Inhibition of Intracellular Clusterin Attenuates Cell Death in Nephropathic Cystinosis

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ABSTRACT

Nephropathic cystinosis, characterized by accumulation of cystine in the lysosomes, is caused by mutations in CTNS. The molecular and cellular mechanisms underlying proximal tubular dysfunction and progressive renal failure in nephropathic cystinosis are largely unclear, and increasing evidence supports the notion that cystine accumulation alone is not responsible for the end organ injury in cystinosis. We previously identified clusterin as potentially involved in nephropathic cystinosis. Here, we studied the expression of clusterin in renal proximal tubular epithelial cells obtained from patients with nephropathic cystinosis. The cytoprotective secretory form of clusterin, as evaluated by Western blot analysis, was low or absent in cystinosis cells compared with normal primary cells. Confocal microscopy revealed elevated levels of intracellular clusterin in cystinosis cells. Clusterin in cystinosis cells localized to the nucleus and cytoplasm and showed a filamentous and punctate aggresome-like pattern compared with diffuse cytoplasmic staining in normal cells. In kidney biopsy samples from patients with nephropathic cystinosis, clusterin protein expression was mainly limited to the proximal tubular cells. Furthermore, expression of clusterin overlapped with the expression of apoptotic proteins (apoptosis-inducing factor and cleaved caspase-3) and autophagy proteins (LC3 II and p62). Silencing of the clusterin gene resulted in a significant increase in cell viability and attenuation of apoptosis in cystinosis cells. Results of this study identify clusterin as a pivotal factor in the cell injury mechanism of nephropathic cystinosis and provide evidence linking cellular stress and injury to Fanconi syndrome and progressive renal injury in nephropathic cystinosis.

The pathogenesis of nephropathic cystinosis, the most common cause of inherited renal Fanconi syndrome, is still poorly understood. Nephropathic cystinosis, characterized by elevated levels of intracellular cystine, is an autosomal recessive disorder caused by mutations in the CTNS gene,1,2 which encodes for cystinosin, a lysosomal cystine carrier. Patients with cystinosis are usually asymptomatic at birth and develop normally during the first 6 months of life, when they often present with failure to thrive, polyuria, excessive thirst, dehydration, and sometimes, rickets.3 These symptoms result from Fanconi syndrome as a consequence of defective renal proximal tubular reabsorption.4 Progressive renal damage leads to chronic renal failure in the patients, resulting in ESRD in the first decade. Cystine depletion therapy and RRT have prolonged the life expectancy of patients but also revealed other extrarenal complications.4 Various reports have indicated the association of processes, such as ATP depletion, mitochondrial dysfunction, autophagy, apoptosis, and cellular stress, with proximal tubule damage in the cystinotic kidney.5–12 Although cystine accumulation is

Received June 3, 2013. Accepted June 10, 2014.

Published online ahead of print. Publication date available at www.jasn.org.

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regarded as the primary defect in cystinosis, increasing evidence indicates that cystine accumulation alone may not be responsible (or may be insufficient) for metabolic alterations in cystinosis. The idea is supported by the findings that renal Fanconi syndrome is not cured after cystine depletion in patients with cystinosis and that, despite high levels of renal cystine accumulation in ctns−/− mice, renal Fanconi syndrome is absent in these mice.13 Although recent observations indicate the involvement of other pathways in addition to the impairment of lysosomal transmembrane cystine transport, the exact mechanisms underlying the renal tubular dysfunction and cell injury leading to disease progress are unknown. Therefore, a major challenge for research in this domain is the discovery of new pathophysiologic and/or diagnostic targets. Our previously published study using high-throughput cDNA microarrays to assay human blood samples from patients with and without nephropathic cystinosis identified various cell death molecules.11 Analyzing the gene expression pattern suggested clusterin (CLU) as a potential target for additional investigation for the pathophysiology of renal injury in nephropathic cystinosis.

CLU (apolipoprotein J) has been found to be differentially expressed and nearly ubiquitous in tissues and body fluids. CLU belongs to a family of abundant extracellular chaperones.14,15 CLU has multiple functions related to apoptosis, oxidative stress, renal injury, clearance of cellular debris, lipid transport, stabilization of misfolded protein, inflammation, and cell differentiation, all playing a role in diseases.16–18 CLU has been implicated in pathologic conditions in which oxidative stress plays a central role, such as ageing, neurodegenerative diseases, and cancer progression.17 Both cytoprotective and cytotoxic roles of CLU have been reported; the predominant secretory form has been shown to protect cells from death, whereas the intracellular form exhibits proapoptotic properties.19–21 Although much of the work on CLU has focused on its role as an extracellular chaperone,22 the protein has a multiplicity of biologic functions.23

In this study, we conducted a series of experiments focused on CLU expression and function in renal proximal tubular epithelial (RPTE) cells and kidney biopsies derived from patients with nephropathic cystinosis. We showed an elevated level of intracellular CLU with altered subcellular localization in cystinosis cells. Our results show that intracellular CLU in cystinosis cells interacts with apoptosis markers (cleaved caspase-3 and apoptosis-inducing factor [AIF]) and autophagy markers, such as LC3 and p62. Finally, our data exhibit a significant attenuation of cell death on effective CLU gene silencing in cystinosis RPTE cells. Hence, on the basis of our data, we suggest that inhibiting intracellular CLU expression in kidney could be beneficial in nephropathic cystinosis.

RESULTS

CLU Is Significantly Enriched in Cell-Death Related Pathways

We have previously used cDNA microarray technology to compare the gene expression patterns of peripheral blood samples from patients with and without nephropathic cystinosis.23 There are 150 genes that were involved in cell death from Gene Ontology, and we identified 133 genes that are overlapped with cDNA microarray platform after reannotation; using an absolute fold change value >1.5, we identified 29 cell death genes of 133 genes. We then further investigated 29 genes by ingenuity pathway analysis to assess the enrichment in specific subcategories under cell death pathway. Intriguingly, CLU was found to be significantly enriched in the key biologic functions, such as cell death of kidney cells and apoptosis of spermatocytes (shown in Tables 1 and 2, Supplemental Figure 1).

Evaluation of CLU Protein Expression in Cystinosis RPTE Cells by Western Blot

We examined the media of cultured cystinotic RPTE cells for the expression of secreted CLU protein. Hence, we conducted Western blot analysis specific for CLU protein using conditioned media of cystinotic RPTE cells. The levels of the 36-kD form, which is the secretory form, were either dramatically reduced or absent in the cystinosis RPTE cells compared with a very high level in normal control RPTE cells (Figure 1A).

Analysis of CLU Protein Subcellular Expression in Cystinosis RPTE Cells

To investigate the intracellular expression of CLU and address the subcellular localization of intracellular CLU, confocal microscopy was performed on cystinotic RPTE cells specific for CLU. Immunofluorescence revealed both cytoplasmic and nuclear staining of CLU in cystinotic RPTE cells, whereas normal RPTE cells mainly exhibited a diffused cytoplasmic staining for CLU (Figure 1, B and C). The intensity of staining for CLU in cystinotic RPTE cells was higher than in normal RPTE cells (Figure 1, D and E). Notably, CLU protein exhibited filamentous staining and a distinct punctate aggregate-like pattern in cystinotic cells, which are shown in the higher magnification images in Figure 1E.

Evaluation of Tissue Expression Pattern of CLU Protein in Human Cystinosis Kidney Specimens by Immunohistochemistry

To further address the question of cellular source and expression pattern of CLU in cystinosis kidney, horseradish peroxidase-3,3′-diaminobenzidine (HRP-DAB) immunohistochemistry specific for CLU protein was performed on kidney biopsies from nephropathic cystinosis and compared with normal kidney biopsies. Cystinosis kidneys exhibited dilated tubules, acute tubular necrosis, tubular ectasia, and simplified epithelium. The immunohistochemistry staining in cystinosis kidneys revealed CLU staining in proximal tubules, the lumen, and cytoplasm of tubules, with a strong staining in the cells shed in the lumen (Figure 2, F–H). Figure 21 shows CLU staining in a cystinosis kidney at a higher magnification, and some cells exhibit nuclear staining for CLU (shown by arrows in Figure 2F). In normal kidneys, essentially negative staining...
for CLU was observed, with little in distal tubules (Figure 2A). A low diffused staining for CLU was observed in kidneys with pyelonephritis, obstructive uropathy, interstitial nephritis, and FSGS (Figure 2, B–E). Fewer tubules stain for CLU and intensity of staining is less compared with cystinosis in all obstructive uropathy, pyelonephritis, interstitial nephritis, and FSGS. This staining pattern is greater in intensity than that observed in normal kidney tissue, but we do not see any nuclear staining. In pyelonephritis, some CLU staining is observed in distal tubules.

Specific Mechanism Associated with Intracellular CLU in Nephropathic Cystinosis

Apoptosis and autophagy, specifically mitophagy, were previously reported to have a significant role in cell injury in cystinosis.13 Therefore, to gain additional insight into the role of intracellular CLU in cystinosis cells, we investigated the expression of CLU in conjunction with apoptosis and autophagy proteins.

Expression Analysis of CLU Protein in Conjunction with Apoptosis Markers by Double Immunofluorescence

AIF has previously been shown to express differentially in cystinosis, and a recent study suggested induction of cleaved caspase-3 activation because of overexpression of nuclear

Table 1. List of pathways significantly enriched in cell death genes

<table>
<thead>
<tr>
<th>Function</th>
<th>Genes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal necrosis/cell death</td>
<td>BCL10, CLU, PSEN1, TNFRSF1B</td>
<td>0.001</td>
</tr>
<tr>
<td>Cell death of kidney cells</td>
<td>CLU, TNFRSF1B</td>
<td>0.004</td>
</tr>
<tr>
<td>Apoptosis of kidney cells</td>
<td>TNFRSF1B</td>
<td>0.01</td>
</tr>
<tr>
<td>Necrosis of kidney cells</td>
<td>CLU</td>
<td>0.03</td>
</tr>
<tr>
<td>Apoptosis of renal tubular epithelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ophthalmic disease</td>
<td>ATXN7, CLU, HTR2A, POLB, PSEN1, SNCA, SOD1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Keratitis</td>
<td>POLB, PSEN1</td>
<td>0.001</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>CLU, HTR2A</td>
<td>0.002</td>
</tr>
<tr>
<td>Retinal degeneration</td>
<td>ATXN7, CLU, SNCA</td>
<td>0.002</td>
</tr>
<tr>
<td>Ocular cicatrical pemphigoid</td>
<td>CLU</td>
<td>0.003</td>
</tr>
<tr>
<td>Lattice corneal dystrophy type I</td>
<td>CLU</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gelationous drop-like corneal dystrophy</td>
<td>CLU</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 2. Fold change values of genes significantly enriched in cell death-related pathways

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLU</td>
<td>−1.74795</td>
</tr>
<tr>
<td>PSE1</td>
<td>1.574642</td>
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<tr>
<td>TNFRSF1B</td>
<td>−2.25788</td>
</tr>
<tr>
<td>BCL10</td>
<td>−1.63454</td>
</tr>
<tr>
<td>ATXN7</td>
<td>1.583974</td>
</tr>
<tr>
<td>HTR2A</td>
<td>−1.64514</td>
</tr>
<tr>
<td>POLB</td>
<td>2.202689</td>
</tr>
<tr>
<td>SNCA</td>
<td>−1.82132</td>
</tr>
<tr>
<td>SOD1</td>
<td>2.227059</td>
</tr>
</tbody>
</table>

Thus, we investigated the expression of CLU with respect to these two apoptosis markers.

Both CLU and AIF exhibited staining mainly in the proximal tubular cells, and a significant overlap was observed between CLU and AIF in cystinosis kidney biopsies; insets in Figure 3A show similar stains on normal tissue. Similarly, cleaved caspase-3–specific staining exhibited a significant degree of overlap with CLU expression in the cystinosis RPTE cells, whereas this specific staining pattern was not observed in normal RPTE cells (Figure 3B).

Evaluation of a Link between Autophagy and CLU in Nephropathic Cystinosis by Confocal Imaging Specific for CLU and Autophagy Markers

To further gain insight into the recently identified role of autophagy in kidney injury in cystinosis,13,14 we studied the expression pattern of CLU with respect to the two well established autophagy markers LC3 and p62.

Intriguingly, the fluorescent confocal images of double immunostaining specific for CLU and LC3 exhibited significant overlap in nephropathic cystinosis RPTE cells (Figure 4A, upper panel). A higher magnification confocal image of a single cystinosis RPTE cell clearly revealed the punctate pattern of LC3 II and a substantial colocalization with CLU (Figure 4A, lower panel). As shown in Figure 4B, immunofluorescence specific for p62 revealed higher expression levels and punctate staining in cystinosis RPTE cells compared with the normal control RPTE cells, which exhibited a diffused low expression. The merged confocal images specific for CLU and p62 showed a substantial overlap in cystinosis RPTE cells, whereas in normal cells, overlap between CLU and p62 was barely detectable (Figure 4B).

Functional Significance of CLU in Nephropathic Cystinosis

Intracellular and nuclear CLU has been reported to have a proapoptotic role and induce cell death. Hence, we evaluated the ability of CLU to affect cell viability and apoptosis in nephropathic cystinosis RPTE cells.
Efficient Silencing of CLU Gene Expression by Using Small Interfering RNA Increases the Cell Viability in Cystinosis RPTE Cells

We analyzed the effect of CLU small interfering RNA (siRNA) transfection on CLU expression in nephropathic cystinosis RPTE cells. Individual cultures of RPTE cells obtained from four different patients with nephropathic cystinosis were subjected to quantitative real-time RT-PCR to measure CLU mRNA levels. Cellular CLU mRNA levels were significantly higher compared with normal RPTE cells ($P=0.02$) (Figure 5A, left panel). Treatment of cystinosis RPTE cells with CLU siRNA induced significant knockdown of the cellular CLU mRNA as measured by quantitative real-time RT-PCR ($P=0.004$) (Figure 5A, right panel), immunofluorescence specific for CLU (Figure 5B), and Western blot (CLU expression in CLU siRNA-transfected cystinosis RPTE cells compared with scrambled control siRNA-transfected cystinosis RPTE cells; $P<0.01$) (Figure 5C).

Nephropathic cystinosis RPTE cells exhibited significantly low cell viability compared with the normal control RPTE cells, which was measured by Trypan Blue exclusion assay ($P=0.001$) (Figure 5D, left panel). However, silencing of CLU gene expression in cystinosis RPTE cells resulted in an increase in cell viability compared with the cystinosis cells transfected with control siRNA ($P=0.03$), whereas no significant difference ($P=0.69$) in cell viability was observed in normal RPTE cells transfected with CLU siRNA compared with control siRNA (Figure 5D, right panel). To further validate the viability data, we conducted an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay on normal and cystinosis RPTE cells transfected with CLU siRNA and CLU siRNA. Silencing of CLU in cystinosis RPTE cells resulted in significant improvement in cell viability ($P<0.01$), which is shown in Figure 5E. Interestingly, cell viability is decreased in normal RPTE cells transfected with CLU siRNA compared with cells transfected with control siRNA, although the difference is not statistically significant (Figure 5, D, right panel and E).

Inhibition of Intracellular CLU Expression Results in Significant Attenuation of Apoptosis in Cystinosis RPTE Cells

An important role of apoptosis has been suggested in cystinosis, and it has been shown that cystinosis cells are more sensitive to apoptosis stimuli compared with the normal cells.$^{9,10,13,14}$ Hence, we investigated the effect of silencing CLU gene expression on apoptosis in cystinosis renal cells. We performed a terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay to evaluate apoptosis in CLU siRNA-transfected cystinosis RPTE cells. As shown in Figure 6A, TUNEL-positive cells exhibiting fluorescein staining were dramatically reduced in cystinosis cells transfected with CLU siRNA compared with the cells transfected with control siRNA.

To further confirm and quantitate the attenuation of apoptosis as a result of silencing of CLU expression in cystinosis RPTE cells, we performed Western blot specific for cleaved...
poly-ADP-ribose polymerase (PARP) in cystinosis cells transfected with either CLU siRNA or control siRNA. Cleaved PARP Ab detects endogenous levels of the large fragment (89 kD) of human PARP1 produced by caspase cleavage and thus, serves as a marker of cells undergoing apoptosis. Other than measuring basal apoptosis, evaluation of apoptosis by PARP Western blot was also performed after treating the cells with Actinomycin D (ActD) and TNF-α to trigger apoptosis.10 The cleaved PARP bands, as shown in the Western blot in Figure 6B, were quantitated by densitometry analysis normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Levels of cleaved PARP normalized to GAPDH in cystinosis RPTE cells were significantly higher than the normal RPTE cells under both basal and stimulated (ActD+TNF-α) conditions (P<0.01) (Figure 6C).

Silencing of CLU gene expression by CLU siRNA transfection in cystinosis RPTE cells under both basal and stimulated (ActD+TNF-α) conditions resulted in significantly low levels of cleaved PARP compared with the control siRNA-transfected cystinosis RPTE cells (P<0.01) (Figure 6D). In contrast, no significant difference in cleaved PARP was observed in the normal cells in both basal stimulated conditions when transfected with CLU siRNA compared with control siRNA transfection.

DISCUSSION

Nephropathic cystinosis is an autosomal recessive disorder caused by mutations in the CTNS gene encoding cystinosin, a
lysosomal membrane cystine transporter. Cystinosis is characterized by elevated levels of intracellular cystine throughout the body. Individuals affected with nephropathic cystinosis develop proximal tubulopathy by 6–12 months of age, and in the absence of treatment, they usually develop progressive renal failure by the end of the first decade. Continuous cystine accumulation eventually leads to multiorgan dysfunction, and patients present with

Figure 3. Double immunofluorescence for CLU and apoptosis markers shows colocalization in cystinosis. (A) Double immunofluorescence specific for CLU and AIF shows colocalization of CLU and AIF in cystinosis kidneys. Original magnification, ×40. Similar stains on normal tissue are shown in the insets. (B) Double immunofluorescence specific for CLU and cleaved caspase-3 reveals colocalization in cystinosis RPTE cells. The immunostained cells were analyzed by confocal microscopy. Scale bar, 47.42 μm. Original magnification, ×63. Similar stains are shown in normal RPTE cells, where these specific staining patterns are not observed.
Figure 4. Double immunofluorescence for CLU and autophagy proteins in nephropathic cystinosis RPTE cells. (A) Staining pattern of CLU and LC3 shows a significant overlap in cystinosis RPTE cells. Higher magnification images show a clear punctate pattern of LC3 II and a significant colocalization with CLU. Original magnification, ×40 in i–iii; ×100 in iv–vi. Scale bar, 47.42 μm. (B) Expression pattern of
hypothyroidism, hypogonadism, photophobia, myopathy and retinal blindness, chronic renal failure, and pulmonary dys función as well as central nervous system calcifications and symptomatic deterioration. Treatment with the drug cysteamine depletes the intracellular cystine, and if used early in the disease and in high doses, it can lower the progression of renal glomerular damage and extrarenal organ injury. However, cysteamine is ineffective against the proximal tubulopathy. Moreover, the side effects, such as persistent odor and digestive intolerance, associated with the drug cysteamine and the need of frequent doses render its regular usage difficult. Hence, there is a need of new therapies, and additional research studies are warranted to address this issue. Recent advances in the field of cystinosis and other lysosomal storage diseases have provided insights into novel mechanisms of pathogenesis in cystinosis.\(^7\) The multifunctional role of CLU has been implicated in several divergent physiologic functions.\(^3\) It may also function as an extracellular chaperone that stabilizes stressed proteins in a folding- competent state.\(^3\) CLU is differentially regulated in many severe physiologic disturbance states, including kidney degenerative diseases, several neurodegenerative conditions, tumorigenesis, inflammation, and cell death.\(^3\) CLU is a ubiquitous protein that is arousing increasing interest owing to its widespread diffusion and multifunctional role. CLU has been implicated in several diseases being characterized by significant oxidant injury.

CLU is a unique heterodimeric glycoprotein that has been suggested to bind misfolded, heat-shocked, or stressed proteins.\(^3\) The predominant form of CLU is a secreted heterodimeric protein of 75–80 kD (secreted CLU protein) reported to be cytoprotective comprised of \(\alpha\) - and \(\beta\)-subunits of approximately 40 kD.\(^4\) A 49-kD CLU isoform discovered in the nucleus has also been reported.\(^4\) Our Western blot data showed a significantly lower level of secreted CLU in conditioned cell culture media of cystinosis RPTE cells. Intriguingly, confocal microscopy revealed a higher level of expression and an altered subcellular localization of CLU in cystinosis RPTE cells. Cystinosis RPTE cells exhibit the presence of a nuclear form of CLU in addition to the punctate and a few filamentous cytoplasmic patterns of expression. We have previously shown abnormal morphologic changes in mitochondria in cystinosis kidney cells.\(^5\) Also, it has been reported that intracellular CLU aggregates cause profound mitochondrial alteration in a cell.\(^5\) Thus, it is tempting to hypothesize that the altered subcellular localization of CLU in cystinosis is correlated with the mitochondrial aberrations reported earlier.

There is also strong evidence that CLU plays a role in renal diseases. It has been shown that CLU colocalizes with the C5b-9 complex within immune deposits in human membranous GN. Immunochemistry on cystinosis kidney specimen revealed that CLU was mainly localized in proximal tubules, and interestingly, the cells shed in the lumen exhibited a strong staining for CLU. Although the exact role of nuclear CLU is still not fully elucidated, it seems to be a prodeath protein form capable of rapidly triggering anoikis cell death characterized by caspase-dependent apoptosis.\(^6\) To gain additional insight into the link between CLU in kidneys and cell death, the cystinosis kidney specimen were immunostained with AIF and cleaved caspase-3 in conjunction with CLU. AIF is a mitochondrial intermembrane flavoprotein that is translocated to the nucleus in response to proapoptotic stimuli, where it induces nuclear apoptosis. The costaining pattern revealed a direct correlation between apoptosis and CLU expression in cystinosis kidney. The tubules expressing CLU also expressed AIF, and the CLU expression in cystinosis RPTE cells overlapped with the activated caspase-3. Thus, these results suggest a proapoptotic role of intracellular CLU in cystinosis kidneys. We have previously discovered abnormal mitochondrial autophagy in the cystinosis kidney. Hence, the next question that we addressed was the correlation of intracellular CLU and autophagy in cystinosis. Immunostaining cystinosis RPTE cells with LC3 and p62 along with CLU elucidated the coexpression of autophagy markers and CLU.

Recently, it became evident that the different functions of CLU depend on its final maturation and localization. Secretory CLU protects cells from death, probably by binding a wide spectrum of hydrophobic molecules, misfolded proteins, and ligands.\(^7\) When secretion of CLU is inhibited, the cell is loaded with CLU, and nuclear targeting of CLU is dominant. It was previously reported that CLU associates with misfolded keratin only if its signal peptide was deleted and its secretion was inhibited. This finding suggests that CLU has ability to bind misfolded proteins, including keratins, but that its physiologic function is restricted to the extracellular space.\(^8\) In this study, cystinosis RPTE cells exhibit overexpression of intracellular CLU with significantly reduced levels of secretory CLU. What causes increased accumulation of intracellular CLU remains to be elucidated. However, previous data showing endoplasmic reticulum stress in nephropathic cystinosis kidneys could be linked to CLU secretion.\(^9\) Intriguingly, apolipoprotein A-I secretion is inhibited by endoplasmic reticulum stress in HepG2 cells.\(^5\) The link between CLU overexpression and cell stress, tissue degeneration, and apoptosis has previously been reported in literature. It has been shown that overexpression of intracellular CLU, localized in cytoplasm, causes cell death in PC-3 prostate cancer cells.\(^5\) Our data reveal overexpression of CLU and p62 reveals (i–iii) a minimal colocalization in normal cells but (iv–vi) a significant extent of overlap in cystinosis RPTE cells. (v) P62 exhibits a distinct punctate pattern in cystinosis RPTE cells. Scale bar, 47.42 μm. Original magnification, × 40.

CLU and p62 reveal (i–iii) a minimal colocalization in normal cells but (iv–vi) a significant extent of overlap in cystinosis RPTE cells. (v) P62 exhibits a distinct punctate pattern in cystinosis RPTE cells. Scale bar, 47.42 μm. Original magnification, × 40.

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intracellular CLU (both cytoplasmic and nuclear) in cystinosis RPTE cells. Hence, the next question that we addressed was if there is a change in the cell viability and cell death if the CLU gene is silenced. Our data show that efficient silencing of the CLU gene increases the cell viability and significantly attenuates apoptosis in both basal and stimulated cystinosis RPTE cells, indicating that the elevated intracellular CLU is toxic to already stressed cystinosis RPTE cells containing aggregates of cystine crystals and autophagic vesicles. Interestingly, DeMattos et al.54 have reported that CLU promotes the formation of toxic oligomeric forms of Ab and is critical for neural toxicity in a mouse model of Alzheimer Disease. Also, the expression of CLU has been shown in Huntington Disease (HD) brain samples with severe atrophy, suggesting its implications in the pathogenesis of HD.55 Cysteamine, a cystine depletion drug for cystinosis, has been shown to significantly extend survival in an HD mouse model.56 Our findings highlight a new role for intracellular CLU in cystinosis, and CLU may be a new target for therapy in nephropathic cystinosis.

**Figure 5.** Knockdown of CLU increases cell viability in cystinosis RPTE cells. (A, left panel) Real-time quantitative PCR analysis of normal and cystinosis RPTE cells shows significantly higher expression of CLU mRNA in cystinosis cells. (A, right panel) Quantitative PCR analysis of cystinosis RPTE cells transfected with control siRNA or CLU siRNA shows knockdown of the CLU gene 48 hours after transfection. 18S was used as the internal control. (B) Immunofluorescence specific for CLU protein in cystinosis RPTE cells treated with control siRNA or CLU siRNA exhibits a significantly reduced level of the CLU protein 36 hours after siRNA transfection. (C) Western blot analysis conducted on cystinosis RPTE cells shows significantly reduced expression of CLU in cells transfected with CLU siRNA compared with control siRNA. The blot shows all four individual samples of RPTE cells obtained from patients with cystinosis. Loading levels are normalized by blotting with 1:20,000 GAPDH and expressed as percent expression relative to control siRNA. **P<0.01. (D, left panel) Cell viability measured by Trypan Blue exclusion assay shows significantly less viability in cystinosis RPTE cells compared with the normal RPTE cells. (D, right panel) Viability of cystinosis RPTE cells transfected with CLU siRNA improved significantly compared with the control siRNA-transfected cells. (E) MTT assay in cystinosis RPTE cells transfected with CLU siRNA shows improvement in cell viability. Data are presented as means±SDs (n=4 except in cell viability experiments, where n=3); P is calculated by two-tailed t test. At least three independent experiments were conducted, and each sample was tested in triplicate. **P<0.01.
CONCISE METHODS

All of the probes on the cDNA microarray platform were reannotated to the most recent National Center for Biotechnology Information gene identifiers (http://ailun.stanford.edu). Genes involved in cell death function were retrieved from the Gene Ontology. Biologic function and pathways were investigated by ingenuity pathway analysis (http://www.ingenuity.com; Redwood City, CA).

Patients and Samples

This study included kidney tissues collected at Stanford Hospital and Clinics and Lucile Packard Children’s Hospital at Stanford. Archival paraffin blocks of 12 unique human kidney tissues were obtained from surgical pathology and examined for this study. They consisted of four native kidneys from patients with nephropathic cystinosis (mean age = 12.3 ± 4.6 years) and four archival paraffin blocks of kidneys with renal injury caused by causes other than cystinosis (FSGS, n = 2; pyelonephritis, n = 2; obstructive uropathy, n = 2; interstitial nephritis, n = 2; mean age = 10.4 ± 6.8 years). Four normal kidney tissue blocks (normal kidney tissue resected from renal tumors) were also obtained.

Cells

Six RPTE cultures were used for this study: two lots of normal primary RPTE cells (both lots performed similarly for all conducted experiments without any specific variations between the two lots; CC-0267; Cambrex Biosciences) and four primary cultures from cystinosis RPTE cells isolated from the urine of patients with nephropathic cystinosis attending the National Human Genome Research Institute at the National Institutes of Health. The study had institutional review board approvals from the National Institutes of Health and Stanford University. All RPTE cells were cultured in renal epithelial growth medium made according to the manufacturer’s instructions (CC-3190; Cambrex). All cells were passaged with trypsin (0.05%) and cultured in a 95% air/5% CO2 Thermo Forma Incubator at 37°C. All of the experiments were performed between passages 2 and 5.

Abs

The primary Abs used were rabbit polyclonal anti-human GAPDH Ab (ab9485; Abcam, Inc.), rabbit polyclonal anti-human p62 (AP 2183B; Abgent), rabbit polyclonal anti-human LC3 (AP 1802A; Abgent), rabbit polyclonal anti-human cleaved caspase-3 (Asp175; Cell Signaling), rabbit polyclonal anti-human AIF (ab1998; Abcam, Inc.), rabbit polyclonal anti-human cleaved PARP (Asp214; Cell Signaling Technology), and goat polyclonal anti-human CLU (Clusterin-α [C-18]; sc-6 419; Santa Cruz Biotechnology). Secondary Abs used were Alexa Fluor 555 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-goat IgG, peroxidase-conjugated goat anti-rabbit IgG, and peroxidase-conjugated rabbit anti-goat IgG purchased from Invitrogen.

Figure 5. Continued.
Figure 6. Knockdown of CLU attenuates apoptosis in cystinosis RPTE cells. (A) In situ detection of apoptosis by use of TUNEL staining detects a decrease in apoptosis in cystinosis RPTE cells transfected with CLU siRNA compared with the cells transfected with control siRNA. (B) Western blot analysis specific for cleaved PARP in normal and cystinosis RPTE cells transfected with CLU siRNA and control siRNA under basal or stimulated conditions (TNF-α and ActD). (C) Graphic presentation of the relative abundance of cleaved PARP.
**Immunohistochemistry for CLU**

Immunohistochemical staining was performed using Abs directed against CLU (dilution of 1:50). Serial sections of 4 μm were obtained, deparaffinized in xylene, and hydrated in a graded series of alcohol. Heat-induced antigen retrieval was carried out with microwave pretreatment in citric acid buffer (10 mM, pH 6.0) for 10 minutes. Endogenous peroxidase was blocked, and the DAKO Envision System (K4011; DAKO Corporation) was used for detection.

**Western Blot**

Cells extracts were prepared, and an equal amount of total protein (30 μg) was subjected to SDS-PAGE. For Western blot assays in the siRNA experiments shown in Figure 6D, 50 μg total protein was used. All primary Ab incubations (CLU, 1:400; cleaved PARP, 1:1000) were done in PBS supplemented with 0.1% (vol/vol) Tween-20 and 3% (wt/vol) milk for a minimum of 1 hour at room temperature followed by washing with PBS-Tween (PBS supplemented with 0.1% Tween). Peroxidase-conjugated secondary abs were diluted 1:10,000 in PBS-Tween, incubated with the blot for a minimum of 1 hour at room temperature, and then, washed with PBS-Tween and developed using ECL Plus Detection Reagent (RPN2124; Amersham). Loading levels were normalized using 1:2000 anti-GAPDH Abs. Band quantification was performed using the ImageJ software (National Institutes of Health).

**Western Blot Analysis of Secretory CLU in Culture Medium**

One milliliter culture medium was reserved from a subconfluent RPTE cells flask. The medium was centrifuged, and the supernatant fraction was precipitated by the addition of ice cold TCA at a final concentration of 15%. Precipitate was washed two times with 95% ethanol, dried, and resuspended in a buffer containing 62 mM Tris-HCl, pH 6.8, 1% SDS, 15% glycerol, 0.04% bromophenol blue, and 0.1% mercaptoethanol. All precipitated proteins were loaded on 10% HCl, pH 6.8, 1% SDS, 15% glycerol, 0.04% bromophenol blue, and ethanol, dried, and resuspended in a buffer containing 62 mM Tris-concentration of 15%. Precipitate was washed two times with 95% ethanol. CLU was tested in triplicate. **Western analysis specific for CLU was performed as described above.**

**Confocal Microscopy**

For immunofluorescence of CLU, cells were plated on chamber slides, washed two times in PBS, fixed in 4% formalin (30 minutes), and permeabilized with 0.5% Triton X-100 (30 minutes). Cells were incubated in blocking buffer (PBS, pH 7.2 and 3% BSA) for 1 hour and then, washed two times in PBS followed by incubation with the primary Ab for 2 hours at room temperature. Alexa Fluor 488 donkey anti-goat IgG was used to detect bound primary Ab for 1 hour at room temperature. Similarly, for coimmunostaining of CLU with cleaved caspase-3, LC3, and p62, cells were processed, and coimmunostaining was performed by incubation with primary Abs for 2 hours at room temperature followed by washing and incubation with secondary Abs (Alexa Fluor 488 and Alexa Fluor 555) for 1 hour at room temperature. Immunofluorescence of kidney tissues was performed using Abs for AIF (1:50) and Alexa Fluor 555 as secondary Ab. Slides were viewed using a Leica SP2 AOBS Confocal Laser Scanning Microscope, and the images were analyzed by Leica Confocal software (version 2.5).

**siRNA Transfection**

Silencer Select CLU siRNA (s3156; GGAAGTAAGTACGTCATA) and Silencer Negative Control siRNA that is shown not to target any gene product (AM4611) were purchased from Ambion. Transfection was performed in cystinosis RPTE cells using the Neon Transfection System from Invitrogen according to the manufacturer’s instructions. Briefly, 1×10⁶ cells and 200 nM siRNA were used for each transfection experiment. After 20 hours, ActD (2.5 μg/ml) and TNF-α (30 ng/ml) were added in the treatment group. Thirty-six hours after transfection, cells were harvested for additional assays, including real-time quantitative PCR, Western blot, and immunofluorescence to monitor CLU expression and cell viability, apoptosis, and Western blot for cleaved PARP.

**Cell Death Measurement**

Apoptosis was examined by TUNEL assay using the TACS 2 TdT-DAB In Situ Apoptosis Detection Kit (4812–30–K; Trevigen) according to the instructions by the manufacturer. The slides were mounted with 4’,6-diamidino-2-phenylindole and viewed using a Leica SP2 AOBS Confocal Laser Scanning Microscope, and the images were analyzed by Leica Confocal software (version 2.5). TUNEL-positive cells exhibited fluorescein staining. Cell viability was determined by Trypan Blue exclusion assay. The cells were scraped and resuspended in the Trypan Blue solution (0.4%), and they were counted in a hemacytometer under a light microscope; the percentage of viable cells was calculated. MTT assay was used to validate cell viability according to the manufacturer’s instructions (Sigma-Aldrich). At least three independent experiments were conducted, and each sample was tested in triplicate.

**ACKNOWLEDGMENTS**

We thank Dr. William Gahl and Dr. Lorraine Racusen for the generous gift of cystinosis RPTE cells. We acknowledge Dr. Neeraja Kambham’s help with immunohistochemistry experiments.

This work was supported by grants from the Cystinosis Foundation Ireland, the Health Research Board Ireland, and the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health.

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normalized to GAPDH in normal and cystinosis RPTE cells under basal and stimulated conditions measured by densitometry analysis of cleaved PARP and GAPDH bands in B. (D) Graphic presentation of relative abundance of cleaved PARP normalized to GAPDH in normal and cystinosis RPTE cells transfected with CLU siRNA and control siRNA treated with and without TNF-α and ActD. Data are presented as means±SDs (n=3); P is calculated by two-tailed t test. At least three independent experiments were conducted, and each sample was tested in triplicate. **P≤0.01.

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