Lysosomal Cystine Storage Augments Apoptosis in Cultured Human Fibroblasts and Renal Tubular Epithelial Cells

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Abstract. Nephropathic cystinosis is a lethal disorder of lysosomal cystine storage due to defective lysosomal cystine transport. How lysosomal cystine causes this multisystemic disorder culminating in end-stage renal disease is not known, because the cystine is isolated from cellular metabolism by the lysosomal membrane. It is here reported that in both normal and nephropathic cystinotic fibroblasts and cultured renal proximal tubule epithelial cells, increased lysosomal cystine causes an increased rate of apoptosis. In nephropathic cystinotic fibroblasts, the rate of apoptosis is 14.8% after exposure to TNF-α versus 7.8% in control normal fibroblasts. Anti-Fas antibodies and UV exposure induced apoptosis in 18.1% and 17.4% of nephropathic cystinotic fibroblasts, respectively, versus 5.2% and 7.1% in normal fibroblasts when analyzed by CaspACE (P < 0.05). Similar results were found when the cells were analyzed by TdT-mediated dUTP nick end labeling (TUNEL). When the cystine content of normal fibroblasts is increased by exposure to cystine dimethylester (CDME), the apoptotic rate is increased to the rate seen in nephropathic cystinotic cells.

Nephropathic cystinosis results from failure of expression of CTNS, located at 17p13, which codes for cystinosin, a 367-amino acid peptide that functions to transport cystine from lysosomes. It is the only lysosomal amino acid transporter yet cloned. The cDNA has 12 exons and is 2.6 kb in length (1). Nephropathic cystinosis has as its major pathophysiologic effects the progressive loss of renal function beginning with the renal Fanconi syndrome at less than 1 yr of age, followed by the onset of glomerular failure after approximately age 7 yr, and reaching ESRD by 10 yr of age. The children are stunted, rarely achieving a height greater than the 50th percentile for a 3-yr-old in the untreated state, and they develop hypothyroidism between the ages of 8 and 10 yr. Renal rickets may also occur secondary to phosphaturia. They display a pathognomonic salt and pepper retinopathy and corneal crystals, which lead to photophobia, corneal ulcerations, and severe debility due to pain as well as impaired vision. Diabetes, esophageal dysmotility, and myopathy may be late complications (2).

The proximate cause of cell death in the cystinotic phenotype is not known, nor is it readily inferred, because the cystine is isolated within lysosomes. The only apparent mode of egress for cystine from lysosomes lacking a functional cystine transporter is exocytosis. After exocytosis, the lysosomal cystine would be deposited at the external face of the plasma membrane, where the amino acid transporter (X̌ gc) is available to transport cystine directly back into the cytosol. There it would be reduced to cysteine, with the concomitant oxidation of GSH to GSSG. NADPH-GSSG reductase functions to reduce GSSG back to GSH, effectively completing the transformation of lysosomal cystine to cytosolic cysteine (3). The cysteine so generated is then available for protein and GSH synthesis (4). Nothing in this sequence suggests a disadvantage for cystinotic cells nor offers an explanation for the lethality of this phenotype.

Recent studies on apoptosis have implicated lysosomes as participants in the critically important process of programmed cell death (5). Lysosomal participation in apoptosis has been documented in a number of studies, but the extent of involvement is still being determined (6,7). It is clear that permeabilization of the lysosomal membrane occurs in this process and...
that cathepsins B and D are released. Movement of cathepsins from granular to cytosolic locations during apoptosis has been found in fibroblasts undergoing an increase in oxidative stress. Induction of apoptosis in macrophage-like cells occurs upon exposure to a lysosomotopic detergent, which causes graded lysosomal leakage (8). Similar effects have been seen after photo-oxidation of lysosomes, leading to increased membrane permeability and resulting in TdT-mediated dUTP nick end labeling (TUNEL) positivity of the nucleus, an accepted measure of apoptosis (9). We here report that lysosomal cystine loading alone causes increased apoptosis in cultured RPTE cells and also causes increased apoptosis in cystinotic and normal fibroblasts after standard apoptotic stimuli. Two fibroblast lines derived from variant forms of cystinosis, intermediate, in which renal death occurs in the teens or twenties, and ocular, in which no renal involvement occurs (2), do not show an increased apoptotic response, even though the lysosomal cystine content is in the same range as in the nephropathic cells.

**Materials and Methods**

**Cell Culture**

Normal and cystinotic fibroblasts were purchased from The Coriell Mutant Cell Repository, and cultured in Coon modification of Ham F12 medium, supplemented with 10% fetal calf serum (FCS). Renal proximal tubule epithelial (RPTE) cells were purchased from Bio-whittaker, cultured in renal epithelial basal medium supplemented with one Singlequots kit per 500 ml to make renal epithelial growth media (REGM, Biowhittaker). Fibroblasts and RPTE were maintained in a 5% carbon dioxide, 95% air, humidified incubator at 37°C (4).

**Induction of apoptosis and assays for its detection** were performed using commercially available reagents. Normal and cystinotic fibroblasts were matched for passage number (± 3 passages) and cell density, and then exposed to one of three apoptotic triggers: TNF-α (2 ng/ml) with actinomycin D (2.5 μg/mL) for 16 h; anti-Fas antibody (500 ng/ml) with actinomycin D (2.5 μg/mL) for 16 h; or UVB light (60 mJ) (10–13). After exposure, the cells were maintained in Coon modification of Ham F12 medium for 16 h before analysis. The cells were then assayed for apoptosis. Serum withdrawal was also used as an apoptotic stimulus (14–17), in which case the cells were incubated

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**Table 1. Genotype and cystine content of cell lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Genotype</th>
<th>Cystine Content (nmol cystine/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM00008</td>
<td>46XX, 65-kb del</td>
<td>7.5</td>
</tr>
<tr>
<td>GM00760</td>
<td>46XY, 753 G→A, premature stop</td>
<td>0.78</td>
</tr>
<tr>
<td>GM00046</td>
<td>46XY, 5-bp del, frameshift</td>
<td>1.51</td>
</tr>
<tr>
<td>GM08761</td>
<td>46XX, not determined</td>
<td>6.29</td>
</tr>
<tr>
<td>GM00379</td>
<td>IVS11+2 T→C</td>
<td>15.7</td>
</tr>
<tr>
<td>GM00010</td>
<td>46XY, apparently normal</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GM05399</td>
<td>46XY, apparently normal</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>RPTE</td>
<td>46XY, apparently normal</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The cystine content was measured by a cystine binding protein assay as described in Materials and Methods. RPTE, renal proximal tubule epithelial cells.*

**Table 2. The apoptosis rate in cystinotic nephropathic, variant, and normal fibroblasts**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Phenotype</th>
<th>% Apoptosis</th>
<th>TNF-α</th>
<th>Anti-Fas</th>
<th>UV</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM008</td>
<td>Nephropathic</td>
<td>14.9±0.6</td>
<td>17.7±2.9</td>
<td>12.8±4.8</td>
<td>2.2±0.2</td>
<td></td>
</tr>
<tr>
<td>GM760</td>
<td>Nephropathic</td>
<td>13.5±4.3</td>
<td>14.4±2.7</td>
<td>26.3±5.4</td>
<td>3.1±0.7</td>
<td></td>
</tr>
<tr>
<td>GM046</td>
<td>Nephropathic</td>
<td>16.1±4.1</td>
<td>22.3±2.9</td>
<td>13.1±2.0</td>
<td>2.2±0.6</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>14.8</td>
<td>18.1</td>
<td>17.4</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>GM08761</td>
<td>Ocular</td>
<td>8.2±0.98</td>
<td>5.9±1.9</td>
<td>6.4±2.0</td>
<td>2.3±0.3</td>
<td></td>
</tr>
<tr>
<td>GM00379</td>
<td>Intermediate</td>
<td>11.4±2.0</td>
<td>7.7±1.0</td>
<td>8.4±0.94</td>
<td>3.1±0.2</td>
<td></td>
</tr>
<tr>
<td>GM010</td>
<td>Normal</td>
<td>9.2±1.9</td>
<td>6.5±2.3</td>
<td>7.0±0.6</td>
<td>2.8±0.2</td>
<td></td>
</tr>
<tr>
<td>GM05399</td>
<td>Normal</td>
<td>6.3±1.9</td>
<td>4.9±0.8</td>
<td>7.2±1.0</td>
<td>1.9±0.1</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>7.8</td>
<td>5.2</td>
<td>7.1</td>
<td>2.4</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were treated with apoptosis inducers as indicated, stained with CaspACE, and analyzed by fluorescence microscopy. A minimum of 250 cells were scored per condition, and this was done in triplicate, thus at least 750 cells were scored per condition. T-statistics: P < 0.001 for average nephropathic versus average normal; P < 0.05 for ocular versus nephropathic; P > 0.05 for ocular versus normal, intermediate versus nephropathic, and intermediate versus normal.*
Table 3. The apoptosis rate in normal and cystinotic fibroblasts

<table>
<thead>
<tr>
<th>Cell</th>
<th>Phenotype</th>
<th>TNF-alpha</th>
<th>Anti-Fas</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM00008</td>
<td>Nephropathic</td>
<td>17.1±2.2</td>
<td>12.3±1.9*</td>
<td>13.7±3.0*</td>
</tr>
<tr>
<td>GM00760</td>
<td>Nephropathic</td>
<td>19.3±3.4</td>
<td>17.7±3.3</td>
<td>21.6±5.8</td>
</tr>
<tr>
<td>GM00046</td>
<td>Nephropathic</td>
<td>15.3±1.1*</td>
<td>15.9±3.2</td>
<td>19.2±2.5</td>
</tr>
<tr>
<td>Average</td>
<td>Nephropathic</td>
<td>17.2</td>
<td>15.3</td>
<td>18.2</td>
</tr>
<tr>
<td>GM08761</td>
<td>Ocular</td>
<td>5.5±0.8</td>
<td>7.1±0.8</td>
<td>6.8±1.9</td>
</tr>
<tr>
<td>GM00379</td>
<td>Intermediate</td>
<td>8.2±1.0</td>
<td>8.5±3.0</td>
<td>8.5±3.0</td>
</tr>
<tr>
<td>GM00010</td>
<td>Normal</td>
<td>12.8±2.4</td>
<td>9.6±2.1</td>
<td>11.9±1.6</td>
</tr>
<tr>
<td>GM05399</td>
<td>Normal</td>
<td>11.4±1.9</td>
<td>10.5±2.6</td>
<td>7.6±0.8</td>
</tr>
<tr>
<td>Average</td>
<td>Normal</td>
<td>12.1</td>
<td>10.1</td>
<td>9.8</td>
</tr>
</tbody>
</table>

* Cells were treated with apoptosis inducers as indicated, stained with TUNEL, and analyzed by fluorescence microscopy. A minimum of 250 cells were scored per condition, in triplicate. P < 0.05 between all pairs of nephropathic cystinotic cells and the intermediate line, and both normal lines except for those indicated by *, which were not significantly different from either normal line. P > 0.05 for ocular versus normal and intermediate; P = 0.01 for ocular versus nephropathic.

Figure 1. The effect of MEA or cystine dimethylester (CDME) on apoptosis in semi-confluent nephropathic cystinotic or normal fibroblasts. Nephropathic cystinotic fibroblasts (passage 7–13) were treated with cysteamine 1 h before exposure to apoptotic triggers, depleting them to a normal lysosomal cystine content (0.01 to 0.13 nmol of cystine per mg of protein). Normal fibroblasts also at passage 7–13 were pretreated with 0.5 mM CDME for 1 h before apoptotic triggers, loading their lysosomes with cystine to the cystinotic range (0.47 to 1.95 nmol of cystine per mg of protein). They were then treated with apoptotic stimuli and incubated for 16 h while in cystine-free or CDME-containing medium. Apoptosis was assayed by CaspACE, and scored as before.

in F₁₂ medium without serum for 24 h and then analyzed for apoptosis as described. Three commercially available apoptosis assays were employed. CaspACE (Promega) is an FITC-conjugated cell-permeable form of the pan-caspase inhibitor zVAD-Fmk, which binds to activated caspases. Cells were incubated in FITC-VAD-Fmk–containing medium (10 μM for 30 min at 37°C), washed, and then fixed in 10% buffered formalin (30 min at room temperature) before analysis. TUNEL employs terminal deoxynucleotidyl transferase (TdT) to label the ends of double-stranded DNA breaks, which occur in apoptotic cells, with FITC-conjugated dUTP. Cells were fixed in 4% buffered formalin, washed, incubated in permeabilization solution (0.1% Triton X-100 in 0.1% Na Citrate) for 2 min on ice and then stained with 25 μl of TdT/45 μl of labeled dNTP mix for 40 min at 37°C. Annexin V-propidium iodide (PI) staining was performed as described (5 and Annexin V Fluos kit directions, Roche Laboratories). The cells were visually enumerated by fluorescence microscopy, with a minimum of five fields (250 to 300 cells) for fluorescence in triplicate followed by counting all cells in the same field by light microscopy, with a minimum of 750 cells scored per condition. The apoptosis rate is the total number of cells that fluoresce divided by the total cells in the field.

Lysosomal cystine depletion of cystinotic fibroblasts was accomplished by treatment with 1 mM cysteamine-HCl (4) (Sigma) in Ham F₁₂ medium lacking cystine (Life Technologies), with 10% FCS, for 1 h followed by exposure to the apoptotic triggers in cystine-free medium to inhibit cystine re-accumulation.

Normal fibroblast lysosomes were loaded with cystine by the addition of 0.5 mM cystine dimethylester (CDME, Sigma) to normal culture medium for 1 h before treatment (18). The cells were treated with apoptotic triggers as described above, leaving CDME in the medium to prevent lysosomal cystine loss, and then analyzed for apoptosis as described. Lysosomal cystine loading of RPTE cells was accomplished by exposure to 0.1 mM or 0.25 CDME in normal REGM for 1 h before treatment with or without apoptotic triggers.

Cystine Binding Protein Assay

Intracellular lysosomal free cystine was determined using a cystine binding protein (CBP) assay as described (19). CBP was procured from Riverside Scientific. The assay has a sensitivity of 0.1 μM.

Total cell protein was determined by a modification of the Lowry method (20). Statistical analyses were performed using paired t test for means with SSPS for Windows. Results are mean ± 1.0 SD; error bars are ± 1.0 SD (Figure 5).
Immunohistochemistry of Normal and Cystinotic Fibroblasts

Cells were stained with Lysotracker Red (Molecular Probes) for 5 min at room temperature, washed twice in PBS, and then fixed in 4% formalin (1 h). Slides were immersed in blocking buffer (PBS, pH 7.2, 0.5% Carnation dry milk, 0.1% Triton X-100) for 20 min at room temperature, washed twice in PBS, and then incubated with primary antibody (anti-cathepsin B, Santa Cruz Biotechnologies) diluted 1:100 for 2 h at room temperature, followed by incubation with secondary antibody (FITC-conjugated rabbit anti-goat, Santa Cruz Biotechnologies), at 4°C overnight. Slides were washed twice with PBS and then sealed with coverslips and viewed using a Leica DMRX deconvoluting microscope.

Results

The cell lines studied, their genotypes, and nominal cystine content are listed in Table 1. The mutations in cell lines GM00008, GM00760, and GM00046 cause typically severe nephropathic cystinosis with ESRD by 10 yr of age. The cystine content shown in the cystinotic lines in Table 1 varies between 0.8 and 15.7 nmol/mg protein, which is that typically seen in cultured cystinotic fibroblasts (2).

Apoptosis induced in three nephropathic cystinotic, two normal, one intermediate cystinotic, and one ocular cystinotic fibroblast line by three separate inducers is shown in Tables 2 and 3. In Table 2, apoptosis in these lines was assessed by CaspACE. The cystinotic cells on average display about 2 to 3 times the apoptotic rate for the three apoptotic triggers compared with the normal cell lines. The cystinosis variant lines do not show increased apoptosis under these conditions. The differences are statistically significant at \( P < 0.05 \) between the averages for cystinotic and normal lines and between nephropathic and ocular cystinotic lines. There is no significant difference in the rate of apoptosis between intermediate and nephropathic lines, ocular cells and normal cells, or intermediate versus normal cells (Table 2). Similar results were obtained when the cells were analyzed by TUNEL, except that...
line GM00008 did not differ significantly from the normal lines' response after anti-Fas or UV exposure, nor did line GM00046 after TNF-α exposure (Table 3). Again, the variant lines did not show augmented apoptosis compared with the normal fibroblast lines.

Modulation of the apoptotic response by altering the lysosomal cystine content of normal or nephropathic cystinotic fibroblasts is shown in Figure 1. The bar graphs show the apoptosis rates induced by exposure of two cystinotic and two normal cell line cells to TNF-α or UV radiation before and after correction of the cystine content of nephropathic fibroblasts to normal levels with MEA and before and after increasing the cystine content of normal fibroblasts to cystinotic levels by pre-incubation with CDME. The mean rate of apoptosis for the cystinotic cells before cystine depletion was 15.6 ± 2.7%; after cystine depletion by exposure to MEA, it fell to 6.1 ± 2.8%. This difference is significant at P < 0.001. The average control apoptotic rate for the normal fibroblast lines was 7.2 ± 1.3%, which rose to 18.7 ± 5.4% after exposure to CDME (P < 0.001). The effect holds whether induction was by TNF-α or UV light. Representative fluorescence micrographs displaying this effect after UV exposure are shown in Figure 2. The high rate of apoptosis in cystinotic fibroblasts with initial cystine content of 4.0 nmol/mg protein is shown in Figure 2A. This fell after treatment with cysteamine, which lowered the cystine content to < 0.1 nmol/mg protein (Figure 2B). The normal rate of apoptosis in normal fibroblasts (cystine content <0.1 nmol/mg protein) is shown in Figure 2C, and this decreases to a rate similar to that seen in cystinotic fibroblasts after pre-exposure of the cells to CDME (Figure 2D), which increased the cystine content to 1.95 nmol/mg protein. Representative phase micrographs of fibroblasts treated with TNF-α, showing characteristic apoptotic morphology, and the effect of lysosomal cystine on apoptosis are shown in Figure 3, in which
Figure 4. Loss of colocalization of cathepsin B in normal and nephropathic cystinotic fibroblasts after TNF-α exposure. Fibroblasts were maintained under normal culture conditions, exposed to TNF-α, loaded with Lysotracker red, fixed, and stained for cathespin B using an anti-cathepsin B antibody as described in Materials and Methods. Panels a through f are normal fibroblasts; a through c are control cells; d through f are treated with TNF-α. Panels g through l are cystinotic fibroblasts; g through i are control; j through l are treated with TNF-α. Note loss of granularity and decreased colocalization in c versus f and i versus l. Photographs via a deconvoluting microscope.
CDME and analysis by Annexin V and propidium iodide (PI) RPTE cells to TNF- 

The RPTE cystine content after exposure to 0.25 mM CDME was 0.6 nmol/mg protein. After 1-h incubation, the cystine content was 2.2 nmol/mg protein.

Each experiment was done in triplicate. The control (time 0) cystine content was equal to that induced by exposure to TNF- 

To substantiate that lysosomes are permeabilized by TNF-α (5) under these conditions, cathepsin B (a lysosomal cysteine protease) was localized by immunohistochemistry in normal and nephropathic cystinotic cells before and after induction of apoptosis. Lysosomes were identified with Lysotracker red dye. In both normal and cystinotic fibroblasts before TNF-α treatment, cathepsin B (green) displays a punctuate pattern that is closely associated with the red lysosomal dye. Induction of apoptosis by TNF-α causes a translocation of cathepsin B from a lysosomal location to a diffuse cytosolic location, with loss of colocalization of color (Figure 4).

Cultured human renal proximal tubule epithelial cells display marked sensitivity to apoptosis after CDME exposure. The concentration employed in fibroblasts (0.5 mM) was toxic to these cells and was decreased to 0.1 or 0.25 mM for the RPTE experiments. The rate of apoptosis produced by 0.25 mM CDME alone was equal to that induced by exposure to TNF-α alone (Figure 5). The time course was accelerated, with these cells attaining a maximum rate of apoptosis within 6 h, as opposed to 17 h in fibroblasts followed by lysis and release from the culture dish. The RPTE cystine content after exposure to 0.25 mM CDME for 1 h was 1.99 nmol/mg protein. Representative fluorescence micrographs at each time point are shown in Figure 6. Exposure of RPTE cells to TNF-α after cystine loading by exposure to 0.1 mM CDME and analysis by Annexin V and propidium iodide (PI) staining yielded 4.3% Annexin V–positive cells and zero PI-positive cells at 1 h after exposure, 9.5% and 1.7% at 3 h, and 25.3% and 9.1% at 6 h. The proportion of necrotic (dead) RPTE cells increases with duration of exposure to CDME, but remains substantially less than the number of those that are apoptotic.

**Discussion**

Four apoptotic triggers (TNF-α, Anti-Fas, UV light, and serum withdrawal) and three standard measures of the apoptotic response (TUNEL, CaspAse activation, and Annexin V) were employed to ascertain the effect of lysosomal cystine on apoptosis in cultured cells. RPTE cells display increased rates of TUNEL reactivity after lysosomal cystine loading without exposure to apoptotic triggers (Figures 5 and 6). CDME is known to traverse both plasma and lysosomal membranes, but it is only concentrated within the lysosomes, where resident hydrolases remove the methylester groups, producing free cystine. The acidic environment produces a net positive charge, inhibiting exit of cystine via the lysosomal cystine transporter cystinosin (18,21). We speculate that under normal circumstances in normal cells, the influx of cystine into lysosomes via proteolysis of disulfide-containing proteins (22,23) proceeds at a rate less than the normal egress rate of cystine via cystinosin. This would yield a small and relatively constant rate of release of cystine into the cytosol, which could be readily reduced to cysteine by glutathione. In cystinotic fibroblasts, which lack functional cystinosin, cystine accumulates to very high levels (2). Apoptotic stimuli lead to lysosomal permeabilization and translocation of cathepsins B and D into the cytosol (5–9) (Figure 4); it is therefore possible that rapid release of cystine into the cytosol occurs during this permeabilization phase and results in a locally altered redox potential, and/or reaction with critical protein thiols, that may sensitize the cells to, or trigger, the apoptotic response (24). In contrast, extracellular cystine deprivation is pro-apoptotic (25–27), and cells can be rescued from apoptosis by extracellular cystine supplementation via the ectomembrane enzyme, glutathione tranpeptidase, which is localized to the plasma membrane and which functions in the synthesis of reduced glutathione (28). Accessibility of extracellular cystine to this plasma membrane complex leads to enhanced GSH synthesis, which may not be the case in cystine originating from lysosomes.

Apoptosis is known to play a role in the renal tubular dysfunction seen in transplantation rejection phenomenon and also in certain forms of retinopathy. The renal tubule and retina are highly sensitive to apoptosis (29,30). These two tissues are the first to be affected in nephropathic cystinosis (2), and it is feasible that the order of tissues involved in the disease reflects the intrinsic sensitivity of each to apoptosis. CDME is known to disrupt renal tubule cell function in animal models and cultured cells (31–33), and it could be that exposure to CDME alone alters the redox potential sufficiently to trigger the apoptotic response (Figures 5 and 6). CDME also causes inhibition of Na⁺–dependent transporters (33). Free cystine has been known to be nephrotoxic in experimental animals since 1925 (34); however, the mechanism remains to be determined. Cystinosis patients display a typical swan neck deformity of typical blebs in the plasma membrane are seen after treatment with TNF-α in both normal and cystinotic cell lines and the modulating effects of MEA and CDME on apoptosis are seen. Serum withdrawal in cultured fibroblasts caused less apoptosis above baseline than the other stimuli employed; however, increasing lysosomal cystine, either naturally due to defective CTNS function or artificially due to CDME loading, again resulted in an increased apoptotic rate (Table 4).
the proximal tubule (35,36), concomitant with development of the Fanconi syndrome and consistent with hypocellularity of that structure.

Enhanced sensitivity to apoptosis due to increased lysosomal cystine loading offers another insight into the interplay of lysosomes and apoptosis as well a new perspective in understanding the pathophysiology of cystinosis. Increased lysosomal cystine in fibroblasts or RPTE cells either as the result of cystinosin deficiency, or induced by incubating normal cells in cystine dimethylester, causes increased apoptosis. This finding is important because it excludes other effects of defective CTNS expression and alternative effects of CDME exposure, because the only common element in the two conditions is elevated lysosomal cystine. Enhanced sensitivity to apoptosis is observed after triggers that stimulate both the intrinsic apoptotic pathway (UV light) and the extrinsic pathway (TNF-α, anti-Fas antibody, or serum withdrawal). The intrinsic and extrinsic pathways converge at the BID/Bcl2 locus and then ultimately activate caspases (37). It is somewhat counterintuitive that lysosomal cystine release increases apoptosis, because the executioner caspases are thiol proteases, which should be inhibited by the disulfide cystine.

Inappropriate apoptotic cell death may occur in cystinotic tissues after extrinsic or intrinsic apoptotic stimuli are presented, which would fail to be executed in normal tissues due to damping of the apoptotic cascade. This aberrant sensitivity could account for the cystinotic phenotype by causing inappropriate cell death throughout embryogenesis and the life of the individual, leading to a hypocellular state in many tissues. The mechanisms causing this augmented rate of cell death remain to be determined. It is possible that the cells may proceed to necrosis in some cases under these circumstances, as suggested by the increase in PI positivity seen in RPTE cells treated with both TNF-α (38) and CDME.

Patients with variant forms of cystinosis are either heterozygous for CTNS mutations, which, in the homozygous state,
cause the nephropathic form of the disease, or they are homozygous for predicted milder mutations (39,40). The leukocyte cystine content overlaps among the various clinical forms of cystinosis, ranging from 1.3 to 11.6 nmol/mg protein in nephropathic, 1.7 to 2.5 in the intermediate form, and between 0.5 and 1.8 in the ocular form. A similar overlap is shown in cultured fibroblasts in Table 1. Distinction among the varieties is based on the age at onset of symptoms and clinical severity (2,39–41).

Two variant cystinotic cell lines did not show enhanced rates of apoptosis despite increased lysosomal cystine (Tables 2 and 3). The combination of a severe nonsense mutation and a splice-site mutation allows for some residual cystine transport activity and, because the splice site mutation in cell line GM00379 causes truncation of cystinosin just distal to the second lysosomal recognition site, permits some lysosomal localization of cystinosin and cystine transport activity (39,42). We speculate however, that these phenotypic cystinosis variants are not due to residual cystinosin activity, as the lysosomal cystine content overlaps that of the nephropathic form; rather, they are due to linkage or to co-segregation of a gene or genes, which confers relative resistance to apoptosis. The cystinosis mutation itself is rare; if a rare allele is also responsible for resistance to apoptosis, then the extreme scarcity of these variants is explained, as is the milder phenotype. The phenotype could also be caused by diminished expression of a pro-apoptotic gene such as APC, as has recently been described in familial adenomatous polyposis (43). This hypothesis raises the possibility of increased neoplastic transformation in these patients, however none has yet been recognized.

We conclude that nephropathic cystinotic fibroblasts enter the pathway for programmed cell death more readily than do normal fibroblasts and that normal fibroblasts and RPTE cells display augmented entry into that pathway after their lysosomal cystine content is artificially increased. Study of the pathway(s) by which cellular perturbations result in modulation of the apoptotic cascade may lead to a clearer understanding of the regulation of apoptosis, and a better understanding of the development of the cystinotic phenotype.

Acknowledgments
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