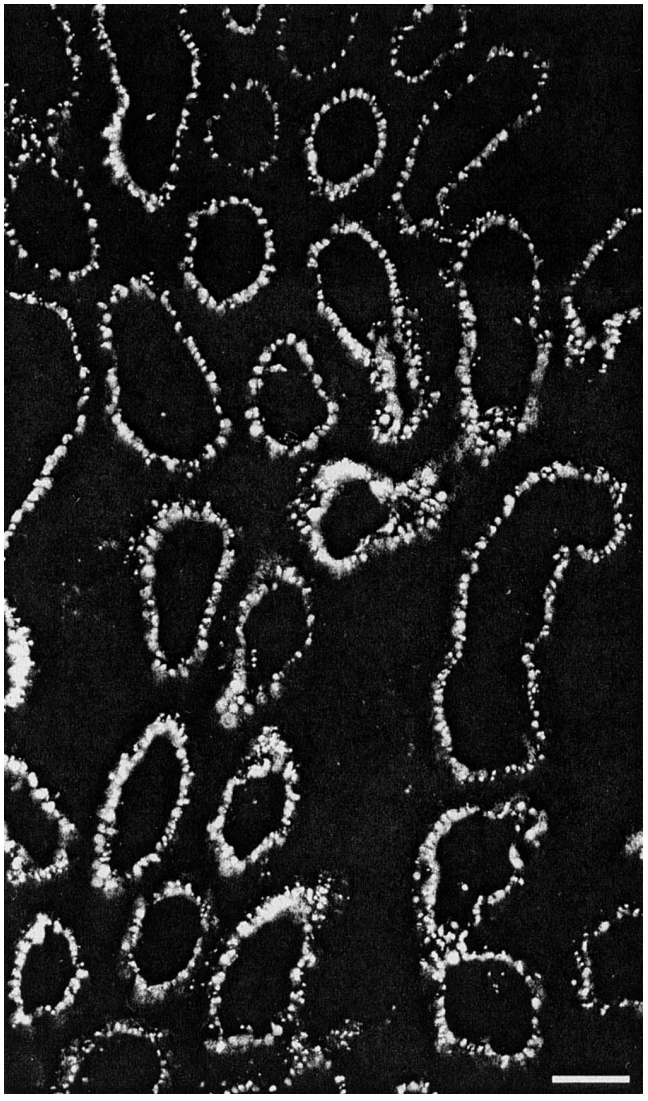

Fig. 2. Endocytosis of FITC-labeled dextran by non-cystic proximal tubules. Systemically administered FITC-labeled dextran is readily taken up by the kidney. At a higher magnification, a punctate pattern of FITC-dextran labeling can be seen, which represents the newly formed endosomes in cortical proximal tubules. Bar, 25 μm.

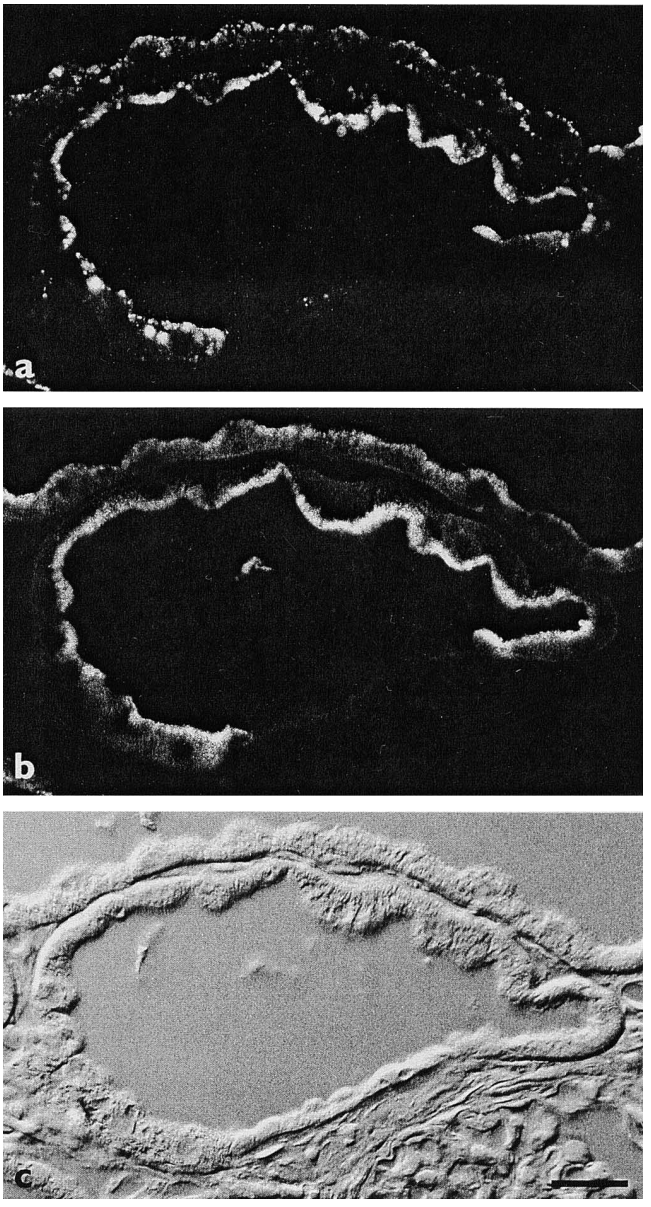

Fig. 3. FITC-dextran uptake and ClC-5 protein expression in cyst-lining epithelial cells. A cyst from a cortical kidney section of a 16 week-old (*cy*/+) rat injected with FITC-labeled dextran shows a mosaic distribution of FITC-dextran uptake (*a*). Immunofluorescence staining with an antibody against ClC-5 (*b*) demonstrates that those cells, which do not endocytose FITC-dextran any longer, also do not express ClC-5. The corresponding interference phase-contrast view is shown in *c*. Bar, 20 μm.

polyethylenimine [43], and oligonucleotides [44, 45], there is at least some evidence that endocytosis plays a role in the uptake of the foreign DNA. Since cystically transformed proximal tubules may lose their differentiation markers [46], it is of great importance to determine whether cyst wall epithelial cells derived from proximal tubules still endocytose properly.

Proteinuria has been reported repeatedly in patients with ADPKD [47–50]. So far, however, it is unclear what factors contribute to the increased urinary excretion of proteins. In our analysis of this problem, we have turned to the (*cy*/+) rat, a model for ADPKD which closely resembles the human disease [46, 51–55]. Four-month-old male (*cy*/+) rats excreted significantly increased amounts of total protein and albumin in their urine when compared with age-matched (+/+) rats (Table 1). When urine samples were analyzed under non-reducing conditions on protein gels, we found no evidence for the excretion of immunoglobulins (data not shown), which argues against a glomerular origin of proteinuria and rather points to a tubular defect. This is in agreement with the origin of cysts in this particular rat model, which are

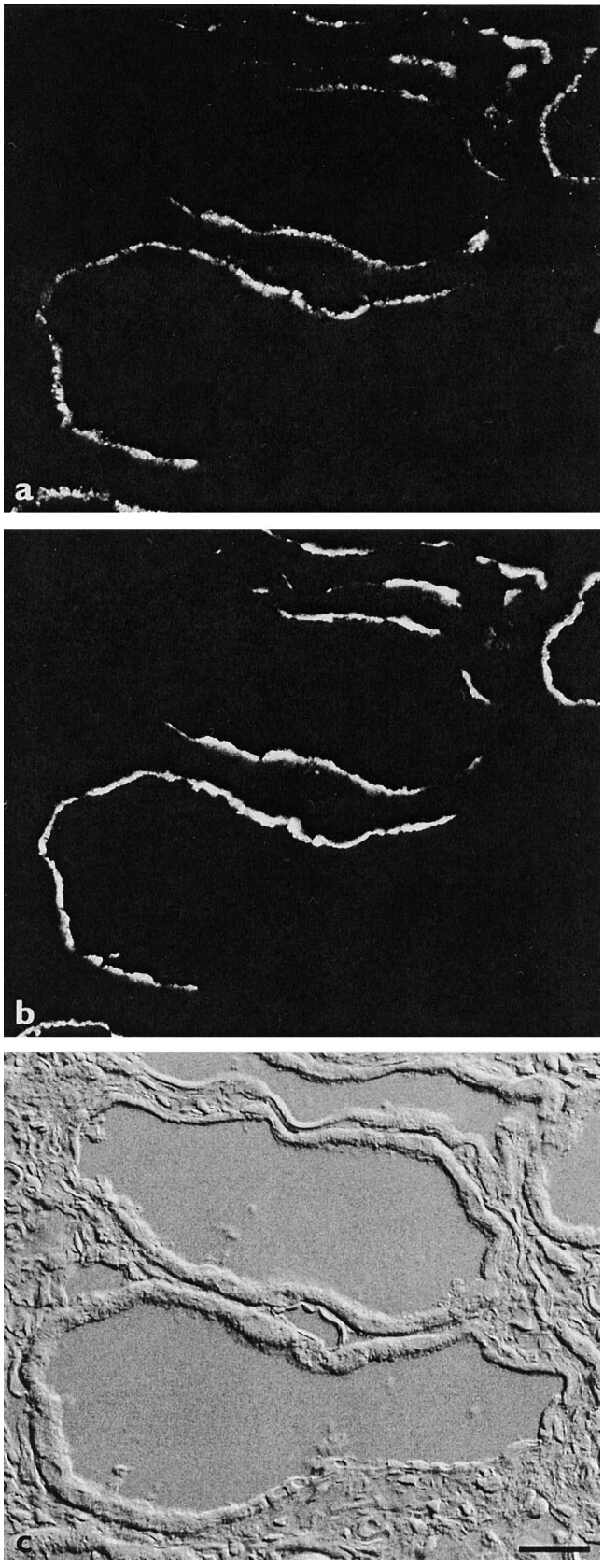

Fig. 4. FITC-dextran uptake and megalin expression in cyst-lining epithelial cells. Two cysts from a cortical kidney section of a 16 week-old (*cy*/+) rat injected with FITC-labeled dextran show a mosaic distribution of FITC-dextran uptake (*a*). Immunofluorescence staining with an antibody against megalin (*b*) demonstrates that those areas, which do not endocytose FITC-dextran any longer, also do not express megalin. The corresponding interference phase-contrast view is shown in *c*. Bar, 25 μm.

derived predominantly from proximal tubules, the nephron segment with the greatest protein reabsorption capacity (Fig. 1).

Initial support for the hypothesis, that cysts suffer from an endocytosis defect, was collected by administering FITC-labeled dextran to (*cy*/+) rats. As demonstrated previously [56], FITC-dextran, which serves as an endocytosis marker, was readily taken up by normal proximal tubules (Fig. 2). We noticed, however, that some cysts did not accumulate any FITC-dextran or showed only a patchy reabsorption of FITC-dextran (Figs. 3a and 4a). In order to gain further evidence for an endocytosis defect in cyst-lining epithelial cells, we performed immunohistochemistry with antibodies against the chloride channel ClC-5 and against megalin. Proteins and peptides, which pass the glomerular filtration barrier, are effectively reabsorbed in proximal tubules. An important first step in the reabsorption process is the binding of albumin and other filtered proteins to receptor proteins such as megalin [57, 58]. Subsequent to the formation of those complexes, endosomes will form and the proteins will be degraded by lysosomal proteases. The acidification of the endosomal compartment is achieved through the action of a V-type H^+-ATPase, whereas the required counterions for the imported H^+-ions are probably provided by the action of the chloride channel ClC-5 [59, 60]. The crucial role of both megalin and ClC-5 for the reabsorption of proteins in the proximal tubule is demonstrated by the inactivation of their respective genes. A null mutation in the gene coding for megalin leads to low-molecular-weight proteinuria in mice [61], and patients with mutations in ClC-5 suffer from Dent's disease and related syndromes, which are also characterized by massive low-molecular-weight proteinuria [62, 63]. Both with the anti-ClC-5 (Fig. 3b) and with the anti-megalin antibody (Fig. 4b), we were able to detect cyst wall epithelia that did not express the respective proteins any longer. The loss of megalin and ClC-5 correlated with the absence of FITC-dextran uptake (Figs. 3 and 4), thus corroborating the endocytosis defect in portions of the cysts and offering an explanation for the proteinuria.

CONCLUSIONS

Polycystic kidney disease presents an important paradigm in the field of renal gene therapy. The cloning of the *PKD1* [10] and *PKD2* [11] genes allows the clear-

cut identification of the underlying gene defect in the affected patients. It should therefore in principle be possible to introduce a wild-type cDNA into the kidneys of patients carrying the mutated gene in order to slow down or even prevent the development of polycystic kidneys. One of the challenges for a successful gene therapy will lie in the efficient targeting of the various nephron segments which, in addition to the collecting duct, can develop into cysts. The proximal tubule clearly is one part of the nephron, which is affected by ADPKD. If the endocytosis defect observed in the (*cy*/+) rat model also is present in polycystic kidneys of patients, it may represent a serious obstacle to gene therapy because of the notion that foreign DNA is taken up by the endocytic pathway. Therefore it would be very important to begin early with gene therapy, when the proximal tubules as yet show no endocytosis defect. Other syndromes such as Dent's disease, which per se are characterized by an endocytosis defect, may require different gene therapy strategies to start with. We also want to point out that the (*cy*/+) rat has already been used as a model for gene therapy. The administration of a recombinant adenovirus into the renal artery resulted in the expression of the β-galactosidase reporter protein in cyst wall epithelia [64]. We would predict that those cyst-lining epithelial cells, which were infected by the adenovirus, were still capable of endocytosis, but of course this assumption has to be proven experimentally.

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