Proliferative Activity of Cyst Epithelium in Human Renal Cystic Diseases

Tibor Nadasdy, Zoltan Laszik, Ginette Lajoie, Kenneth E. Blick, Don E. Wheeler, and Fred G. Silva

T. Nadasdy, Z. Laszik, G. Lajoie, K.E. Blick, D.E. Wheeler, F.G. Silva. Departments of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, and University of Texas, Southwestern Medical Center, Dallas, TX


ABSTRACT

Increased proliferative activity of the renal tubular epithelium is thought to be a prerequisite for renal cyst formation by many investigators. However, in humans, the exact in vivo proliferation rate of epithelial cells lining these cysts is not known. In this study, which used immunohistochemical methods with an antibody to proliferating cell nuclear antigen (PCNA), the proliferation index (PI) (percentage of PCNA positive cell nuclei among epithelial cells lining the renal cysts) was determined in 10 cases of autosomal dominant polycystic kidney disease (ADPKD), 8 cases of autosomal recessive polycystic kidney disease (ARPKD), and 8 cases of acquired cystic kidney disease (ACKD). Cysts with proximal and distal nephron phenotype and cysts with markedly thickened basement membranes, as well as cysts lined by atrophic (flattened), "regular" (cuboidal or cylindrical), and hyperplastic epithelium, were evaluated separately. The overall PI of cyst epithelium (excluding hyperplastic cysts) was 2.58 in ADPKD, 10.5 in ARPKD, and 3.61 in ACKD. Overall, there were only minor differences in the PI between the various types of cysts. Cysts with hyperplastic epithelium in ACKD (unlike in ADPKD) showed a high PI (9.1). For comparison, the PI of two renal cell carcinomas occurring in two ACKD cases was also determined (13.70 and 8.67%). The PI of tubular epithelium in normal kidneys was only 0.22 to 0.33%, depending on the tubule segment. In contrast, in polycystic kidneys, those noncystic segments of the nephron from which the cysts are thought to originate (distal nephron specifically collecting duct) in ARPKD, primarily distal in ADPKD, proximal and distal in ACKD) had PI values similar to those of the cyst epithelium. In summary, these results suggest that (1) increased renal tubular epithelial cell proliferation might precede cyst formation; and/or that (2) increased epithelial cell proliferation by itself may not be sufficient for cyst formation to occur. Hyperplastic renal cyst epithelium may be a precursor of renal epithelial neoplasia in ACKD.

Key Words: Polycystic kidney disease, human, cell proliferation, immunohistochemistry

Human renal cystic diseases can be acquired or hereditary. Acquired cystic kidney disease (ACKD) has emerged as a separate entity after patients have been on maintenance dialysis for several years (1). The hereditary forms include the most common renal cystic disorder, autosomal dominant polycystic kidney disease (ADPKD), affecting approximately 500,000 people in the United States (2). The other major, but less common, form of hereditary cystic disease is autosomal recessive polycystic kidney disease (ARPKD), affecting infants and young children with a frequency estimated between 1:10,000 and 1:40,000 (3).

The pathogenesis of renal cyst formation is still unknown. Human and experimental data suggest three major pathogenetic elements: (1) increased cell proliferation (and possible subsequent intratubular obstruction); (2) enhanced fluid secretion by the tubular epithelium; and (3) abnormalities in the extracellular matrix (2,4). The abnormal proliferation of tubular epithelial cells, whether primary or secondary, is thought to play a crucial role in cyst development and growth. The presence of cysts with hyperplastic epithelium, the altered expression of several proto-oncogenes, growth factors, and growth factor receptors (e.g., c-myc, c-fos, c-Ki-ras, c-erb B-2, c-Jun, transforming growth factor-Alpha, epidermal growth factor receptor), associated with renal cysts, and experimental thymidine incorporation studies all emphasize the importance of epithelial proliferation in cyst growth (2.4–18). The determination of mitotic index has been used to assess cell proliferation in a mouse model for ARPKD and was found to show only a moderate and transient increase over normal levels (19). However, the mitotic phase is only one, and indeed, the shortest phase of the cell cycle; thus, the number of mitotic figures represents only a small portion of cycling cells. To date, besides counting mitotic figures, there has not been a reliable and reproducible method to identify cells undergoing proliferation in human tissue sections.

Recently, antibodies to proliferation-associated nuclear proteins, such as proliferating cell nuclear antigen (PCNA), have become commercially available and can be applied to routinely formalin-fixed, paraffin-embedded archival material (20,21). PCNA, a 36-kd
acidic, nonhistone nuclear polypeptide (an auxiliary protein of DNA polymerase $\delta$) is expressed mainly in the S phase of the cell cycle (22). Antibodies to PCNA stain nuclei of cells engaged in the cell cycle, thus, the proliferation index (PI; PCNA positive nuclei/total number of counted nuclei \( \times 100 \), i.e., the percentage of PCNA-positive nuclei) for PCNA can be calculated even in routinely formalin-fixed, paraffin-embedded archival tissues. We have previously determined the PI values of normal intrinsic renal cell populations in histologically normal human kidneys (23), as well as of tubular epithelial cells in ESRD and in acute tubular necrosis (24.25). The objective of this study was to determine the PI of epithelium in cysts with various morphologic appearances in three different human polycystic kidney diseases (ADPKD, ARPKD, ACKD) and to compare these data with the PI of adjacent noncystic tubules in these polycystic kidneys and with the PI of tubular epithelium in normal kidneys.

MATERIALS AND METHODS
Ten cases of ADPKD (adults), eight cases of ARPKD (1 day- to 6-wk-old infants), and eight cases of ACKD (adults) were collected from the archival material of the pathology departments at the University of Oklahoma Health Sciences Center, Oklahoma City, OK, the University of Texas Southwestern Medical Center at Dallas, TX, and the Baylor College of Medicine, Texas Children’s Hospital Houston, TX. All cases had been routinely formalin fixed and paraffin embedded. Four ARPKD cases were autopsy kidneys; the remainder were nephrectomy specimens. Only renal tissues without apparent histologic signs of autolysis were included in the study.

Immunohistochemistry and Lectin Histochemistry
For the use of the monoclonal antibody to PCNA (Dako, Carpinteria, CA), the microwave pretreatment method suggested by Shi et al. (21) was used with slight modifications (23). Briefly, after deparaffinization and endogenous peroxidase blocking, slides were placed in 10 mM citric acid buffer (pH 6.0) and microwaved in a 700-W commercial microwave oven at 50% power level for 15 min. After microwaving, slides were cooled for 20 min at room temperature in the buffer. After a phosphate-buffered saline wash, the blocking serum (normal rabbit) was applied for 20 min. Slides were then incubated with the anti-PCNA antibody (1:50 dilution) for 60 min at room temperature. The remainder of the staining procedure was performed by the use of standardized peroxidase-antiperoxidase methodology. Diaminobenzidine was used as the chromogen.

To evaluate cell proliferation in cyst epithelium with proximal and distal tubular phenotypes separately, double-labeling studies with the antibodies to epithelial membrane antigen (EMA, a distal nephron, distal convoluted tubule, and collecting duct marker) (Dako) and PCNA, as well as with the lectin Tetrogonolobus purpureus (TP) (a proximal tubule marker) (Sigma, St Louis, MO) and the antibody to EMA, were performed. First, the immunostaining for PCNA was accomplished with the above-described peroxidase-antiperoxidase method (diaminobenzidine chromogen). After this procedure was completed, the immunostaining for EMA was performed with a streptavidin-conjugated alkaline phosphatase (Dako)-based system, where the secondary horse anti-mouse antibody (Vector, Burlingame, CA) was labeled with biotin. Paast Red TR (Biogenex, San Ramon, CA) was used as the chromogen. The procedure for the TP lectin staining has been described previously (see Reference 26).

Briefly, deparaffinized sections were treated with 1.25% H$_2$O$_2$ in methanol for 30 min to block the endogenous peroxidase activity. The concentration for TP was 0.3 mg/mL. Sections were incubated with the peroxidase-labeled lectin for 60 min at room temperature. The reactions were developed with 3-amino-9-ethylcarbazol. The lectin specificity of the renal tubule markers (TP lectin and the antibody to EMA) have been determined in our previous studies (24–31).

Evaluation of Slides
To calculate the PI of the renal epithelium in various cysts, epithelial cell nuclei in the cysts lined by EMA-positive and TP-positive epithelium were counted separately. Cysts were also divided according to the histologic appearance of the lining epithelium, as suggested by Bernstein (32): cysts lined by thin, atrophic-appearing, flattened epithelium, by cuboidal or columnar ("regular") epithelium (resembling epithelium of normal tubules), and by hyperplastic epithelium (cysts with multilayered epithelium and papillary projections in the lumen) were evaluated separately. Because disturbances in cell-matrix interactions may play an important role in the pathogenesis of polycystic kidney diseases, we also tested whether the number of proliferating cells in the cyst epithelium correlates with the thickness of the cyst basement membranes: PCNA-positive nuclei in cysts with thick, basement membranes (thicker than half of the diameter of epithelial cell nuclei) were counted. Epithelial cell nuclei were manually counted in each type of cyst (500 to 2,000 nuclei in each subtype of cyst), and the PI (percentage of positive nuclei with the antibody to PCNA) for each subtype of cyst was calculated. To achieve representative data, the nuclei of the epithelium in at least five cysts (if available) were counted and the numbers were added. The PI for noncystic tubules showing proximal and distal phenotypes was also determined in all three examined renal cystic diseases (ADPKD, ARPKD, ACKD) by counting 500 to 2,000 nuclei of TP lectin-positive and EMA-positive epithelial cells in separate sections. Only clearly definable nuclei with high signal/noise (image/background) ratio were identified as PCNA positive.

Statistics: Data base and Spreadsheet Analysis
All data were keyed into a data base (BASE IV, Version 1.5; Ashton-Tate, Torrance, CA) program run on an IBM PC Model 55/59 equipped with 16 megabytes of RAM memory and 400 megabytes of disk storage. Subsets of data were ported to Lotus 1-2-3. Release 2.01 (Lotus Development Corp., Cambridge, MA) for statistical comparison studies. For comparison of means, we used the means along with standard deviations of sample populations to compute $t$ values. Levels of significance were determined from the latter.

RESULTS
The cysts in ARPKD were always and exclusively EMA (distal nephron marker) positive. In ARPKD, the cysts had a fairly uniform, "regular" epithelium; no hyperplastic cysts or clearly atrophic cysts were identified. The great majority of cysts in ADPKD were also EMA...
positive; in 4 of the 10 cases, no TP lectin (proximal tubular marker)—positive cysts were identified at all. Hyperplastic cysts occurred in four ADPKD cases. No TP lectin reactivity was noted in the epithelium of these hyperplastic cysts; they were diffusely or focally EMA positive. Cysts in ACKD were positive either with the TP lectin or with the EMA antibody. Hyperplastic cysts in ACKD stained usually with TP lectin, but not with the EMA antibody. Occasional cysts in ACKD (and in ADPKD as well) were negative for both TP and EMA. Atrophic (flattened) and "regular" (cylindrical) cyst epithelium stained either for TP or for EMA.

The PI values of the examined cyst types in ADPKD, ARPKD, and ACKD are indicated in Table 1. The highest PI values were detected in ARPKD (Figure 1). Excluding the hyperplastic cysts, the number of PCNA-positive cells in the cyst epithelium in ARPKD was significantly higher than in ADPKD (<0.005) and in ADKD (<0.005). The PI of cysts in ACKD was higher than in ADPKD; this difference was also statistically significant (<0.05).

Hyperplastic cysts were noted in five of the eight ACKD cases (Figure 2A). The number of PCNA-positive cells in the epithelium of these hyperplastic cysts was considerably higher than in any other type of cyst in ACKD or ARPKD (Table 1); these differences were all statistically significant. Two renal cell carcinomas occurred in two cases of ACKD. The PI values of these two carcinomas (13.70 and 8.67%) were similar to the PI of hyperplastic cysts in ACKD (Figure 2B). Unlike in ACKD, hyperplastic cysts in ADPKD did not show substantially higher PI values than other cyst types. Only minor differences were noted in the PI of cyst epithelium positive with the TP lectin, the EMA antibody, and atrophic and "regular" cyst epithelium. The majority of cysts did not have substantially thickened basement membranes under the light microscope. The cysts that had a thickened basement membrane showed only a slightly higher PI compared with the total number of cysts in ADPKD and ACKD. There was a great variation in the number of PCNA-positive nuclei between individual cysts with thick basement membranes (see high standard deviations in Table 1).

The PI of noncystic proximal (TP lectin-positive) tubules in ADPKD was slightly lower than the PI of the cyst epithelium, whereas the PI of noncystic EMA-positive tubules (distal tubules and collecting ducts) was somewhat higher than that of the cysts (Table 1; Figure 3). The lowest PI of all examined cell populations was noted in the noncystic proximal tubules in kidneys with ARPKD. In contrast, the noncystic distal nephron in ARPKD showed a high PI, similar to that of the cysts in this disease (Table 1; Figure 4). The difference between the PI of noncystic proximal and distal nephron segments was statistically significant in both ARPKD and ADPKD (<0.005). The PI of noncystic tubules in ACKD has been determined in one of our previous studies (24). The PI of tubules with proximal nephron phenotype varied between 3.00 and 2.12, whereas the PI of tubules with distal nephron phenotype was between 1.68 and 0.83, depending on the type (morphologic appearance) of tubule (24). In summary, in ACKD, noncystic tubules with proximal nephron phenotype had a higher PI than tubules positive with distal nephron markers. The clustering of PCNA-positive epithelial nuclei in noncystic tubules

![Figure 1. Several PCNA-positive nuclei in the cyst epithelium in a case of ARPKD. Original magnification, ×200.](image)

**Table 1. PI (±SD) of epithelium lining cysts and noncystic tubules in ADPKD, ARPKD, and ACKD**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Cysts</th>
<th>Tubules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP + (1)</td>
<td>EMA + (1)</td>
</tr>
<tr>
<td>ADPKD</td>
<td>N = 6</td>
<td>N = 10</td>
</tr>
<tr>
<td></td>
<td>2.57 ± 1.30</td>
<td>2.40 ± 0.62</td>
</tr>
<tr>
<td>ARPKD</td>
<td>N = 8</td>
<td>N = 8</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>10.5 ± 3.50</td>
</tr>
<tr>
<td>ACKD</td>
<td>N = 8</td>
<td>N = 8</td>
</tr>
<tr>
<td></td>
<td>3.78 ± 1.45</td>
<td>2.40 ± 0.73</td>
</tr>
</tbody>
</table>

*Abbreviations and symbols: *, The PI of noncystic tubules varied between 2.12 and 3.00, depending on the type of atrophic tubule (24); **, The PI of noncystic tubules varied between 0.83 and 1.68, depending on the type of atrophic tubule (24); TP +, T. purpureas lectin—positive cysts; EMA +, epithelial membrane antigen—positive cysts; (1), does not include hyperplastic cysts; Atroph., cysts lined by flattened epithelium; Regular, cysts lined by cuboidal or columnar epithelium; Thick BM, cysts lined by markedly thickened basement membrane; Hyperpl., cysts lined by hyperplastic epithelium; PRX. Tubs., noncystic proximal renal tubules; Dist. Tubs., noncystic distal tubules and collecting ducts; NA, not applicable.
occurred not infrequently. Some tubular cross-sections contained many PCNA-positive nuclei (Figure 3), whereas neighboring tubules (even though positive with the same nephron-specific marker) showed no or only scattered nuclear PCNA staining.

DISCUSSION

As evaluated with the anti-PCNA antibody, the PI of the normal tubular epithelium in adult and pediatric kidneys, respectively, is 0.12 and 0.27 in the proximal tubules, 0.16 and 0.43 in the thin limb of Henle, 0.18 and 0.54 in the thick ascending limb of Henle, 0.17 and 0.17 in the distal convoluted tubules, and 0.39 and 0.39 in the medullary collecting ducts (23). The PI of cyst epithelium is substantially higher in all three cystic diseases studied than in any segment of the nephron of normal kidneys. As will be discussed below, this was not the case if we compared the PI of cysts with the PI of noncystic tubules in the cystic kidneys.

It had been shown that cysts in ARPKD derive from the collecting duct system and that the cyst epithelium carries a distal nephron phenotype (28,33-35). Cysts in ADPKD are thought to arise from all portions of the nephron (36,37); however, lectin and antibody marker studies indicate that the majority of cysts have a distal nephron phenotype (27,38). In ACKD, more cysts appear to be of proximal than of distal origin (29,39,40). Interestingly, in this study, the PI of the noncystic nephron segment from which the cysts are thought to primarily originate (distal nephron in ARPKD, primarily distal in ADPKD, predominately proximal in ACKD) equaled or was very close to the PI of cyst epithelium in all three human cystic kidney diseases.
differences studied. In contrast, the PI of the remainder of the noncystic nephron (proximal in ARPKD and ADPKD and distal in ACKD) was lower than the PI of the cyst epithelium. This difference in the PI between the proximal and distal portions of the uninvolved nephron was particularly striking in ARPKD, where all cysts showed a distal nephron phenotype and the PI in the noncystic distal tubules was much higher than in the noncystic proximal tubules. These data suggest that tubule segments, genetically predisposed for cyst formation, show increased cell proliferation even without cystic transformation. This segmental effect could be termed a "field effect" in the nephron in the sense that a certain segment of the nephron seems primarily to be involved in increased epithelial cell proliferation, which appears to precede cyst formation.

Of course, the question arises whether the noncystic tubules with large numbers of PCNA-positive epithelial cell nuclei will ever undergo cystic transformation. It can be postulated that the high number of PCNA-positive nuclei in the cystic epithelium is the consequence of an increased cell turnover, i.e., the epithelial cells proliferate in order to replace cells lost, e.g., because of apoptosis. Indeed, recent studies suggest that apoptosis is increased in polycystic kidney disease (41,42).

Disturbances in cell-matrix interactions may also play an important pathogenetic role in the development of renal cysts (4), and it has been shown that the disruption of epithelial cell-matrix interactions induces apoptosis (43). This could be particularly true for cysts with markedly thickened basement membranes, where occasionally, clusters of PCNA-positive epithelial cell nuclei were noted. The above data suggest that increased epithelial cell proliferation in polycystic kidney disease is an epiphenomenon, rather than a primary pathogenetic factor.

Renal intratubular epithelial polypoid hyperplasia with subsequent occlusion of the tubular lumina has been implicated in the pathogenesis of human and experimental renal cysts (18,44,45). The many PCNA-positive nuclei in certain segments of noncystic tubules seem to support this hypothesis. We did not note polypoid epithelial structures in these tubules; however, we did not perform serial sectioning. Polypoid structures were easily identified in hyperplastic cysts, with especially large number of PCNA-positive nuclei in the routinely sectioned ACKD kidneys.

With the exception of hyperplastic cysts, no major differences in the proliferative activity of the human renal cyst epithelium between ADPKD and ACKD were noted. The high PI of cysts in ARPKD is most likely explained by the rapid progression (rapid cyst growth) of the disease compared with ADPKD and ACKD.

There was a variability in the number of PCNA-positive nuclei between cysts, even within the same kidney. This variability may be due to local differences in the speed of epithelial cell turnover and/or to the variability of the growth rate of individual cysts. The clustering of PCNA-positive nuclei also occurred in the noncystic renal tubules, a phenomenon that can be occasionally observed in the normal renal tubular epithelium as well (23). It would be interesting to examine whether cyst size (small early cysts versus large cysts) accounts for some of these differences in the PI. Such a study unfortunately would be very difficult to perform in tissue sections, because of their two-dimensional nature. A cyst can be several centimeters in diameter; thus, even serial sectioning would be unreliable to define the size of a cyst, unless it is small and one can follow its appearance and disappearance in step sections.

Among the histologic types of cysts ("atrophic," "regular," or hyperplastic), only hyperplastic cysts in ACKD showed a significantly increased PI relative to other cyst types. Whether a cyst is lined by flattened epithelium or by epithelium of normal height ("regular" epithelium) might simply be determined by the intracystic fluid pressure. Certainly, cysts with flat ("atrophic") epithelium cannot be considered end-stage, atrophic, or quiescent cysts, at least not in terms of cell proliferation. This is in concordance with our previous study in end-stage kidneys where tubules showing prominent atrophy had a surprisingly high PI (24).

The finding that the PI of hyperplastic cysts in ACKD is similar to the PI of renal cell carcinomas developing in these kidneys suggests that carcinomas in ACKD might originate from these hyperplastic proliferating cysts. The increased frequency of renal cell carcinoma in ACKD is a well-recognized phenomenon (1,46). In contrast, the possible association between renal cell carcinoma and ADPKD is controversial (47,48). Despite a high incidence of adenomas and intracystic polypoid hyperplasias, most studies have failed to demonstrate an increased risk of renal cell carcinoma in patients with ADPKD (48). The high PI values of hyperplastic cysts in ACKD compared with ADPKD may reflect the difference in the incidence of renal cell carcinoma between ACKD and ADPKD.

In summary, we sought to characterize the proliferative activity of cyst epithelia in three different forms of polycystic kidney diseases using an antibody to PCNA. By determining the PI values of various cysts, our study clearly suggests that at least some cysts in patients with polycystic kidney diseases demonstrate an increased risk of renal cell carcinoma. Our results suggest that hyperplastic cysts in ACKD might share at least some pathogenetic factors with renal cell carcinomas developing in ACKD kidneys.
ACKNOWLEDGMENT

The authors thank Debbie L. Johnson for her excellent technical assistance.

REFERENCES


