Albumin overload induces apoptosis in LLC-PK1 cells

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Erkan, Elif, Maryely De Leon, and Prasad Devarajan. Albumin overload induces apoptosis in LLC-PK1 cells. Am J Physiol Renal Physiol 280: F1107–F1114, 2001.—The degree of albuminuria is a well-known adverse prognostic indicator in human glomerular diseases. However, the mechanisms by which albuminuria by itself contributes to tubulointerstitial injury and progression of renal disease remain unclear. We tested the hypothesis that apoptosis may represent one of the mechanisms by which tubule epithelial cells are damaged after albumin overload in vitro. Cultured LLC-PK1 proximal tubule cells were incubated with varying concentrations of BSA. This resulted in a dose- and duration-dependent induction of apoptosis, as evidenced by internucleosomal DNA cleavage (DNA laddering) and nick-end labeling, externalization of plasma membrane phosphatidylserine (annexin labeling), and characteristic morphological changes (cell shrinkage and nuclear condensation). Albumin overload also resulted in a dose-dependent upregulation of Fas and Fas-associated protein with death domain (FADD), and activation of caspase 8. Incubation with the caspase 8 inhibitor IETD ameliorated the albumin-induced apoptosis. Collectively, our results indicate that albumin overload induces apoptosis of cultured LLC-PK1 cells, mediated at least in part by the Fas-FADD-caspase 8 pathway.

proteinuria; programmed cell death; progression of renal disease; tubular damage; cultured proximal tubule epithelial cells

THE RENAL GLOMERULUS PREVENTS the passage of albumin and many other plasma proteins by forming a semipermeable size- and charge-selective barrier between the capillary lumen and the urinary space. Proteinuria is the hallmark of glomerular pathology, and the magnitude of proteinuria is a well-known adverse prognostic factor in a wide variety of kidney diseases (7, 27). In addition, there is now a significant body of evidence demonstrating that persistent proteinuria by itself contributes to tubulointerstitial injury and progression of renal disease (5, 6, 26). Proteinuria has been shown to cause direct proximal tubule cell injury in animals (2, 11, 18) and humans (20). Albumin, the predominant protein in the glomerular filtrate, is taken up by proximal tubule cells via receptor-mediated endocytosis (4) and can induce the expression of several proinflammatory molecules, such as monocyte chemoattractant protein-1 (12, 31), osteopontin (12), regulated on activation normal T cell expressed and secreted (RANTES) (34), and endothelin-1 (35). Although these pathways provide attractive hypotheses for the interstitial inflammation and fibrosis that characterize progressive renal diseases, the pathophysiological mechanisms underlying the proximal tubule cell death and tubular atrophy in albuminuric states remain unclear.

Apoptosis or programmed cell death is characterized by distinct morphological changes consisting of cell shrinkage, nuclear condensation, and internucleosomal DNA fragmentation (19). It has been observed in an increasing array of renal disorders (22) and particularly mediates renal tubule cell death in polycystic kidney disease (32), ureteral obstruction (9), ischemia (13), nephrotoxic injury (21), and transplant rejection (24). Thus renal tubule cell apoptosis has emerged as a final common pathway in response to a wide variety of cellular insults that are applied at an intensity below the threshold for necrotic cell death, as exemplified by the majority of clinical nephrological conditions. Indeed, a recent study in an animal model of albumin-overload proteinuria has also documented the presence of tubular cell apoptosis (29), but the underlying mechanisms remain unknown.

In general, the caspase family of proteases constitutes the final effectors of programmed cell death, and it is convenient to classify the major intracellular apoptotic pathways based on the type of initiator pro-caspase activated (1, 30). Thus activation of the initiator pro-caspase 8 usually results from signaling via death receptors, which are integral membrane proteins that transduce apoptotic signals initiated by specific ligands to downstream molecules that possess a conserved “death domain” (3). The two best-characterized death receptors are Fas and TNF receptor 1 (TNFR1). Engagement of Fas receptor to Fas ligand results in recruitment of the Fas-associating protein with death domain (FADD) and activation of caspase 8 (3). The Fas-FADD-caspase 8 pathway has been recently implicated in the renal tubule cell apoptosis after endotoxin treatment (25), ischemic injury (13), transplant rejection (24), and in the tubular atrophy of chronic renal failure (28). Evidence for a role of TNFR1 or TNFR1-associated death domain protein (TRADD) in renal cell apoptosis is still preliminary (22). On the other hand,
activation of the initiator procaspase 9 is dependent on mitochondrial signaling pathways involving members of the Bcl-2 family (1). Both caspases 8 and 9 participate in a cascade that culminates in the activation of the effector caspase 3 (30). Caspase 3 cleaves several substrates, including poly (ADP) ribose polymerase (PARP), lamins and cytoskeletal proteins, with resultant chromosomal DNA fragmentation and cellular morphological changes characteristic of apoptosis (30).

In this study, we have modified a previously described protocol of in vitro albumin overload in cultured proximal tubule epithelial (LLC-PK1) cells (31, 34) to study the mechanisms underlying albumin-induced cell injury. Our results indicate that albumin induces apoptosis of proximal tubule cells, mediated at least in part by the Fas-FADD-caspase 8 pathway.

METHODS

Cell culture. LLC-PK1 cells, a well-characterized porcine proximal tubule cell line (American Type Culture Collection, Rockville, MD), were cultured in minimal essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (GIBCO, Gaithersburg, MD).

Albumin overload protocol. LLC-PK1 cells were seeded in plastic six-well tissue culture plates (Costar, Cambridge, MA). Confluent cells were washed with sterile PBS and incubated in α-MEM with BSA (Sigma, St. Louis, MO) at varying concentrations (0, 5, 10, or 20 mg/ml) for an additional 24, 48, or 72 h. This preparation of albumin has been shown to be essentially endotoxin free by the manufacturing company. For some experiments, delipidated endotoxin-free BSA (Sigma) was used instead, at the same concentrations. This range of albumin concentrations is similar to that used in previous studies (31, 34) and is representative of the range of albuminuria encountered in clinical practice. The maximum incubation period of 72 h was chosen because gross morphological changes suggestive of cell death (nonadherence of cells, appearance of gaps in the monolayer) begin to appear by this time. In separate experiments, cells were incubated with either bovine transferrin or bovine immunoglobulin G (both at 20 mg/ml for 72 h, both from Sigma) to determine whether the effects of albumin are specific or are shared by other proteins that may be encountered in the tubular lumen.

Albumin immunofluorescence assay. Albumin uptake by LLC-PK1 cells was documented by immunofluorescence using a monoclonal antibody to albumin (Sigma). Briefly, cells grown on coverslips to confluence were incubated with 0 or 20 mg/ml BSA for an additional 72 h, washed twice with cold 1 × PBS, fixed in 4% formaldehyde at room temperature for 30 min, and blocked with nonantigenic goat serum at room temperature for 60 min. After a 60-min incubation with primary antibody at room temperature, the cells were washed extensively with PBS and exposed to rhodamine-conjugated goat anti-mouse secondary antibody (GAM-Cy3, vector, Burlington, MA). Confluent cells were washed with sterile PBS and incubated with 0 or 20 mg/ml BSA for an additional 48 or 72 h, washed twice with cold 1 × PBS, and incubated with annexin V-FITC antibody for 15 min at room temperature in the dark. Cells were visualized by fluorescent microscopy with fluorescein filters as above. The double-stained cells were visualized with rhodamine filters to detect albumin uptake and with fluorescein filters to identify the fluorescent nuclei characteristic of apoptotic cells.

Because internucleosomal DNA cleavage may also be observed in necrotic cells, it was important to confirm the presence of apoptosis by additional assays (13, 22). The morphological changes of apoptosis, including cell shrinkage and nuclear condensation, were examined by direct light microscopy of cells stained with hematoxylin. In addition, we used the annexin V-FITC cell membrane-labeling assay (ApoAlert Annexin V Kit, Clontech). This method detects the translocation of phosphatidylserine from the inner face of the cell membrane to the outer surface, where it binds an annexin V-FITC conjugate and serves as an early marker of apoptosis (28). Briefly, cells grown on coverslips to confluence were incubated with 0 or 20 mg/ml BSA for an additional 48 or 72 h, washed twice with cold 1 × PBS, and incubated with annexin V-FITC antibody for 15 min at room temperature in the dark. Cells were visualized by fluorescent microscopy with fluorescein filters as above.

Other methods. To assess the role of caspases, we incubated LLC-PK1 cells with both albumin (20 mg/ml) and the specific caspase 8 inhibitor IETD-fmk (50 μM, Clontech) for 72 h and tested for DNA laddering. Furthermore, we detected caspase 8 activity by using a fluorescent assay kit (Clontech). For SDS-PAGE, both nonadherent and adherent cells were solubilized in 2 × sample buffer and boiled for 10 min. Monoclonal antibodies to Fas, FADD, TNFR1, and TRADD were from Transduction Laboratories (Lexington, KY), monoclonal antibody to tubulin was from Sigma, and the caspase 8 polyclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Immunodetection of transferred proteins was performed by using enhanced chemiluminescence (Amer sham, Arlington Heights, IL).

Statistics. Statistical analysis was performed using the SPSS statistical software (Version 9.0). Results are expressed as means ± SD of three to five separate experiments. Mann-Whitney tests were used for nonparametric distributions. P < 0.05 was considered significant.

RESULTS

Cultured proximal tubule cells uptake albumin. To document albumin uptake in an in vitro model of albu-
min overload, cultured LLC-PK₁ cells were incubated with BSA (0 or 20 mg/ml) for 72 h and immunostained with a monoclonal antibody to albumin. Fluorescence microscopy revealed intensely positive albumin staining in cells exposed to albumin overload (Fig. 1). In addition to a diffuse cytoplasmic staining, a distinct punctate distribution of albumin was also noted, consistent with endolysosomal staining. Others (4, 10) have reported similar findings. Cells incubated in medium lacking albumin showed only a barely detectable diffuse cytoplasmic staining.

**Albumin overload results in a dose-dependent apoptosis of LLC-PK₁ cells.** Cultured LLC-PK₁ cells were incubated with varying concentrations of BSA for 24, 48, or 72 h and subjected to several apoptosis assays. In multiple experiments, internucleosomal DNA fragmentation was clearly evidenced by the characteristic 180-bp laddering pattern in cells subjected to albumin overload (Fig. 2). The DNA laddering was not detected after 24 or 48 h of BSA but was clearly evident after 72 h of BSA at 20 mg/ml. These results indicate that the proapoptotic effects of BSA are duration dependent. We then examined the effect of varying concentrations of BSA, using the 72-h incubation period. The DNA laddering was just detectable in cells exposed to 5 mg/ml BSA but was easily evident in cells exposed to larger concentrations of BSA, indicating that the effect was also dose dependent. Because albumin-borne lipids have been shown to play a role in the development of proteinuria-associated renal damage in vivo (18), we examined in a separate set of experiments the effects of delipidated albumin on LLC-PK₁ cells. The results were similar to those observed with BSA. Internucleosomal DNA laddering was seen in cells incubated with delipidated BSA at 5 mg/ml for 72 h and was even more evident at higher concentrations of delipidated BSA (Fig. 2). These results indicate that albumin induces apoptosis of LLC-PK₁ cells in a dose- and duration-dependent manner, independent of any effect of lipids. Furthermore, because proximal tubules in vivo may also be exposed to other proteins such as IgG and transferrin (35), we examined the effects of incubating LLC-PK₁ cells with these proteins. At the same dose and duration (20 mg/ml for 72 h), neither transferrin nor IgG resulted in apoptosis as assayed by DNA laddering (not shown). Thus the proapoptotic effect of albumin was specific and not shared by other proteins.
that may be present in the tubular lumen in proteinuric states.

Several additional assays were utilized to confirm these new findings. LLC-PK1 cells incubated with 0 or 20 mg/ml BSA for 72 h were stained with hematoxylin and examined by light microscopy. Whereas control cells appeared healthy and in a confluent monolayer (A), whereas albumin-overloaded cells (20 mg/ml for 3 days) showed gaps in the monolayer, with the adjacent cells appearing shrunken with condensed nuclei (D). These changes are consistent with apoptosis. Furthermore, control cells were negative for annexin staining (B and C), whereas the plasma membranes of albumin-overloaded cells were intensely positive after 2 days (E), heralding the onset of apoptosis. After 3 days, annexin staining was noted throughout the cytosol (F), indicating a later phase of apoptosis. Bars, 10 μm.

Albumin-induced apoptosis is associated with upregulation of Fas and FADD. The Fas-FADD axis has been shown to be an important mechanism for the recognition and transduction of a variety of apoptotic stimuli in renal epithelial cells (13, 24, 25, 28). It was therefore of interest to determine whether this pathway was activated in albumin-induced apoptosis. LLC-PK1 cells incubated with varying concentrations of BSA were analyzed by SDS-PAGE and Western blotting with monoclonal antibodies to Fas and FADD. These proteins were detectable in control LLC-PK1 cells, but their expression was significantly and coordinately upregulated in a dose-dependent manner after incubation with BSA (Fas at 50 kDa and FADD at 25 kDa, Fig. 5). Densitometric analysis of multiple blots (five for Fas, four for FADD) showed that at lower concentrations of BSA (5 mg/ml), both proteins were overexpressed by ~2.5-fold (P < 0.05 vs. control), as shown in Fig. 6. At 20 mg/ml BSA, their expression was increased even further (by ~3.5-fold). The expression of TNFR1 at 55 kDa and TRADD at 35 kDa were unchanged at all concentrations of BSA (Fig. 5), indicating that the effect on Fas and FADD expression is specific and that the TNFR1-TRADD pathways are not upregulated by BSA in these cells.

Albumin-induced apoptosis is associated with the activation of caspase 8. Because albumin overload resulted in upregulation of the Fas-FADD axis, it was of...
interest to examine the activity of caspase 8, which is the most proximate initiator pro-caspase activated by signaling from death receptors (1, 30). LLC-PK1 cells incubated with varying concentrations of BSA were analyzed by SDS-PAGE and Western blotting with a polyclonal antibody to caspase 8, which detects only the inactive, parent form of the protein. As shown in Fig. 5, control cells display a strong reactivity to the antibody at 55 kDa, which represents the inactive pro-caspase 8. Cells incubated with albumin displayed a dose-dependent decrease in the pro-caspase immuno-reactivity, indicating a dose-dependent activation of caspase 8. These findings were corroborated by employing fluorescent assays that are specific for the detection of activated caspase 8, which revealed a significant increase in caspase 8 activity in a dose-dependent manner, as illustrated in Fig. 6. Analysis of these results from three separate fluorescent assays showed a 1.5-fold increase in caspase 8 activity at 5 mg/ml BSA ($P < 0.05$ vs. control) and an even greater (2.5-fold) increase at 10 or 20 mg/ml BSA. This increase in caspase 8 activity at 20 mg/ml BSA was completely abrogated by pretreatment of the cells with the specific caspase 8 inhibitor IETD-fmk at the dose of 50 μM (Fig. 6).

**Caspase 8 inhibitor ameliorates albumin-induced apoptosis.** Because our results indicated that albumin-induced apoptosis was mediated by the Fas-FADD-caspase 8 pathway, it was of significant interest to examine the effects of specific caspase 8 inhibition. Coincubation of cells with BSA (20 mg/ml) and the caspase 8 inhibitor IETD-fmk (50 μM) for 72 h was followed by the DNA laddering assay. As illustrated in Fig. 7, cells treated with caspase 8 inhibitor were remarkably protected from apoptotic internucleosomal cleavage. These results were consistent in three separate experiments and lend significant strength to our findings that albumin-induced apoptosis of LLC-PK1 cells involves up-regulation of the Fas-FADD-caspase 8 pathway.

**DISCUSSION**

The present study shows that albumin overload has a direct cytotoxic effect on cultured renal proximal
tubule cells. We have shown for the first time that incubation of LLC-PK₁ cells with either BSA or delipidated BSA results in a dose- and duration-dependent induction of apoptosis, as evidenced by morphological, biochemical, and molecular criteria. We have also demonstrated for the first time that albumin-induced apoptosis is associated with a dose-dependent overexpression of Fas and FADD and activation of caspase 8. Indeed, caspase 8 inhibitors markedly ameliorate this apoptosis. Collectively, our results point to a potential role for the Fas-FADD-caspase 8 pathway in albumin-induced apoptosis of LLC-PK₁ cells.

Proteinuria is the hallmark of glomerular injury, and the degree of proteinuria is an important predictor of both the severity and progression of a variety of kidney disorders (7, 27). In addition, proteinuria in itself has recently been recognized as a direct mediator of tubulointerstitial injury, independent of the cause of proteinuria (5, 6, 26). Excessive protein causes direct proximal tubule cell injury in animals (2, 11, 18) and humans (20) in proportion to the degree of proteinuria and has been implicated in the tubular atrophy characteristic of progressive renal insufficiency (28, 29). Albumin, the predominant protein in the glomerular filtrate, is taken up by proximal tubule cells via receptor-mediated endocytosis (4) and can induce the expression of several proinflammatory molecules such as monocyte chemoattractant protein-1 (12, 31), osteopontin (12), RANTES (34), and endothelin-1 (35). Although these important findings provide attractive hypotheses for the interstitial inflammation and fibrosis that characterize progressive renal diseases, the pathophysiological mechanisms underlying the proximal tubule cell death and tubular atrophy in albuminuric states remain unclear.

We hypothesized that apoptosis may represent one of the mechanisms underlying albumin-induced tubular cell injury. The hypothesis partly stems from recent observations of apoptotic tubular cells in an animal model of protein-overload proteinuria (29). In addition, apoptosis has now emerged as an important general mechanism by which renal tubule cells are injured, when subjected to a variety of stimuli at an intensity below the threshold for necrotic cell death (22). The list of such stimuli or disease states is rapidly expanding and particularly includes ischemia (13), nephrotoxins (21), obstruction (9), polycystic disease (32), and transplant rejection (24). Other inducers of renal tubule cell apoptosis in vitro include ATP depletion (13), serum starvation (33), steroids (14), and stimulatory Fas antibodies (13). To test whether albumin excess results in apoptosis, we modified a previously described protocol of in vitro albumin overload in LLC-PK₁ cells (31, 34). The cells were incubated with varying concentrations of albumin, based on previous studies and on the range of albuminuria encountered in clinical practice. Albumin uptake was documented by immunofluorescence microscopy with specific monoclonal anti-albumin antibody. Albumin in these proximal tubule cells predominantly localizes in a punctate distribution, reminiscent of endolysosomal staining, as has been previously reported (4, 10).

Albumin overload caused a dose- and duration-dependent induction of apoptosis in cultured LLC-PK₁ cells, as evidenced by internucleosomal DNA fragmentation (DNA laddering and TUNEL assay), morphological changes (cell shrinkage, nuclear condensation), and plasma membrane alterations (externalization of phosphatidylserine detected by annexin V staining). Multiple complementary assays were used because DNA fragmentation may also be evident in cells undergoing necrosis (13, 22). Colocalization studies were performed to demonstrate that the cells undergoing apoptosis were also positive for albumin staining. Because albumin-borne fatty acids may contribute to proteinuria-associated renal damage in vivo (18), we used delipidated albumin to confirm that its proapoptotic activity was independent of lipids. Furthermore, we showed that the proapoptotic effect of albumin was specific and not shared by other proteins that may be present in the tubular lumen in proteinuric states, such as IgG and transferrin (35).

Even the earliest and most subtle signs of apoptosis (plasma membrane staining with annexin) became apparent only after prolonged (48 h) incubation with a high dose (20 mg/dl) of albumin. Indeed, the typical morphological and DNA changes of apoptosis became evident only after 72 h of incubation with high-dose albumin. Our prolonged time-dependent studies are in contrast to earlier reports, in which albumin incubation for 8 h was devoid of toxic effects in cultured proximal tubule cells (8). Our results are also in contrast to previous elegant studies demonstrating that albumin is a survival factor in a primary culture of mouse proximal tubule cells (16). However, it should be emphasized that those studies utilized only the lowest doses of delipidated BSA that were used in our experiments (5 mg/ml). Also, the absence of apoptosis was inferred purely on morphological appearance, without other apoptosis assays (such as DNA laddering and annexin assays) that may be more sensitive. Collectively, our results along with these previous studies suggest that albumin in low or “physiological” doses may indeed constitute a major antiapoptotic survival factor. In contrast, prolonged exposure to high- or “nephrotic-range” doses can result in proximal tubule cell apoptosis.

Although the presence of apoptotic tubular cells has been previously noted (by demonstration of nuclear fragmentation) in rats subjected to albumin overload (29), the proximal pathways involved in the stimulus recognition, signal transduction, and effector phases of albumin-induced apoptosis are unknown. We examined the role of the Fas-FADD pathway, because it has recently been implicated in the renal tubule cell apoptosis after endotoxin treatment (25), ATP depletion (13), transplant rejection (24), and in the tubular atrophy of chronic renal failure (28). Our results indicate that albumin overload results in a significant and coordinate upregulation of Fas and FADD in a dose-dependent manner. Because the Fas-FADD pathway
results in activation of the pro-caspase 8, we sought evidence for this effector phase event. We documented a dose-dependent activation of caspase 8 in response to albumin overload by using complementary techniques including Western analysis for reduction in pro-caspase 8 protein abundance and specific fluorescent assays for caspase 8 activity. Additional direct evidence for the role of caspase 8 was obtained using the specific caspase 8 inhibitor IETD-fmk, which significantly ameliorated apoptosis when co-cubated with albumin. Caspase inhibitors have also been shown to protect cultured renal tubule cells from ischemic injury (13, 17). Thus our results lend support to the notion that inhibition of apoptosis may offer a novel approach to cytoprotection of renal tubule cells from a variety of stimuli.

In summary, we have shown that albumin overload induces apoptosis in LLC-PK1 cells, mediated at least in part by the Fas-FADD-caspase 8 pathway. We speculate that renal tubule cell apoptosis in the analogous in vivo scenario in proteinuric states may play a direct pathogenetic role in the initial tubule cell death. We favor the notion that apoptosis in these situations is a “packaging” mechanism, allowing for cells sublethally injured by albumin to be removed by neighboring cells in a Fas-dependent manner (28). Alternatively, the apoptotic cells may be phagocytosed by professional inflammatory cells recruited by cytokines such as monocyte chemoattractant protein-1 and RANTES (12, 31, 34). If proteinuria is self-limited, it is conceivable that cell proliferation (28) and tubular regeneration will prevail over apoptosis, leading to the return of normal structure and function. On the other hand, we speculate that chronic severe proteinuria will tilt the balance toward ongoing cell death, leading to progressive tubular atrophy. It will be important in future studies to confirm the role of the Fas-FADD-caspase 8 pathways in the albumin-induced tubule cell injury in vivo. It will also be of significant interest to examine the role of mitochondrial pathways (such as the Bcl-2 family and cytochrome C) in the programmed cell death induced by albumin. Elucidation of such stimulatory and inhibitory pathways may lead to a better understanding of the mechanisms by which proteinuria by itself contributes to tubular cell atrophy and may reveal clues for the rational design of novel therapeutic interventions.

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