Apoptosis in polycystic kidney disease: involvement of caspases

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Polycystic kidney disease (PKD) is a genetic disorder characterized by the formation of large renal cysts ultimately leading to end-stage renal disease. The mechanisms involved in the pathogenesis of PKD are thought to involve cellular proliferation with no differentiation or morphogenesis, resulting in the formation of cysts, altered epithelial cell polarity and mislocation of Na+/K+ ATPase pump, secretion of cyst fluid, and cyst enlargement and remodeling of the matrix (8, 9).

Autosomal recessive (ARPKD) and autosomal dominant PKD (ADPKD) are the two major inheritable forms of PKD in humans. ARPKD is a rare defect, and infants born with ARPKD die from renal failure shortly after birth. In ADPKD, cyst formation also occurs during development, however, the disease is usually only detected in the third decade of life. By late middle age, patients with ADPKD may also suffer from renal failure.

The exact mechanism involved in the pathogenesis of PKD has not been identified. Recently, two genes, one on chromosome 16 (ADPKD1) and another on chromosome 4 (ADPKD2), have been identified (17, 33). The ADPKD1 gene encodes a large integral membrane protein named polycystin, which is believed to be involved in cell-cell matrix interactions (3, 10, 14, 42). The ADPKD2 gene encodes a similar protein with sequence similarity to Ca2+- and Na+-channel proteins and may function as a channel or pore (26). Several mutations of polycystin have been identified and linked to PKD. The most recent report suggests that, in vivo, PKD1 may regulate activity of PKD2 by a homotypic interaction via their COOH termini to form a functional pore (31). PKD1 and PKD2 may therefore be involved in a common signaling cascade involved in tubular morphogenesis. Any mutations in the COOH termini of these proteins can therefore lead to altered tubulogenesis. Frequently, altered cell-cell interactions or signaling leads to death of the cell.

Evidence is growing to suggest that apoptosis is involved in PKD. Significant quantities of apoptotic cells have been reported both in ARPKD and in ADPKD (21, 43); however, whether this apoptosis is involved directly in cyst formation and/or loss of renal function is undetermined. The mechanisms involved in the progressive loss in renal failure in patients with PKD are unclear, because cysts develop in only a few nephrons and end-stage renal failure does not occur in all patients. It has been suggested that, in some patients, the cysts participate in the destruction of the renal parenchyma, and this involves accumulation of interstitial mononuclear cells and the resulting induction of interstitial scarring in the neighborhood of the cysts (11). It is possible that apoptosis plays a role in these processes.

Caspases are a family of cysteine proteases that are the key components of the apoptotic cell-death machinery (1, 27). There are three main subfamilies of caspases, the interleukin-1β-converting enzyme (ICE)-like, the initiators, and the effectors. They are synthesized as proenzymes in normal cells and are activated when the cells receive apoptotic stimuli. Their substrates include proteins that have important functions in cell regulation, cell signaling, DNA repair, homeostasis, and cell survival (29, 35). It has been demonstrated that activa-
tion of one or more caspases can commit the cell to undergo apoptosis (16).

Bcl-2 and its related proteins function as either positive or negative regulators of apoptosis (19, 32). Interestingly, bcl-2 knockout mice develop renal cysts, further suggesting that apoptosis may contribute to cyst formation (41). Bcl-2 can protect cells from apoptosis by inhibiting the process of caspase activation (18, 48). Overexpression of bax, which is a bcl-2 family member, is known to promote apoptosis (47). Dimerization among the bcl-2 family members has been shown to be necessary for these proteins to exert their roles in regulating apoptosis (28, 36, 49).

Cpk mice carry an autosomal recessive mutation that arose spontaneously (7). Although the identity of the cpk mutant allele is unknown, it maps to mouse chromosome 12 (6). The inheritance pattern and the disease manifestation of this model resemble human ADPKD, although the mouse allele is not syntenic with either human gene (2, 4). The development of renal cysts in cpk homozygous mice begins in the fetal and newborn stages with the dilation of proximal tubules. After birth, these mice begin to develop cysts, which results in kidney enlargement. The animals eventually die of renal failure between 3–4 wk of age.

In the present study, we have further evaluated the role of apoptosis in PKD using cpk mice. Our data indicate that apoptosis occurs in the same proximity as cyst formation, suggesting its role in renal cyst formation. In addition, the expression of caspase 4, bax, and bcl-2 was abnormally increased.

MATERIALS AND METHODS

Animal preparation. Twelve cpk heterozygous breeding pairs were obtained from Jackson Laboratories and housed in an isolated room. Litters were monitored daily and killed either after maximum cyst formation (on day 21) or on days 5, 10, and 15. The litter size varied from three to 10 pups. At least three litters were examined at each time point. Because the gene responsible for the cpk phenotype has not been identified, genotyping was not possible. Homozygous cpk pups were therefore identified by the presence of cysts. No visible changes in the kidney morphology were observed at days 5 or 10. At day 15, three pups had cystic kidneys and were designated as homozygous (cpk/cpk). All pups were killed and their kidneys frozen in liquid nitrogen and stored at −80°C until use. One kidney was used for the preparation of RNA and the other for histological and enzyme activity assays.

Caspase mRNA expression. Total cellular RNA was prepared by extraction in RNA-STAT-60 (Tel-Test). Twenty micrograms of RNA were electrophoresed on a 1.2% agarose gel after glyoxalation for 1 h at 55°C. RNA from the gel was transferred to NYTRAN nylon membranes (Schleicher & Schuell, Kenee, NH) and immobilized by ultraviolet cross-linking. The quality of the RNA was examined by staining the blot with methylene blue (0.04% methylene blue in 0.5 M sodium acetate, pH 5.2) and washing several times with water. Prehybridization was carried out for at least 4 h at 42°C in 50% formamide, 5× sodium chloride-sodium phosphate-EDTA (SSPE), 5× Denhardt's, and 0.5% SDS. Filters were probed with murine cDNAs for caspase 4 (a 250-bp fragment), caspase 2, caspase 7 (483 bp), caspase 3 (1.4 Kb), and hBax (600 bp) cDNAs. The cDNAs were radioactively labeled with α-32P-dATP using Prime-it II random primer labeling system (Stratagene, La Jolla, CA). Hybridization was performed with the radiolabeled probe at 42°C overnight in 50% formamide, 5× SSPE, 2.5× Denhardt’s, 0.1% SDS, and 100 μg/ml salmon sperm DNA. The filter was washed three times for 10 min each in 1× SSPE/0.1% SDS followed by two final 30-min washes in 0.1× SSPE/0.1% SDS at 65°C. The filters were exposed to the PhosphorImager overnight and scanned the next day.

The blots were subsequently hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ascertain quantitative loading of RNA in all lanes. The GAPDH probe was a 452-bp fragment for rat GAPDH coding sequence (586–1037), which were PCR-cloned using internal primers obtained from Clontech Laboratories (Palo Alto, CA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining. Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining was carried out using the ApopTag in situ apoptosis detection kit (Oncon, Gaithersburg, MD). Frozen sections were fixed in 10% formaldehyde/PBS for 10 min at room temperature and washed in PBS twice. The slides were then postfixed in ethanol and acetic acid (2:1) for 5 min at 20°C and again washed in PBS. Without drying, 75 μl of a 0.05% diaminobenzidine (DAB) solution (100 mg/ml 3,3’-diaminobenzidine tetrahydrochloride in 0.01 M Tris-HCl, pH 7.4) was applied for 5 min followed by 54 ul of working-strength TdT-enzyme mix, which consisted of fluorescein-conjugated dUTP, tailing buffer, and terminal deoxynucleotidyl transferase. A Parafilm (American National Can, Chicago, IL) coverslip was applied, and slides were incubated in a humidified chamber at 37°C for 1 h. Subsequently, the slides were washed in working-strength stop/wash buffer for 10 min at room temperature. The slides were then counterstained with propidium iodide and viewed under a microscope using fluorescent excitation and emission filters.

Induced caspase activity in the apoptotic kidney extracts was quantitated and characterized by comparing with substrate profiles generated using recombinant enzymes and their respective, preferred fluorogenic peptide substrates: Ac-YVAD-AMC and Ac-LEED-AMC, tetrapeptide substrate, and recombinant caspase 1 or kidney extract. Caspase 3 assays contained 25 mM K+ HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 3-(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate, 2 mM 1,4-dithiothreitol (DTT), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A. Caspase 8 assays contained 25 mM K+ HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 3-(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate, 2 mM 1,4-dithiothreitol (DTT), 5 mM EDTA, tetrapeptide substrate, and recombinant caspase 8 or kidney extract. Caspase 4 assays contained 25 mM K+ HEPES (pH 5.8), 10% sucrose, 0.1% CHAPS, 1 mM EDTA, tetrapeptide substrate, and recombinant caspase 4 or kidney extract. Caspase 4 assays contained 25 mM K+ HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 1 mM EDTA, tetrapeptide substrate, and recombinant caspase 4 or kidney extract. Caspase 8 assays contained 75 mM Na+ MOPS (pH 7.5), 10% glycerol, 1 mM DTT, 0.25 mM EDTA, tetrapeptide substrate, and recombinant caspase 8 or kidney extract. The concentration of each of the substrates was as follows: Ac-YVAD-AMC and Ac-LEED-AMC,
100 µM; Ac-DEVD-AMC, Ac-DQMD-AMC, and Ac-VETD-AMC, 25 µM. The assays were conducted in 96-well microtitre plates in a total volume of 100 µl. After a 10-min equilibration of the incubation mixture (containing extracts) at 30°C, the reaction was initiated by the addition of substrate. Peptide cleavage was measured using a Cytofluor 4000 fluorescent plate reader (PerSeptive Biosystems) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

Western blot analysis. Tissue extracts were prepared in a similar way as described above for determination of caspase activity. Approximately 30 µg of protein from each sample were subjected to SDS-PAGE, and immunoblot analysis was performed using an anti-bcl-2 monoclonal antibody (Transduction Laboratories) followed by incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Transduction Laboratories). Immunobilized antibodies were detected using chemiluminescence (Pierce). The same blot was stripped and reprobed with monoclonal antibody anti-GAPDH (Research Diagnostic).

RESULTS

The presence of apoptosis in the kidneys of 21-day-old pups was detected using the ApopTag labeling system and propidium iodide staining. Apoptotic cells were observed only in the interstitium of cystic kidney and displayed typical morphological features, including bright and fragmented nuclei (Fig. 1). No apoptotic cells were identified in noncystic kidneys.

The mRNA expression of various caspases in the cpk mouse kidney was determined using Northern blot analysis. Total RNA was hybridized with mouse-specific probes for caspases 2, 3, 4, and 7. The expression of all caspases evaluated was higher in cystic kidneys taken from 21-day-old pups (Fig. 2); however, the greatest increase was observed with caspase 4. To evaluate whether the increase in caspase 4 mRNA preceded or paralleled cyst formation, RNA blots were prepared from kidneys of 5-, 10-, 15-, and 21-day-old pups and probed with the radiolabeled murine caspase 4 cDNA. The data indicate that caspase 4 mRNA expression was elevated only at day 21 when maximum cyst formation had occurred (Fig. 3). Despite the presence of cysts at day 15, no obvious increase in caspase 4 expression was detected (Fig. 3).

Caspase activity was higher in cystic kidneys taken from 21-day-old pups than in noncystic control kidneys (Fig. 4). The use of substrates with preferential specificity for various caspases (Fig. 4B) indicated that the greatest increases in activity appeared to be associated with caspase 4 and possibly caspase 3. Thus there

Fig. 1. Detection of apoptotic cells by in situ fluorescein-nucleotide end labeling of DNA in cpk kidney of a 21-day-old mouse. All images were taken with ×40 magnification and filters (except B). A: section of kidney showing clustering of apoptotic bodies (bright yellow staining) located in interstitium beneath epithelial cells. B: same field as A visualized by FITC filter showing clustering of apoptotic bodies only. C: section of kidney showing 2 apoptotic bodies (bright yellow staining) located in interstitium. D: section of normal kidney cortex. No apoptotic bodies were found.
was a sevenfold increase in caspase 4-like activity (Ac-LEED-AMC), and a sixfold increase in caspase 3-like activity (Ac-DQMD-AMC). The assignment of the increased DQMDase activity could not be unequivocally assigned to caspase 3, because the selectivity comparisons used recombinant human as opposed to murine caspases. A modest threefold induction of caspase 1-like activity (Ac-YVAD-AMC) was also observed (Fig. 4).

The expression of the apoptosis regulator bax in the cpk mouse kidney was determined using Northern blot analysis with a human bax cDNA probe. Total RNA isolated from 21-day-old cpk/cpk mice demonstrated an elevation of bax expression compared with the control mice (Fig. 2). Because bcl-2 and bax are known to have opposing roles in the regulation of apoptosis, we investigated whether the expression of bcl-2 was also affected in these mice. Expression of bcl-2 protein was determined by Western blot analysis using 21-day-old kidney tissues collected from cpk/cpk mice, siblings of cpk/cpk, which appear to have normal kidneys, and C57BL mice. Expression of bcl-2 was increased dramatically only in the cpk kidneys, as indicated by the presence of a ~26-kDa band (Fig. 5). There was also a small elevation of expression in the cpk liver compared with the normal ones. No expression of bcl-2 was detected in the kidneys of normal C57BL mice, and only negligible expression was observed in some of the normal siblings.

**DISCUSSION**

Apoptosis is an intrinsic death program that must be precisely regulated to maintain the integrity and homeostasis of a multicellular organism. Apoptosis has been implicated in a number of human diseases including PKD (21, 43). Several growth factors and protooncogenes have altered expression in PKD and have been linked to this group of disorders (4); however, the primary mechanisms involved in the disease progression are as yet unknown.

The cpk mouse is a useful model for studying human ADPKD. The main features of this model are the early onset of the disease and the involvement of the collecting ducts, making it very similar to the human disease. In the present study, we have demonstrated, using in

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**Fig. 2.** Northern blot analysis of caspase and bax expression in normal and cystic kidneys from 21-day-old mice. Blots were probed with murine caspase 2, 3, 4, and 7 cDNA and human bax cDNA. All blots were later reprobed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to demonstrate equal loading of RNA.

**Fig. 3.** Time course of caspase 4 mRNA expression in cystic kidneys (†).
situ end labeling, the presence of apoptosis in the kidneys of 3-wk-old cpk mice. Consistent with the observation first made by Woo (43), we observed that apoptosis occurred in the same proximity as the aggressive proliferation of epithelial cells and cyst development. Our observations differ somewhat from previous reports in that we only observed apoptosis in the interstitium surrounding the cysts. We found no evidence of apoptosis in cyst epithelium or noncystic tubules as has been observed by others (43). It is not clear why our observation is different, although it is possible that apoptosis occurred in the epithelial cells and these cells were phagocytosed into the interstitium; however, we have no direct evidence for this.

Our observation that apoptosis occurred primarily in the interstitium, however, is supportive of the concept that accumulation of interstitial mononuclear cells and the resulting induction of interstitial scarring contributes to the loss of renal function (11). We also observed that the abnormal apoptotic death observed in the kidneys of cpk mice may involve a caspase-dependent pathway. Thus we observed that caspase 4 expression as well as caspase 4 activity to be upregulated. In addition, the activity of several other caspases was also elevated.

There is convincing evidence indicating that caspases play a critical role in apoptosis. Mice deficient in caspase 3, 8, or 9 have striking developmental defects that are attributed to the deficiency in apoptosis (12, 20, 40, 46). Proteolytic processing of the highly insoluble cytoskeleton is necessary to dismantle the cells committed to apoptosis. Disassembly of microtubules has been shown to be an early step in the execution phase of apoptosis (25). Other cytoskeletal proteins, such as fodrin, lamins A, B, and C, keratins, and vimentin are found to be cleaved by caspases during apoptosis (30). Thus the activation of caspases probably has major effects on the organization of the cytoskeleton in the kidneys of cpk mice. It is interesting to note that taxol, a microtubule stabilizer, is able to significantly inhibit the progression of polycystic kidney disease and prolong the survival of cpk/cpk mice (24, 44, 45).

Although bcl-2 is known to be one of the most important apoptosis regulators that acts to inhibit cell death, we found that overexpression of bcl-2 was not sufficient to protect the cells of cpk kidneys from apoptosis. This is probably due to the simultaneous increase of bax expression. It has been established that the expression ratio of bcl-2 to bax determines the relative cellular sensitivity or resistance to the apoptotic stimuli (32, 36, 49). Previous studies have demonstrated that c-myc expression is increased to 25- to 30-fold in 3-wk-old cpk mice (5). Transgenic mice that overexpress both bcl-2 and c-myc, as well as bcl-2 knockout mice, demonstrated increased apoptosis with...
a PKD phenotype (38, 39). It appears that in cpk mice, there is also a c-myc-mediated apoptotic pathway that is bcl-2/bax independent and that is likely to be involved in the formation of renal cysts (21, 39). Therefore, blockade of this abnormal apoptotic pathway might prevent the loss of renal function in polycystic kidney disease. Caspases represent potential targets because numerous studies have demonstrated that blockade of caspases can indeed prevent apoptosis (13, 15, 22, 23, 34, 37).

Perspectives

Evidence demonstrating that cystic kidneys occurred when the apoptosis-regulated bcl-2 is knocked out in mice, and the demonstration of a programmed cell death in patients with PKD as well as animal models suggest that apoptosis may be an important process in this disease. Whether apoptosis contributes directly towards cyst formation and/or the progressive loss in renal function observed in some patients with PKD is unclear; however, identification of the cellular mechanism involved in the apoptosis process may provide a mechanism to evaluate this further.

The authors are grateful to Laura Davis and colleagues in the Dept. of Laboratory Animal Sciences, Maria McDewitt and Lisa Contino.

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Received 14 July 1999; accepted in final form 6 October 1999.

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