

## APOPTOSIS AND LOSS OF RENAL TISSUE IN POLYCYSTIC KIDNEY DISEASES

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**Abstract Background.** Polycystic kidney disease is characterized by the enlargement of renal cysts, interstitial fibrosis, and gradual loss of normal renal tissue in association with progressive deterioration of renal function. The process causing the progressive loss of renal tissue is unknown, but it could be the result of a form of programmed cell death known as apoptosis.

**Methods.** We assayed apoptotic DNA fragmentation in normal and polycystic kidneys biochemically by gel electrophoresis and histochemically by in situ end-labeling. A DNA-specific dye, Hoechst 33258, was used to detect morphologic apoptosis in renal samples from patients with normal kidneys, polycystic kidney disease, and other kidney diseases.

**Results.** Apoptotic DNA fragmentation was detected in polycystic kidneys from 5 patients without renal failure and 11 patients with renal failure but not in kidneys from

12 patients with no renal disease. In situ end-labeling revealed apoptotic cells in glomeruli, in cyst walls, and in both cystic and noncystic tubules of the polycystic kidneys. No tubular apoptosis was detected in renal-biopsy specimens from five patients with IgA nephropathy, three patients with nephrosclerosis, two patients with focal glomerulosclerosis, one patient with diabetic nephropathy, six patients with acute tubular necrosis, or four patients with acute and four patients with chronic renal-transplant rejection. The capacity of polycystic kidney cells to undergo apoptosis was retained in vitro in the absence of uremia, ischemia, and other confounding pathologic conditions.

**Conclusions.** Apoptotic loss of renal tissue may be associated with the progressive deterioration of renal function that occurs in patients with polycystic kidney disease. (N Engl J Med 1995;333:18-25.)

THE polycystic kidney diseases are a group of disorders characterized by the presence of numerous cysts throughout grossly enlarged kidneys. In humans, they are inherited as autosomal dominant or autosomal recessive disorders. Autosomal dominant polycystic kidney disease is a major cause of chronic renal failure, accounting for 10 percent of all cases requiring long-term dialysis or renal transplantation. Despite the recent cloning of the gene responsible for 80 to 90 percent of the cases of autosomal dominant polycystic kidney disease, the primary pathogenic mechanism of polycystic kidney disease remains unknown.

Three types of defects have been implicated in the process whereby renal tubules enlarge to become cystic: abnormalities in basement-membrane components,<sup>1,2</sup> abnormal proliferation of cystic epithelia,<sup>3</sup> and abnormal development and polarization of cystic epithelia.<sup>4,6</sup> Renal insufficiency is attributed to the compression of normal renal tissue and interstitial disease.

Each human kidney is made up of approximately 1 million nephrons. In all types of polycystic kidney disease examined to date, the cysts numbered in the hundreds or thousands. Microdissection studies indicate that each cyst arises from the focal dilatation of a small segment of a nephron.<sup>7,8</sup> The loss of a small percentage of nephrons due to cyst formation should not result in renal insufficiency, because many functional nephrons should remain.

Apoptosis is a type of physiologic cell deletion that occurs during embryonic development and in the renewal of mature tissues. In this study, we evaluated the possibility that the loss of noncystic renal tubules in polycystic kidneys could be caused by apoptosis.

## METHODS

Polycystic human kidneys were obtained from the surgical pathology department of the UCLA Medical Center, the International

Institute for the Advance of Medicine (Exton, Pa.), the National Disease Research Interchange (Philadelphia), and the Polycystic Kidney Research Foundation (Kansas City, Mo.). Five of the 16 kidneys from patients with autosomal dominant disease (Polycystic Kidney Specimens 1, 3, 5, 8, and 13) were originally removed from brain-dead organ donors for transplantation but were not used because they were polycystic. These organs were not subjected to warm ischemia, and the donors had normal serum creatinine concentrations. Polycystic kidneys were also collected from 10 patients with end-stage renal disease who were undergoing renal transplantation. A polycystic kidney was obtained from a one-day-old infant with terminal autosomal recessive polycystic kidney disease. Twelve normal human kidneys obtained from either the International Institute for the Advance of Medicine or the National Disease Research Interchange had been obtained for transplantation, but suitable recipients were not found. These kidneys were not subjected to warm ischemia.

The kidney tissues were trimmed into cubes measuring 1 to 3 mm and were processed for the isolation of genomic DNA, cryopreserved in a 1:1 mixture of DMEM and F12 medium containing 10 percent dimethyl sulfoxide, or processed further for in vitro culture. In addition, fine-needle-biopsy specimens of renal tissue in Bouin's fixative from five patients with IgA nephropathy, three patients with nephrosclerosis, two patients with focal glomerulosclerosis, one patient with diabetic nephropathy, six patients with acute tubular necrosis, and four patients with acute and four patients with chronic renal-transplant rejection were obtained from Dr. Arthur Cohen (Cedars-Sinai Medical Center, Los Angeles). These fine-needle-biopsy samples were used only for the histologic detection of apoptotic cells.

We obtained *cpk* mice<sup>9</sup> from the Jackson Laboratory (Bar Harbor, Me.); they were maintained by mating known heterozygotes. We obtained *pcy* mice<sup>10</sup> from Dr. H. Takahashi (Fujita Health University, Toyoake, Japan) through Dr. Jared Grantham (University of Kansas, Kansas City); they were maintained by back-crossing heterozygous female mice with homozygous polycystic male mice.

Cells from 1-mm<sup>3</sup> fragments of normal and polycystic human kidneys were prepared for in vitro culture as described elsewhere.<sup>11</sup> Both primary cultured cells and cryopreserved primary cells revived and cultured for up to three passages were studied. The cells were plated at a density of 10<sup>6</sup> cells per 10-mm culture plate (Corning, Cambridge, Mass.) and incubated at 37°C in 5 percent carbon dioxide.

Genomic DNA from tissues and cultured cells was prepared with the proteinase K-sodium dodecyl sulfate digestion method.<sup>12</sup> Oligonucleosome-length DNA fragments were detected by electrophoresis on 1 percent agarose gels containing 0.5 µg of ethidium bromide per milliliter.

For in situ detection of apoptotic nuclei, normal and polycystic kidney cells and tissues were fixed with 1 percent paraformaldehyde and embedded in paraffin. Tissue sections on ProbeOn Plus slides (Fisher Scientific, Pittsburgh) were processed for in situ end-labeling as de-

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Supported by grants from the National Institutes of Health (DK40700 and DK45663).

scribed below. Cultured cells were grown in four-compartment glass chamber slides (Nunc, Naperville, Ill.) and fixed overnight before in situ end-labeling.

For in situ end-labeling, the ends of DNA fragments generated during apoptosis in the nuclei of apoptotic cells were enzymatically labeled in situ with biotin-16-deoxyuridine triphosphate (Boehringer–Mannheim, Indianapolis) or biotin-21-deoxyuridine triphosphate (Clontech, Palo Alto, Calif.) and terminal transferase (Promega, Madison, Wis., or Boehringer–Mannheim) with a modification of the homopolymer tailing method.<sup>13</sup> The reactions were carried out in the capillary space between a pair of ProbeOn Plus slides. First,

endogenous peroxidase activity was stopped by incubation with 3 percent hydrogen peroxide in phosphate-buffered saline for 15 minutes. The slides were washed and then incubated at 37°C for one hour with 200  $\mu$ l of a solution containing 50 units of terminal transferase and 10  $\mu$ M biotin-16-deoxyuridine triphosphate (or biotin-21-deoxyuridine triphosphate) in 100 mM cacodylate (pH 6.8), 1.5 mM cobalt chloride, and 0.1 mM dithiothreitol. The biotin incorporated in the sections was coupled to horseradish peroxidase with Vectorstain Elite ABC reagents (Vector Laboratories, Burlingame, Calif.). The slides were washed and then developed with a diaminobenzidine substrate kit (Vector Laboratories) for 10 minutes. The

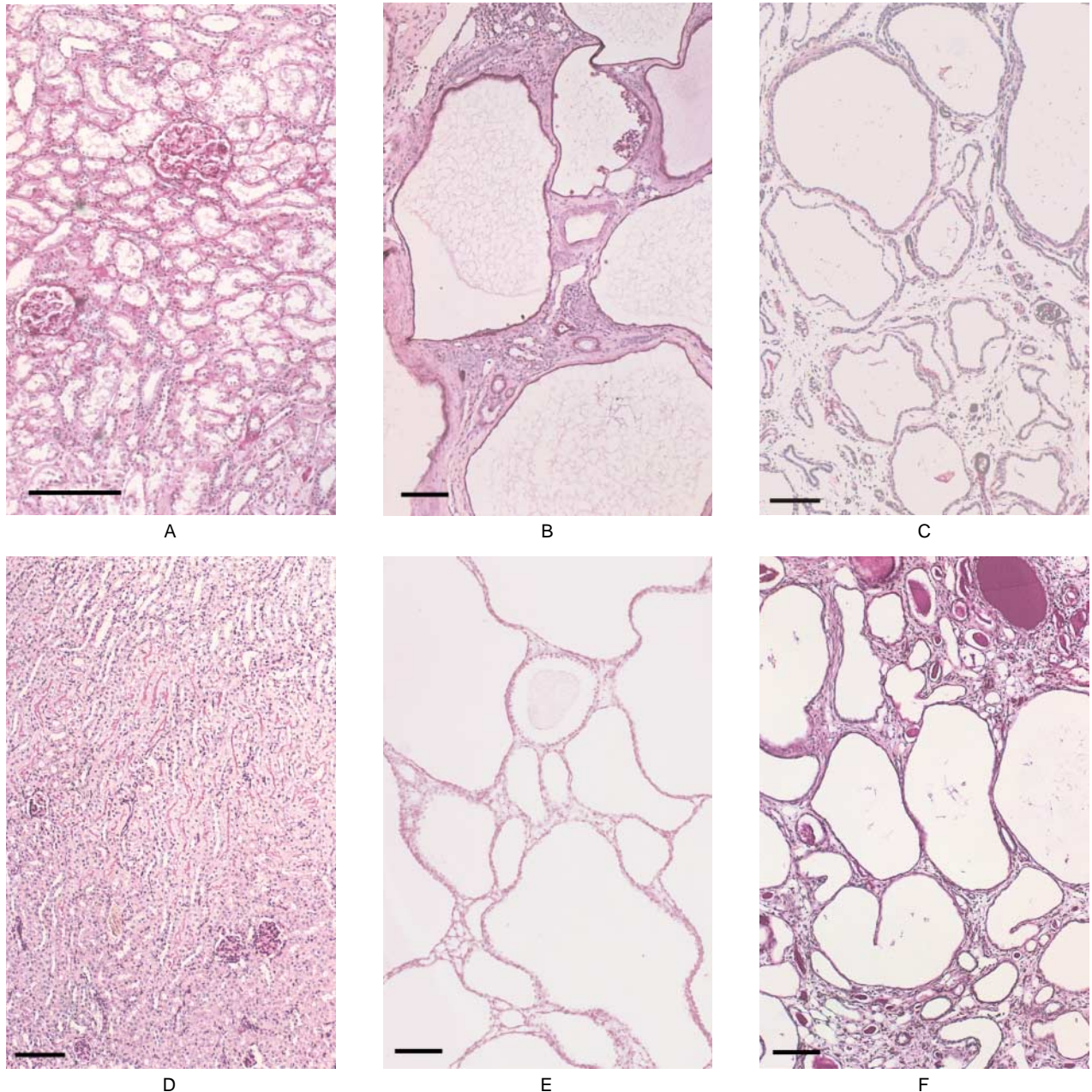


Figure 1. Loss of Renal Tissue in Polycystic Kidney Disease.

There are abundant glomeruli and tubules in a normal human kidney (Panel A) and a normal mouse kidney (Panel D). In contrast, there is a lack of glomeruli and renal tubules and there are multiple cysts in kidneys from patients with end-stage autosomal dominant (Panel B) or autosomal recessive (Panel C) polycystic kidney disease, a kidney from a *cpk* mouse with end-stage polycystic kidney disease (Panel E), and a kidney from a *pcy* mouse with end-stage polycystic kidney disease (Panel F). Interstitial fibrosis is apparent in the kidneys from patients with end-stage polycystic kidney disease (Panels B and C) and in the kidney from a six-month-old *pcy* mouse with polycystic kidney disease (Panel F). Bars represent 0.1 mm. Sections were stained with periodic acid–Schiff stain and counterstained with hematoxylin.

slides were then stained with periodic acid–Schiff reagent and counterstained with hematoxylin.

To detect apoptotic nuclei in histologic sections of fine-needle-biopsy samples of kidney tissue from the patients with other renal diseases, the sections were deparaffinized, rehydrated in phosphate-buffered saline, and stained with 10  $\mu\text{g}$  of the DNA-specific bisbenzimidazole dye Hoechst 33258 (Molecular Probes, Eugene, Oreg.) per milliliter in 100 mM sodium chloride, 10 mM TRIS, and 1 mM EDTA (pH 7.5) for 10 minutes and mounted in VectaShield mounting medium for fluorescence microscopy. Apoptotic nuclei are identified by their condensed appearance and intense blue fluorescence as compared with normal nuclei.<sup>14</sup>

## RESULTS

Progressive loss of renal function is a common feature of polycystic kidney disease and other chronic renal diseases. Kidneys from most patients with nonpolycystic end-stage renal disease contain some remnant nephrons and abnormal glomeruli. However, within grossly enlarged kidneys of patients with end-stage

polycystic kidney disease, fibrotic tissue filled the interstitium and few or no nephrons remained (Fig. 1). These observations prompted the search for evidence of apoptosis as a possible mechanism underlying the progressive loss of renal tissue in polycystic kidney diseases. The range of disease progression represented in the 16 kidneys from patients with polycystic kidney disease is shown in Figure 2.

In contrast to necrosis, which is the passive result of traumatic cell death, apoptosis is a type of programmed cell death in which each cell actively synthesizes new RNA and proteins to mediate its own demise.<sup>15</sup> In apoptosis, cell death is preceded by the cleavage of chromatin into oligonucleosomes. The DNA in these oligonucleosomes can be detected by agarose-gel electrophoresis as a ladder of DNA fragments with lengths in multiples of about 180 base pairs.<sup>16</sup> Oligonucleosome-length DNA ladders indicative of apop-

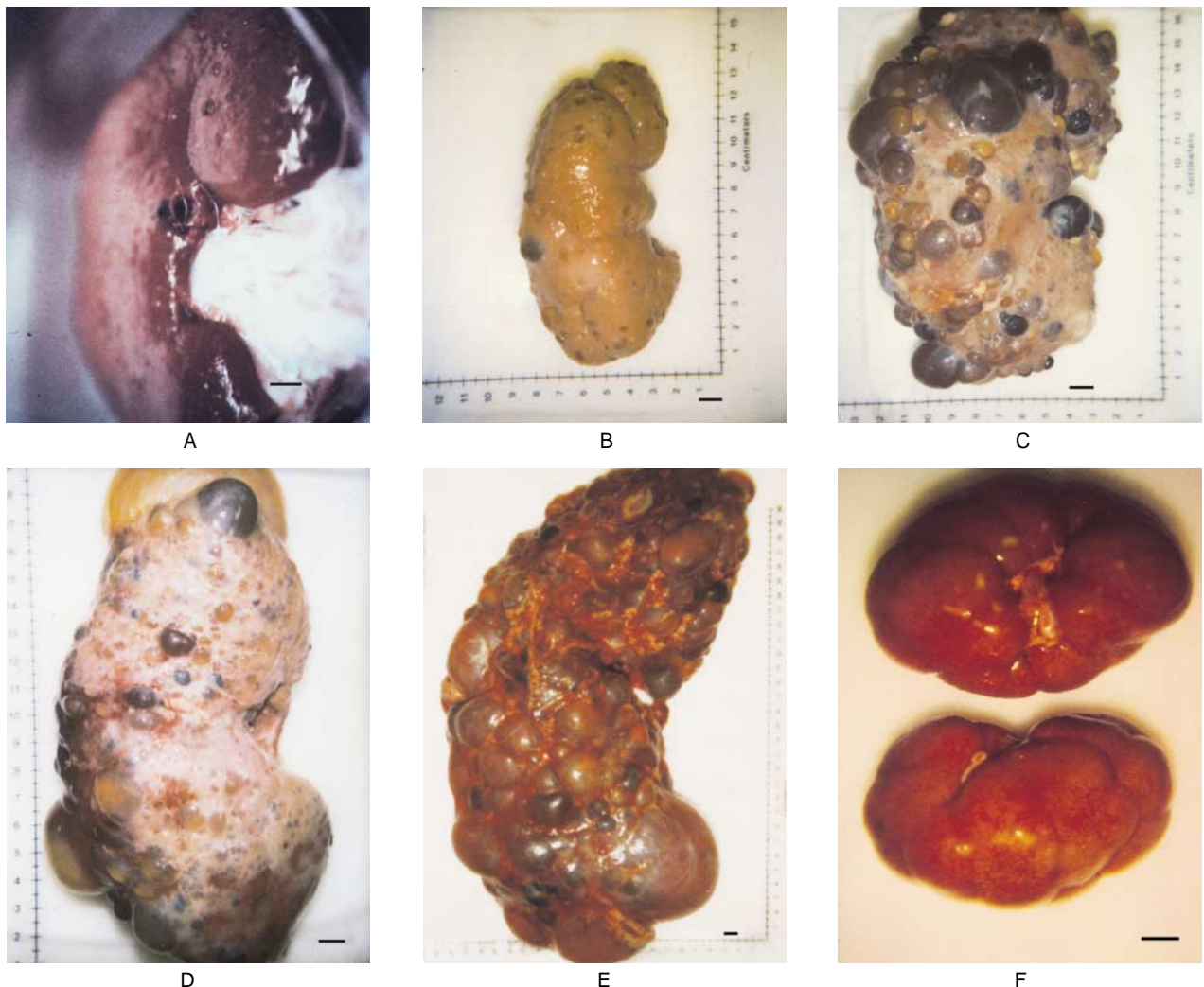
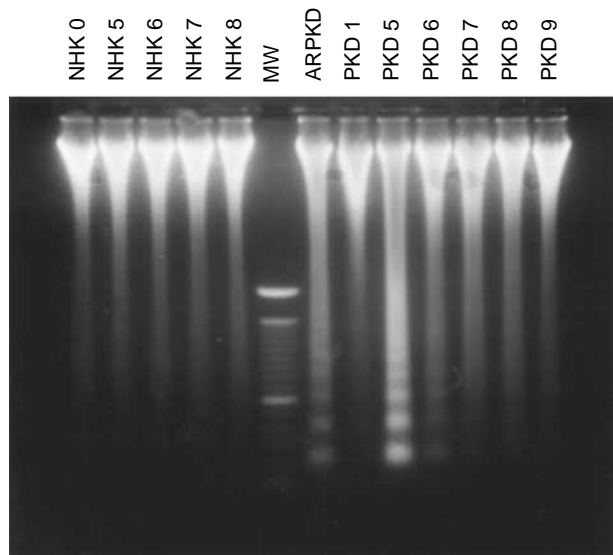
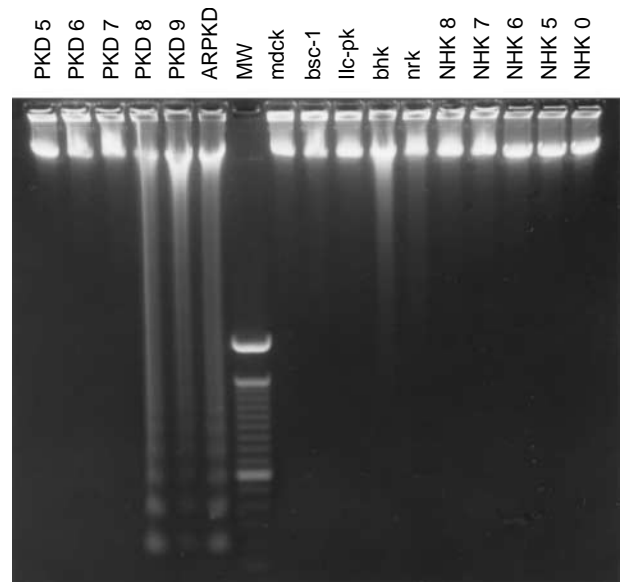


Figure 2. Range of Disease Progression in Polycystic Kidney Disease in Humans.

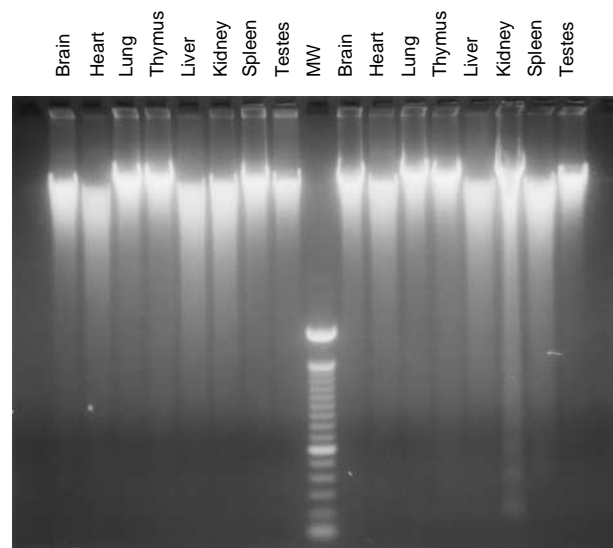
The full range of cystic involvement was represented in the 16 kidney specimens from patients with autosomal dominant polycystic kidney disease that were examined in this study (5 of which are shown). Polycystic Kidney Specimens 1, 5, 8, and 13, shown in Panels A, B, C, and D, respectively, were functionally normal. A kidney from a patient with end-stage polycystic kidney disease (Specimen 9) is shown in Panel E. A pair of kidneys from an infant who died of polycystic kidney disease 24 hours after birth is shown in Panel F. Bars represent 1 cm.



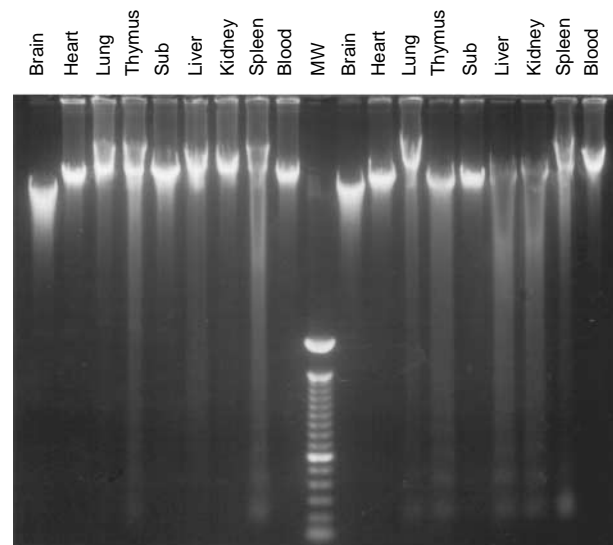
A



D



B Normal Littermates *pcy* Mice



C C57/BL6 Mice *cpk* Mice

Figure 3. Apoptotic Fragmentation of DNA in Kidneys from Patients and Mice with Polycystic Kidney Disease.

In Panel A, DNA extracted from five normal human kidneys (NHK 0, 5, 6, 7, and 8) shows no signs of apoptotic fragmentation. DNA extracted from a kidney from a patient with autosomal recessive polycystic kidney disease (ARPKD) and six kidneys from patients with autosomal dominant polycystic kidney disease (PKD 1, 5, 6, 7, 8, and 9) had various degrees of apoptotic DNA fragmentation. The patients from whom Polycystic Kidney Specimens 1, 5, and 8 were obtained had normal renal function, whereas those from whom Polycystic Kidney Specimens 6, 7, and 9 were obtained had end-stage renal failure. Although apoptotic nuclei were detected in all examples of early polycystic kidney disease in humans with use of the *in situ* homopolymer tailing technique (as shown in Fig. 4), no apoptotic DNA fragmentation was detected in Polycystic Kidney Specimen 1. The level of DNA fragmentation in Polycystic Kidney Specimens 7, 8, and 9 did not correlate with the stage of disease. In Panel B, apoptotic DNA fragmentation is evident in the preuremic polycystic kidneys of three-month-old *pcy* mice. Apoptotic DNA fragmentation cannot be detected in other organs tested in these mice at three months of age or later. No apoptotic DNA fragmentation was detected in the organs of normal littermates. In Panel C, apoptotic fragmentation of DNA is evident in the thymus and spleen of normal 10-day-old C57/BL6 mice. In 10-day-old preuremic *cpk* mice, apoptotic DNA fragmentation was detected in the lung, liver, and kidneys, in addition to the thymus and spleen. Sub denotes DNA samples from the submaxillary gland. In Panel D, apoptotic fragmentation of DNA in cells cultured from Polycystic Kidney Specimens 8 and 9 and ARPKD can be seen. No apoptotic fragmentation of DNA was detected in cells cultured from normal human kidneys (NHK 8, 7, 6, 5, and 0); from the established cell lines mdck, bsc-1, llc-pk, bhk, or nrk; or from Polycystic Kidney Specimens 5, 6, and 7. All cultured human cells were cryopreserved, revived, and passaged twice in culture. All lanes were loaded with 50  $\mu$ g of total genomic DNA from each sample. MW denotes the molecular-weight marker, a DNA ladder of 100 base pairs (BRL, Gaithersburg, Md.).

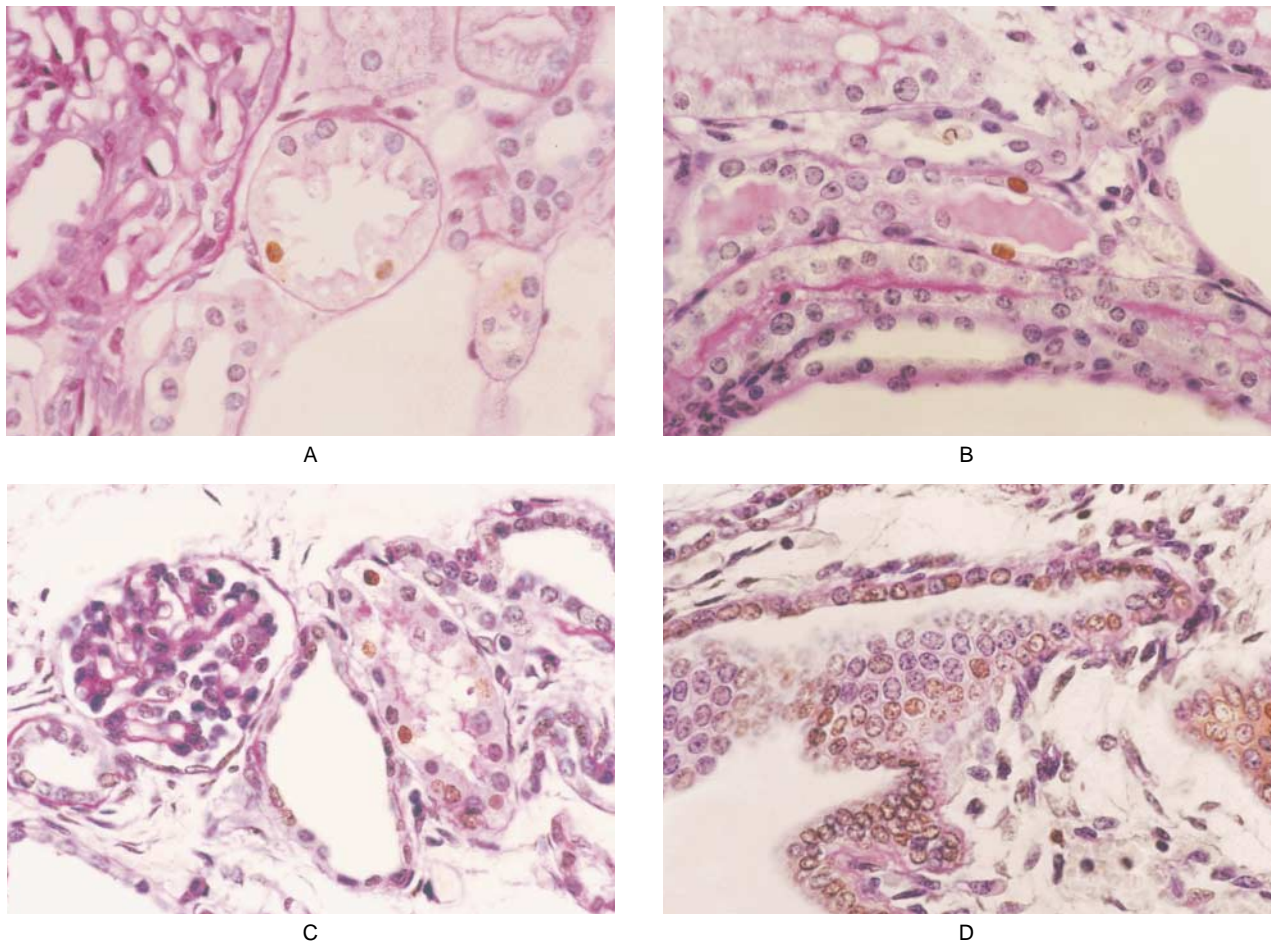


Figure 4. In Situ Demonstration of Apoptotic Nuclei in Polycystic Kidneys.

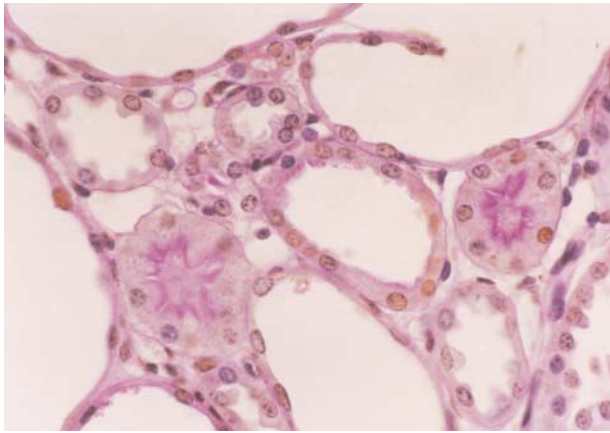
With the use of in situ end-labeling, apoptotic nuclei are stained brown by the horseradish peroxidase–diaminobenzidine reaction, whereas normal nuclei are stained purple by the hematoxylin counterstain (in all panels except Panel G). Panels A and B show apoptotic nuclei within the tubules and a glomerulus of a kidney from a patient with autosomal dominant polycystic kidney disease (Specimen 1) ( $\times 470$ ). Panels C and D show cells with apoptotic nuclei next to cells with normal nuclei in undilated tubules and in cells lining the expanded cyst wall of a kidney from a patient with autosomal recessive polycystic kidney disease ( $\times 470$ ). Apoptotic nuclei are apparent in normal tubular cells and cells lining cysts within the polycystic kidneys of a 10-day-old *cpk* mouse (Panel E;  $\times 470$ ) and a 3-month-old *pcy* mouse (Panel F;  $\times 470$ ). In Panel G, after Hoechst-dye staining, apoptotic nuclei within tubules of a kidney from a patient with early polycystic kidney disease (Specimen 1) appear condensed (pyknotic) and bright blue ( $\times 470$ ). Panel H shows apoptotic nuclei in cultured kidney cells from a patient with autosomal dominant polycystic kidney disease ( $\times 950$ ).

otic DNA fragmentation were detected in DNA prepared from all the kidneys we studied from patients with autosomal recessive and autosomal dominant polycystic kidney disease (Fig. 3A), except for one (Specimen 1) in which the disease was at a very early stage. An apoptotic DNA ladder was not found in the 12 samples of normal human kidneys. Apoptotic DNA fragmentation was evident in the preuremic kidneys of *pcy* mice (Fig. 3B) and *cpk* mice (Fig. 3C), but not in kidneys of age- and sex-matched normal littermates. In addition, apoptotic DNA fragmentation was detected in the lung and liver of *cpk* mice. Apoptotic DNA fragmentation in the thymus and spleen of 10-day-old mice is normal.<sup>17</sup>

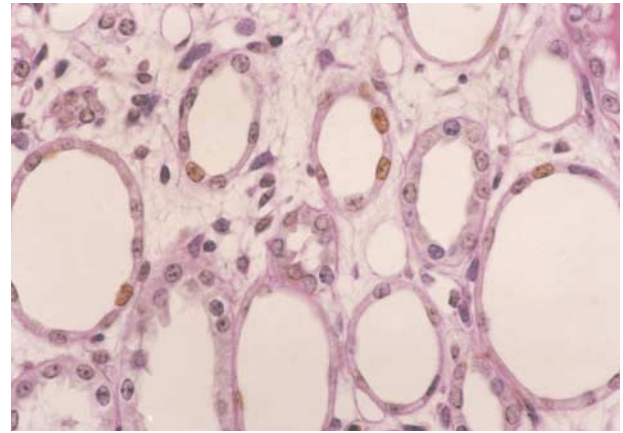
Apoptotic DNA fragmentation was detected in most cultures of polycystic kidney cells from humans but was not found in any culture of normal human kidney cells (Fig. 3D). The absence of DNA fragmentation in some

cultures of polycystic kidney cells probably reflects variations in sampling, because DNA fragmentation could be demonstrated in other cultures from the same polycystic kidneys (data not shown). Apoptotic fragmentation of chromosomal DNA was apparent in cultured polycystic kidney cells from *cpk* and *pcy* mice but not from normal littermates (data not shown).

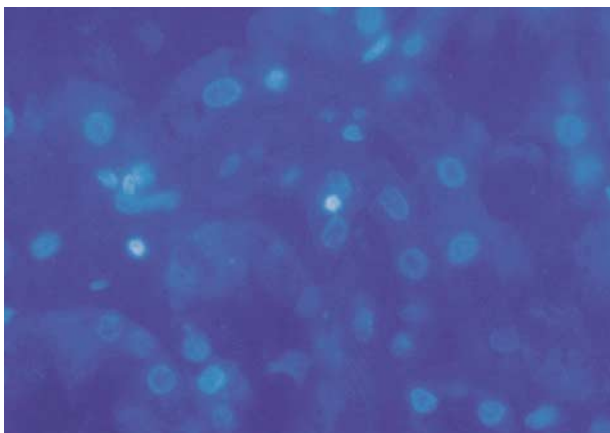
The in situ end-labeling technique labels the large number of DNA ends in oligonucleosomes that are generated within apoptotic nuclei. Figure 4 shows apoptotic nuclei in kidney tissue from patients with autosomal dominant and autosomal recessive polycystic kidney disease and mice with congenital polycystic kidney disease. Apoptotic nuclei can be detected in noncystic tubular epithelial cells, in cells within glomeruli, and in cells lining renal cysts. With the use of this technique, all the polycystic human kidneys but none of the normal human kidneys we examined had evidence of ap-



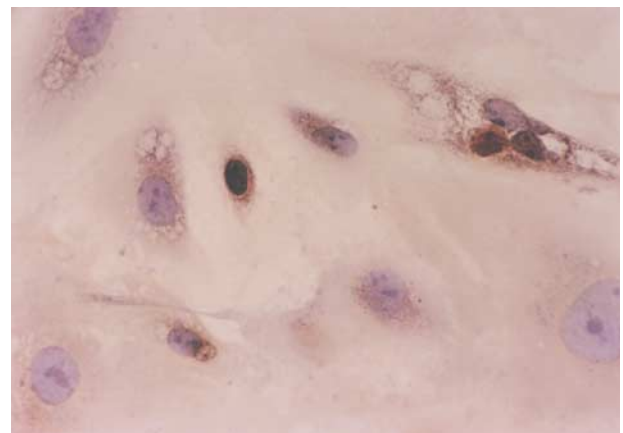
E



F



G



H

optotic nuclei. In addition, apoptosis was readily detected in all proliferating cultures of human and murine polycystic kidney cells.

The detection of apoptosis in undilated nephrons in preuremic polycystic human kidneys and in polycystic *cpk* and *pcy* mouse kidneys and the contrasting absence of apoptosis in kidneys from normal humans and mice strongly suggest that apoptosis may be associated with the progressive loss of renal tissue in polycystic kidney disease. However, the reported detection of apoptosis in rats with obstructive hydronephrosis<sup>18,19</sup> and ischemic renal atrophy<sup>20</sup> raised the possibility that renal apoptosis may be a common manifestation of uremia and ischemia associated with renal disease. Therefore, kidney-biopsy specimens from patients with various types of nephropathy, acute tubular necrosis, and acute and chronic transplant rejection were analyzed for the presence of apoptotic nuclei. Because low pH induces random strand breaks in DNA and Bouin's fixative contains 5 percent acetic acid, samples in Bouin's fixative produced unacceptably high levels of background nuclear staining in the terminal transferase labeling assay.

All histologic samples of normal kidneys, polycystic kidneys, and kidneys with other types of diseases were stained with the DNA-specific dye Hoechst 33258 and examined with ultraviolet fluorescence microscopy to

detect the morphologic features of apoptotic nuclei. With the use of this technique, apoptotic nuclei were detected in all polycystic kidney samples (Fig. 4G). No tubular apoptosis was seen in normal kidneys or in any of the samples of tissue from patients with noncystic renal diseases (100 to 200 tubules studied per sample), indicating that, if present, tubular apoptosis is not easily detected in these forms of renal diseases. Apoptotic nuclei were seen in two glomeruli in one tissue sample from a patient with IgA nephropathy and in a single glomerulus in one tissue sample from a patient with acute transplant rejection. In the absence of tubular apoptosis, glomerular apoptosis may be associated with recovery rather than injury.<sup>21,22</sup>

#### DISCUSSION

Programmed cell death occurs during normal embryonic development and morphogenesis, in the maturation of the immune system, and in the turnover of renewing tissues,<sup>23</sup> and it may have a role in tumor formation<sup>24</sup> and degenerative diseases such as the acquired immunodeficiency syndrome and Alzheimer's disease.<sup>25,26</sup> This study shows that in addition to cyst enlargement and interstitial fibrosis, apoptosis is a pathological feature of polycystic kidney disease.

Within solid tissues, apoptotic cells are phagocytized within a few hours by neighboring cells or by phago-

cytes in a process involving the vitronectin receptor or the phosphatidylserine receptor.<sup>27,28</sup> Thus, apoptosis can be demonstrated histologically only during the interval before the apoptotic cell is phagocytized and digested. This limits the detection of apoptosis to samples in which apoptosis is occurring continually. Except in *cpk* mice with polycystic kidney disease and in the most severe forms of autosomal recessive polycystic kidney disease, in which there is rapid loss of renal tissues, renal insufficiency usually develops very gradually in polycystic kidney disease. In the more slowly progressive forms, in which loss of nephrons is slow, apoptosis was nevertheless detected before the onset of uremia. The failure to detect renal tubular apoptosis in several nonpolycystic forms of chronic renal disease suggests that either cell death occurs in polycystic kidney disease more than in other renal diseases with comparable or higher rates of loss of renal function, or, if cell death occurs in these other chronic kidney diseases, a nonapoptotic form of programmed cell death may be involved.<sup>29,30</sup>

The finding of extrarenal apoptosis in the *cpk* mice is in agreement with the well-known fact that polycystic kidney disease is not a kidney-specific disease.<sup>31</sup> The ability of the lung and liver to maintain cell-number homeostasis by cellular proliferation may explain the absence of abnormalities in these organs. Apoptotic degeneration of vessel walls could lead to the aneurysms that are frequently associated with polycystic kidney disease. The finding of apoptosis in both primary cultures and serially passaged polycystic kidney cells in vitro, in the absence of uremia, ischemia, obstruction, compression, or other undefined pathologic conditions associated with the in vivo disease state, suggests that apoptotic cell death may be an innate abnormality of cells in polycystic kidney disease.

The mature mammalian kidney is a quiescent organ with little or no mitotic activity, and little or no apoptosis was found in adult human kidneys. Although the mature kidney is capable of cellular proliferation in special circumstances, such as after acute tubular necrosis,<sup>32</sup> renal cells, like differentiated neurons, seldom divide. Usually, the kidney compensates for loss in mass and function or responds to increased functional demands by hypertrophy and not by hyperplasia.<sup>33</sup> The evolution of this hypertrophic response is dictated by the complex cellular and functional architecture of the individual nephrons. Developmentally, each nephron results from the amalgamation of the inducing ureteric bud, which differentiates into collecting tubules, and of the responding metanephric mesenchyme, which differentiates into the remainder of the nephrons.<sup>34</sup> Large-scale apoptosis occurs during metanephric development,<sup>14,35</sup> which may serve to match the numbers of collecting ducts developed from the ureteric bud to the number of tubules developing from the metanephric mesenchyme. As a result, after all the differentiated nephrons are formed, the mature kidney can no longer generate new nephrons. In polycystic kidneys, uncompensated apoptosis and the in-

ability to regenerate new nephrons would result in the progressive loss of renal tissue.

The progressive apoptotic death of rod cells in retinitis pigmentosa could result from mutations in several different genes,<sup>36</sup> and so too the various mutations in polycystic kidney disease could directly or indirectly trigger the aberrant activation of one of the apoptotic pathways. For example, basement-membrane components regulate the proliferation, differentiation, and apoptosis of epithelial cells.<sup>37</sup> Since polycystic kidneys have abnormal basement membranes,<sup>1,2</sup> the apoptotic loss of intact nephrons, the increased proliferative potential of cystic epithelia, and the aberrant epithelial-cell polarization and differentiation in polycystic kidneys could be a result of defective basement-membrane components.

In mice with a homozygous deletion of the *bcl-2* gene, polycystic kidney disease develops and is fatal<sup>38,39</sup>; *bcl-2* is a member of a family of genes that regulate apoptosis. Polycystic kidneys have also been reported in transgenic mice expressing *c-myc*<sup>40</sup> or SV40-LT.<sup>41</sup> The fact that all these divergent models of polycystic kidney disease can be linked to the aberrant expression of regulators of apoptosis provides circumstantial support for the hypothesis that apoptosis may be central to the pathogenesis of polycystic kidney diseases.

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