The pathogenesis of cystinosis: mechanisms beyond cystine accumulation

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1Laboratory of Genetic Endocrine and Metabolic Diseases, Department of Laboratory Medicine, Radboud University Nijmegen Medical Centre, The Netherlands; 2Laboratory of Pediatrics, Catholic University, Leuven; 3Department of Nephrology and Urology, Division of Nephrology, Bambino Gesù, Rome, Italy; and 4Department of Pediatrics, University Hospitals Leuven, Belgium

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Wilmer MJ, Emma F, Levtchenko EN. The pathogenesis of cystinosis: mechanisms beyond cystine accumulation. Am J Physiol Renal Physiol 299: F905–F916, 2010. First published September 8, 2010; doi:10.1152/ajprenal.00318.2010.—Renal proximal tubules are highly sensitive to ischemic and toxic insults and are affected in diverse genetic disorders, of which nephropathic cystinosis is the most common. The disease is caused by mutations in the CTNS gene, encoding the lysosomal cystine transporter cystinosin, and is characterized by accumulation of cystine in the lysosomes throughout the body. In the majority of the patients, this leads to generalized proximal tubular dysfunction (also called DeToni-Debré-Fanconi syndrome) in the first year and progressive renal failure during the first decade. Extrarenal organs are affected by cystinosis as well, with clinical symptoms manifesting mostly after 10 yr of age. The cystine-depleting agent cysteamine significantly improves life expectancy of patients with cystinosis, but offers no cure, pointing to the complexity of the disease mechanism. In this review, current knowledge on the pathogenesis of cystinosis is described and placed in perspective of future research.

cystine transport; Fanconi syndrome; glutathione

Clinical and fundamental research in the past decades has significantly increased our knowledge of nephropathic cystinosis (MIM 219800) and has improved the quality of life of patients suffering from this very severe disease. Nephropathic cystinosis is an autosomal recessive disorder caused by mutations in the CTNS gene (46, 135) and characterized by elevated levels of intracellular cystine. The estimated incidence of the disease is ~1 in 100,000–200,000 live births (46). Although the pathogenesis is not completely elucidated, research in the field of cystinosis has been spurred in the late 1990s by the identification of the CTNS gene, which encodes for cystinosin, a lysosomal cystine carrier.

Pioneering Studies

During the autopsy of a child that died at 21 mo, the Swiss biochemist Emil Abderhalden found cystine crystals accumulating in the spleen and liver (1). Two siblings of this child had previously died with similar symptoms. After this report in 1903, which is generally regarded as the first description of cystinosis, other pediatric patients suffering from rickets, failure to thrive, glucosuria, and phosphaturia were reported in the 1920s and 1930s (28, 29, 33, 77). This clinical picture was subsequently termed DeToni-Debré-Fanconi syndrome, and for several years it remained unclear whether Fanconi syndrome and cystinosis represented two separate clinical disorders or the clinical spectrum of a single disease (34).

In the 1960s, Schneider et al. (118) showed for the first time increased intracellular cystine levels in granular fractions of leukocytes from patients, identifying cystinosis as a cystine storage disease that was associated with Fanconi syndrome. Nowadays, other genetic and nongenetic causes of Fanconi syndrome have been recognized. However, cystinosis remains the most common cause of inherited renal Fanconi syndrome and should be suspected in all infants presenting with failure to thrive, polyuria, and dehydration (84).

Clinical Features

Cystinotic patients are usually asymptomatic at birth and develop normally during the first 6 mo of life, when they often present with failure to thrive, vomiting, constipation, polyuria, excessive thirst, dehydration, and sometimes rickets (13). These symptoms result from Fanconi syndrome, which is characterized by inappropriate urinary losses of water, amino acids, phosphate, bicarbonate, glucose, sodium, potassium, low-molecular-weight (LMW) proteins, and other solutes as a consequence of defective renal proximal tubular reabsorption (46). Usually, patients develop chronic renal failure during early childhood, leading to end-stage renal disease in the first decade of life. Renal replacement therapy and treatment with the cystine-depleting agent cysteamine have prolonged the life expectancy of patients but have also revealed other extrarenal complications, recently reviewed by Nesterova and Gahl (88).

In addition to infantile nephropathic cystinosis, which represents the most frequent form of the disease, two milder variants have been identified. These include the “juvenile” or “intermediate” form (MIM 219900), which is usually diag-
nosed during childhood or adolescence and is characterized by less severe renal symptoms, and a third form, that has been reported primarily in adults that is characterized only by ocular symptoms and has been thereby termed “ocular” or “nephropathic” cystinosis (MIM219750) (45). In most cases, the severity of the disease cosegregates within family members. Co-occurrence of intermediate and nephropathic cystinosis was recently reported in one family (121).

Studies of Proteinuria in Cystinosis

Similarly to other proximal tubular disorders, such as Dent’s disease and Lowe syndrome, LMW proteinuria represents a key feature of nephropathic cystinosis. Reabsorption of LMW proteins in the proximal tubular epithelium is mediated by the multiligand receptors megalin, cubulin, and amnionless (22, 25). Ligands present in the glomerular ultrafiltrate bind to the receptors and are internalized via clathrin-coated pits, which transform into endosomes and subsequently fuse with the lysosomal compartment. Acidic pH in the late endosomal compartment promotes the dissociation of ligands from their receptors, which are recycled to the brush-border membrane (BBM).

Defects in the endocytic apparatus of proximal tubules have also been demonstrated in other diseases such as Dent’s disease (23, 104), which is characterized by a defect in the endosomal CLC-5 chloride/proton exchanger. Typically, Dent’s disease (Dent 1; MIM 300009) is caused by mutations in the CLCN5 gene located on the X chromosome. However, mutations in OCRL1, which are usually associated with Lowe syndrome (MIM 309000), have been reported in Dent’s patients as well (Dent 2; MIM 300555) (11, 58). The OCRL1 gene, which is also located on the X chromosome, encodes for a phosphatidylinositol 4,5-biphosphate-5-phosphatase that is involved in membrane trafficking between the Golgi apparatus and other cell compartments (16, 21). Decreased urinary megalin excretion in patients with Lowe syndrome or Dent’s disease strongly suggests that impaired recycling of receptors to the plasma membrane is responsible for the LMW proteinuria that is observed in these two genetic conditions (94). Megalin and cubulin expression in the BBM have been shown to be decreased in CLCN5−/− mice and in patients with Dent 1 disease (23, 117). However, a recent study failed to show impairment of megalin-mediated endocytosis in OCRL1 knockout renal cells (26).

Similarities among the renal phenotypes of Dent’s disease, Lowe syndrome, and nephropathic cystinosis (e.g., LMW proteinuria) recently led us to investigate megalin and cubulin function and expression in cystinosis (141). The histological examination of a renal specimen obtained from a cystinotic patient surprisingly showed that megalin and cubulin were normally expressed in the BBM and endocytic vesicles, suggesting that the mechanism of LMW proteinuria in cystinosis may be dissimilar to that observed in related tubular disorders (Fig. 1). The presence of megalin in urine of cystinotic patients further suggests expression of the receptor in the plasma membrane, as opposed to patients with Dent’s disease and Lowe syndrome that do not shed megalin in their urine (94).

The concomitant observation of abundant LMW proteins and albumin in cystinotic urine and in intracellular vesicles located throughout the proximal tubule points toward a defect in the mechanisms of renal reabsorption of proteins. Hypothetically, glomerular proteinuria in cystinosis can saturate the ligand binding sites of endocytic receptors, causing LMW proteinuria. This concept is supported by the observation of increased amounts of IgG (HMW proteins) in urine of cystinotic patients starting from infancy (141). Increased glomerular permeability in cystinosis also raises questions on the role of the CTNS gene in podocytes. However, it should be emphasized that the magnitude of LMW proteinuria in cystinosis is higher compared with other proteinuric glomerular diseases (133), suggesting that other factors, probably of tubular origin, play an important role. In this respect, abundant presence of the endocytic receptors on the brush border of cystinotic proximal tubular cells and increased megalin excretion into cystinotic urine might indicate defective receptor retrieval, contributing to proteinuria. Excessive amounts of albumin and other solutes in renal tubular lumens can trigger inflammatory responses and the production of chemokines and cytokines by proximal tubular epithelia (140, 147, 148). Consequently, inflammatory cells are attracted to the renal interstitium, initiating tubulointerstitial fibrosis (53, 141). Recent investigations using a mouse model of diabetic nephropathy have shown cell damage in proximal tubules with prominent albumin staining (68). Similarly, using a megalin knockout mosaic mouse, it has been shown that tubular cells expressing megalin selectively express markers of tubular injury (87). Taken together, these experimental data combined with our observations on cystinotic patients highlight the potential contribution of glomerular proteinuria in mediating renal damage in nephropathic cystinosis and support the use of renin-angiotensin system (RAS) inhibitors to diminish the progression of renal disease (73). Drugs that inhibit the RAS should be used cautiously in cystinosis, as they may cause hypotension and renal failure in patients that may be salt depleted. Whether lysosomal cystine accumulation initiates inflammatory responses in cystinotic cells and whether this process can be mitigated by cysteamine remain unclear.

Impaired Lysosomal Cystine Transport in Cystinosis

Following the seminal studies by Schneider et al. (98) in the 1960s, electron microscopy of lymph nodes of patients with cystinosis suggested that cystine accumulation occurred in lysosomes. Lysosomal localization of cystine was confirmed in cystinotic leucocytes by Schulman et al. (120) using sucrose density gradient centrifugation. Goldman (51) and Reeves (108) developed a method using dimethyl esters to artificially load isolated lysosomes with amino acids (51, 108). In the following years, this method was adapted by several research groups to reproduce the lysosomal cystine accumulation that is observed in cystinosis to study the mechanism underlying the disease’s pathogenesis. Using cystine dimethyl ester (CDME), isolated lysosomes extracted from cystinotic leucocytes and fibroblasts were loaded with radiolabeled cystine and the kinetics of cystine clearance was analyzed (61, 123). This allowed the demonstration that impaired lysosomal cystine efflux represents the primary defect that causes cystine accumulation (44, 47).

Following these observations, several compounds were tested for their ability to decrease intracellular cystine levels as candidate drugs to treat patients with cystinosis. Moderate successes were obtained using 1,4-dithiothreitol (DTT) or
ascorbic acid (50, 69), but the breakthrough experiments were performed using the aminothiol cysteamine, which remains to this date the main drug for treatment of cystinosis (131). Cysteamine (Cystagon) depletes lysosomal cystine by a disulfide exchange reaction, resulting in the equimolar generation of a cysteine-cysteamine molecule and a molecule of cysteine (48). Both compounds can exit lysosomes via “system c” transporters, bypassing the defective cystinosin pathway (103). The efficacy of cysteamine can be monitored in clinical practice by measuring intracellular cystine levels in polymorphonuclear (PMN) leukocytes and is considered to be a reflection of tissue cystine content. Over the years, several methods have been developed to measure intracellular cystine levels in polymorphonuclear (PMN) leukocytes and is considered to be a reflection of tissue cystine content. Over the years, several methods have been developed to measure intracellular cystine levels for the diagnosis of cystinosis and for monitoring cysteamine treatment. Currently, HPLC and liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) are the most widely used (15, 52). LC-MS/MS is the most sensitive method (allows detection of as little as 0.02 μmol cystine/l, compared with 0.15 μmol cystine/l using HPLC) and has recently been developed for cystine determination in granulocytes (15). Unfortunately, Fanconi syndrome of cystinosis is not cured by cysteamine, although one single report showed milder proximal tubular dysfunction in two patients treated since the first months of life (66). Lack of efficacy of cysteamine on Fanconi syndrome is unlikely to be explained by defective proximal reabsorption of the drug, as almost no cysteamine is detected in the urine of cystinotic patients (74).

Of note, lysosomal cystine accumulation is also observed in mucolipidosis type II or I-cell disease (MIM 252500) (132). This autosomal recessive disorder is caused by a deficiency of N-acetylglucosamine-1-phosphotransferase, preventing mannos-6-phosphorylation (67). As a consequence, several lysosomal enzymes are not correctly targeted to the lysosomal compartment. The clinical symptoms are heterogeneous, but usually include dwarfism, coarse facial features, and mental retardation (6). At the renal level, proximal tubular dysfunction has been reported, including LMW proteinuria, aminoaciduria, hyperphosphaturia, and high urinary calcium excretion (10).

Several studies have investigated the source of cystine in cystinotic lysosomes, which is generated by the oxidation of two molecules of cysteine. Experimental data using radiolabeled cystine in cystinotic fibroblasts have shown that part of the lysosomal cystine pool originates from the uptake of extracellular cystine (27). Of note, lysosomal cystine levels do not increase in cystinotic fibroblasts after incubation with cysteine (130). Proteolysis within lysosomes also contributes

Fig. 1. Immunohistochemical staining of control and cystinotic renal tissue. Semithin sections were treated with antibodies against megalin, cubilin, albumin, and β2-microglobulin (β2m). Both the receptors megalin and cubilin are expressed on the brush border of the convoluted and straight proximal tubules in control and cystinotic tissue. In convoluted proximal tubules of the cortical labyrinth, the ligands albumin and β2m are present in apical vesicles in both control and cystinotic kidney. Staining for the ligands in straight proximal tubules in the medullary ray is more abundant in cystinotic tissue. Adapted from and reprinted from Am J Kid Dis with permission.
significantly to cystine accumulation, as it has been demonstrated by incubation of cystinotic fibroblasts, preincubated with cysteamine for cystine depletion, with culture medium containing BSA (129). Cystine accumulation after incubation with BSA could be inhibited by the lysosomotropic drug chloroquine, indicating that cystine accumulation within lysosomes was caused by lysosomal proteolysis of albumin. In renal proximal tubules, most of cystine uptake is mediated by the apical heterodimeric transporter $b_0^{10}\cdot$AT-rBAT, encoded by the $b_0^{10}\cdot$AT1/SLC7A9 and rBAT/SLC3A1 genes (35, 105). Mutations in these genes cause cystinuria (MIM 220100), which is characterized by urinary losses of cystine and dibasic amino acids. Transporters involved in cystine reabsorption in different tissues have not yet been completely elucidated. A thorough review of renal amino acid transport pathways has been recently published by Camargo et al. (14). To what extent apical uptake of cystine vs. the hydrolysis of reabsorbed proteins contributes to proximal tubular cystine accumulation in cystinosis remains unclear. Recently, Nielsen et al. (91) demonstrated a novel pathway of proximal tubule lysosomal biogenesis, namely, the apical reabsorption of lysosomal hydrolases. Whether this pathways is altered in cystinosis, and how it influences renal cystine accumulation, has not been studied so far.

Following breakthrough observations in cystinotic fibroblasts, Jonas et al. (61) have demonstrated that lysosomal cystine efflux was dependent on the activity of a proton pump ATPase. These authors showed that cystine efflux from CDME-loaded lysosomes was stimulated by the hydrolysis of exogenous ATP and that this effect was absent in lysosomes isolated from cystinotic cells. Stimulation of proton-ATPase activity resulted in the acidification of lysosomes from both control and cystinotic cells and correlated with cystine efflux in normal lysosomes, indicating the cooperation between two distinct transporters (60).

In the cytosol, most cystine is readily reduced into free cysteine by cell reducing systems, mainly by the glutathione ([GSH/GSSG] redox couple. In proximal tubular cells, cytosolic cysteine is also generated de novo from methionine (transsulfuration pathway) and is a substrate for several transporters located in the apical and basolateral membrane (see below) (36). A significant part of the intracellular cysteine pool contributes to the synthesis of glutathione (GSH) through the γ-glutamyl cycle. Interestingly, 5-oxoproline, one of its intermediate metabolites, is increased in the urine of cystinotic patients who are not treated with cysteamine (110). Figure 2 summarizes the main pathways involved in the regulation of lysosomal and cell thiol pools.

Fig. 2. Simplified overview of epithelial cystine metabolism and transport. Cystine can enter the lysosomes via cystine-containing proteins (protein-cys) or enter the cell via $b_0^{10}\cdot$AT/rBAT transporters at the apical membrane. After protein degradation, cystine efflux from the lysosomes via CTNS is mediated by a proton gradient. Defects in the CTNS gene lead to the characteristic elevation of cystine levels observed in cystinosis. One mole of cytosolic cystine is reduced to two moles of cysteine, which is the limiting substrate for the γ-glutamyl cycle. This pathway is responsible for the production of GSH, a major antioxidant of the cell. GSH levels are also maintained via SDCT2 and OAT1/OAT3 transporters located at the basal membrane or via reabsorption of filtered GSH at the apical membrane. Additionally, cysteine can be produced de novo from methionine or transported via LAT-2.
The Molecular Basis of Cystinosis

The Mendelian inheritance of cystinosis was recognized very early after the initial descriptions. By linkage analysis the gene defect was mapped to the region of chromosome 17p spanning the D17S1583 and D17S796 markers (128). In 1998, the CTNS gene was mapped to the 17p13.2 region and was cloned (135). Molecular diagnosis in the following years have allowed the identification of >90 different mutations in patients with cystinosis (2, 4, 37, 39, 134). The CTNS gene is composed of 12 exons, of which the first two represent non-coding regions (39). The gene spans 23 kb and encodes for a 367-amino acid protein, containing 7 putative transmembrane domains and two lysosomal targeting motifs. The functional relevant part of the CTNS promoter has been restricted to the region encompassing nucleotides −316 to +1 with respect to the start codon (102). The most common mutation, accounting for ∼75% of the affected alleles in Northern Europe, is a 57-kb deletion (3, 134) removing the first 9 exons and a part of exon 10 of the CTNS gene, the upstream 5′ region that encodes for the CARKL gene and the first two noncoding exons of the TRPV1 gene (Fig. 3A). The function of the CARKL gene has been identified in the phosphorylation of sedoheptulose, an intermediate metabolite of the pentose phosphate pathway (138). Consequently, patients with a homozygous 57-kb deletion have elevated blood and urinary levels of sedoheptulose, which can be used as a fast screening method in families carrying this mutation. Phosphorylation of sedoheptulose is connected to the pentose phosphate pathway and therefore might contribute to alterations in cellular redox status. Since the pentose phosphate pathway is responsible for the production of NADPH, which has similar antioxidant functions as GSH, it could be speculated that defects in CARKL might influence the NADPH levels, hence, intracellular redox status (138).

The transient receptor potential channel, vanilloid subfamily member 1 (TRPV1), encodes for an ion channel that is primarily expressed in sensory nerves and is activated by a wide range of chemical stimuli, including capsaicin, the active ingredient in chili peppers (100). TRPV1 is implicated in various biological processes, such as heat sensing, mechanical and thermal hyperalgesia, and anxiety behavior (92). It has been suggested that TRPV1 activation protects against salt-induced renal damage (139). The effective contribution of the CARKL or TRPV1 genes in the pathogenesis of the disease in patients with a homozygous 57-kb deletion has not yet been fully studied, although it has been suggested that these patients have a more severe extrarenal phenotype and higher mortality (43). Larger studies based on international registries are needed to elucidate these aspects. These registries are in the process of being constituted. In addition to the 57-kb deletion, smaller deletions, insertions, nonsense mutations, missense mutations, mutations within the promoter region, and splice site mutations have been reported. Recently, Taranta et al. (127) also identified intronic mutations in two separate families in one or both CTNS alleles that affect splicing of exon 5 or exon 9. These results indicate that cystinosis is probably a monogenic disorder and that cDNA sequence analysis should be performed when gene sequencing does not allow identification of mutations (127).

In vitro studies of residual cystine transport activity have shown that infantile cystinosis generally results from severe mutations that lead to complete loss of cystine transport activity (63). Of notice, cystine levels increase up to 100-fold in affected individuals compared with control subjects, whereas heterozygous carriers of CTNS mutations demonstrate only a slight increase in cystine levels without clinical consequences. This indicates that expression of one normal allele is sufficient to prevent significant cystine accumulation, despite that in vitro studies have shown that cystine efflux in lysosomes isolated from heterozygous carrier cells is intermediate to the efflux measured in control and cystinostic lysosomes (47).

Recently, a transcript variant of the CTNS gene has been reported by Taranta et al. (126), who identified a CTNS isoform, termed CTNS-LKG. This isoform originates from an alternative splicing of exon 12, which replaces the lysosomal targeting motif GYDQL at the C terminus by a longer amino acid sequence (Fig. 3B). Overexpression of the LKG transcript in renal HK-2 cells showed expression of cystinosin LKG in the plasma membrane, in lysosomes, in the endoplasmatic reticulum, in the Golgi apparatus, and in small intracellular vesicles. The role of this isoform in cells is currently under investigation.
The identification of the CTNS gene has also allowed confirmation that the CTNS gene encodes for a proton-driven lysosomal cystine carrier (62). Kalatzis et al. (62) have deleted the lysosomal targeting motif, redirecting cystinosin to the cell membrane with the intralysosomal domains facing the extracellular medium. Using this model, they were able to measure specific cystine transport when they applied a proton gradient by lowering the pH of the extracellular medium. Furthermore, the lysosomal localization was proven by colocalization studies using green fluorescent protein-tagged cystinosin and the lysosomal marker LAMP-2 (17). Overall, these studies have fully confirmed the results obtained by the pioneering studies of the late 1970s and early 1980s (44, 61, 118).

Models for Studying Cystinosis

Although the basic mechanism of lysosomal cystine accumulation in cystinosis has been unraveled, the pathogenesis of the disease is not yet fully understood. To gain more insights into underlying mechanisms leading to cell dysfunction, several research models have been developed.

Animal models. Since cystinosis is a multisystemic disease, animal models have been developed to study the pathogenesis of the disease in affected organs. Lysosomal loading with CDME in adult rats has been used to mimic the cystinotic phenotype (38). Parenteral administration of 400 μmol CDME twice a day cause symptoms of Fanconi syndrome, such as polyuria, phosphaturia, glucosuria, and aminoaciduria. Remarkably, during the initial 4 days following CDME injections, creatinine clearance and renal intracellular cystine levels did not change significantly, as opposed to increased cysteine levels. This raises the question of whether cystine accumulation is directly responsible for development of Fanconi syndrome, at least in this model, and whether CDME exerts a specific toxic effect that is unrelated to cystinosis.

The first ctns−/− knockout mouse model was generated in a mixed 129Sv and C57BL/6 strain by replacing the last four ctns exons (18). This mutation was reported to result in complete abolishment of cystinosin-mediated cystine transport (5). Although cystine accumulation resulted in ocular, muscular, neurological, and bone abnormalities, 129Sv (5–9 mo old) mice did not develop proximal tubular dysfunction or renal failure. Interestingly, when further bred for 10 generations with C57BL/6 strains, ctns−/− mice aged 15 mo developed incomplete tubulopathy and renal failure, as opposed to crossing them with FVB/N mice, which did not lead to the development of significant renal disease (89). These results indicate that the renal phenotype is dependent on the genetic background in mice and raise the hypothesis that modifier genes may influence the human phenotype (82). Ocular defects in mice, on the other hand, are very similar to those observed in humans (64).

To test whether gene transfer can reverse the cystinotic phenotype, Hippert et al. (56) have transduced the liver of ctns−/− mice backcrossed on a C57BL/6 background with adenovirus vectors expressing the wild-type human CTNS gene. Their results show that gene transfer can restore, at least in part, lysosomal cystine transport in young mice (2–3 mo old) but not in older mice (5–9 mo old), despite the equal transduction efficiency of 20–75% (56). Very promising data were also recently reported in C57BL/6 ctns−/− mice, after syngeneic bone marrow cell (BMC) transplantation using cells harvested from wild-type mice (2–4 mo of age) (125). After BMC transplantation, intracellular cystine levels decreased by >50% in all tested organs, including kidneys, brain, and liver. In addition, progression of renal failure was prevented and corneal cystine crystals markedly decreased. These results are poorly explained by the number of engrafted cells in tissues (<15%) and require further studies to understand these observations. Nevertheless, they open new perspectives for future treatment of cystinosis.

In vitro models for studying cystinosis. Much of our current knowledge on the pathogenesis of cystinosis has been obtained using in vitro cell models. The pioneering studies of the 1960s and 1970s were performed mainly on human cystinotic leukocytes, fibroblasts, or lymph node cells, allowing the establishment of lysosomal cystine accumulation as the basic defect in cystinosis (98, 118, 119).

To further study in vitro the biochemical pathways involved in the pathogenesis of the renal phenotype of cystinosis, renal cell models are desirable. Currently, many renal epithelial cells are available, each of which have advantages and limitations that have been recently reviewed by Bens and Vandewalle (9). Commerically available cell lines from pig (LLC-PK1), opossum (OK), or dog (MDCK) have proved their utility for membrane transport studies, especially since these cells are polarized when cultured on permeable supportive membranes (32, 49, 81). To study more specifically human transport systems in the proximal tubule, human proximal tubular cell lines have also been developed such as the HK-2 cell line, which are immortalized using, e.g., HPV 16 E6/E7 genes, to maintain proliferation (111). Alternatively, isolated perfused tubules have been used.

For cystinosis research, many investigators have used these cell lines and models after loading lysosomes with CDME. Preincubation of isolated renal tubules with CDME results in a significant increase in cystine levels, mimicking the cystine accumulation observed in cystinosis (38). Salmon and Baum (113) have perfused rabbit proximal tubules with CDME and documented decreased bicarbonate and glucose absorption. Furthermore, decreased ATP levels were demonstrated after CDME loading (24). However, direct toxicity of CDME has also been demonstrated in control fibroblasts and in renal HK-2 cells (144). Specifically, CDME inhibits mitochondrial ATP production and generates superoxide radicals in control cells. These effects are not observed in cystinotic fibroblasts with comparable cystine levels, which severely question the validity of the CDME model in cystinosis.

To overcome these limitations, attempts have been made to culture cells directly from cystinotic kidneys. Primary renal cell lines have been generated from autopsy specimens obtained from two deceased cystinotic patients, demonstrating cystine accumulation, as observed in cystinotic fibroblasts (99). Unfortunately, these cells could only be cultured for a maximum of seven passages.

Alternatively, human urine can be used to establish epithelial renal cell cultures, as first reported by Sutherland and Bain using newborns samples (124). This method was further refined in the 1980s (30, 55). Exfoliated renal cells collected from urine of a cystinotic patient have been used by Racusen et al. (106, 107) to establish a proximal tubular cell line. These cells presented epithelial characteristics and had cystine levels that were increased 100-fold compared with control cells. Using a similar approach,
primary cystinotic proximal tubular cells (71) and cell lines transfected with the HPV 16 E6/E7 genes (145) were generated to study cell metabolism in cystinosis. The advantage of the latter method is that cells maintain proliferation allowing one to obtain sufficient and homogeneous materials. However, cystine levels in HPV 16 E6/E7 immortalized cells are only 10-fold higher compared with control cells, probably due to their high proliferation rate, while levels in human cystinotic kidney tissue are increased by 60- to 350-fold (45).

To limit the proliferation rate, a novel proximal tubular epithelial cell (PTEC) model was developed, using a temperature-sensitive vector, SV40T tsA58 (SV40T), that stimulates proliferation only at lower temperatures (33°C). These cells are referred to as conditionally immortalized PTEC (ciPTEC) (142). When ciPTEC are transferred to 37°C, the expression of the SV40T antigen ceases and proliferation is inhibited, allowing cells to differentiate. We have successfully used this strategy in combination with transfection of the human telomerase reverse transcriptase (hTERT) gene to prevent the cells from undergoing replicative senescence (12) and have generated ciPTEC from control and cystinotic urine samples (75). When grown at 37°C, ciPTEC maintain proximal tubular characteristics, including expression of aminopeptidase N, zona occludens 1, aquaporin-1, dipeptidyl peptidase IV, multidrug resistance protein 4, and alkaline phosphatase activity (142). Furthermore, the expression and functional activity of the apical P-glycoprotein transporter and basolateral organic cation transporter 2 were demonstrated, together with endocytic uptake of albumin and sodium-dependent phosphate uptake, allowing the use of this model to study in vitro proximal tubular cell metabolism and transepithelial transport. Compared with cystinotic HPV 16 E6/E7 cells, cystine levels are about sixfold higher in cystinotic ciPTEC. Compared with control cells, levels are on average 37-fold higher, approaching results observed in vivo (75).

Studies are underway using cystinotic ciPTECs to delineate the mechanisms involved in the tubular cell dysfunction observed in cystinosis. In particular, polarized ciPTEC grown on permeable supports should allow the study of transcellular transport under various physiological and pharmacological stimuli and intracellular protein trafficking. Table 1 summarizes available models that can be used for investigating the pathogenesis of nephropathic cystinosis.

**Pathogenesis of Nephropathic Cystinosis**

Despite the fact that the CTNS gene was cloned more than 10 years ago, the pathogenesis of nephropathic cystinosis is not yet fully understood. In particular, no convincing explanation has been documented linking lysosomal cystine accumulation to cell dysfunction and renal Fanconi syndrome. Herein, we will summarize the three major hypotheses that have been proposed over the past years, namely, altered ATP metabolism, increased apoptosis, and cell oxidation. For the most part, these hypotheses have been formulated using cell models and are not necessarily mutually exclusive.

**ATP metabolism in cystinosis.** Initial studies performed after exposure of perfused renal tubules or cell lines to CDME have led to the hypothesis that altered ATP metabolism is the primary cause for diminished tubular reabsorption in Fanconi syndrome (8, 24, 85, 113). Inhibition of Na-K-ATPase activity as a consequence of decreased ATP levels was suggested to reduce the transcellular sodium gradient, leading to decreased sodium-dependent transport. In line with this hypothesis, impaired ATP metabolism causing Fanconi syndrome has been reported in patients with mitochondrial disorders (31, 54, 90, 136). Additionally, histological studies in cystinotic renal tubular cells have revealed swollen mitochondria, suggesting a defect in cell metabolism related to decreased mitochondrial oxidative phosphorylation (59).

In the CDME model, however, cystine accumulates in lysosomes, but cystine efflux is not compromised, as opposed to cystinotic cells lacking a functional cystine transporter. Furthermore, CDME may exert a direct toxic effect on cells, as already mentioned (144). Therefore, results obtained after loading of cells with CDME should be viewed critically and most likely do not mimic the pathophysiology of cystinosis in vivo.

Table 1. Pros and cons of models for studying the pathogenesis of cystinosis

<table>
<thead>
<tr>
<th>Model Type</th>
<th>Pro</th>
<th>Con</th>
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<tr>
<td><strong>Mouse</strong></td>
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<tr>
<td>129Sv − C57BL/6</td>
<td>Multisystemic; ocular, muscular, neurological, and bone abnormalities.</td>
<td>No proximal tubulopathy, no end-stage renal disease.</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Multisystemic; mild tubulopathy, end-stage renal disease.</td>
<td>Selective mild tubulopathy compared with humans.</td>
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<tr>
<td>Blood cells</td>
<td>Easy to obtain; cystine accumulation due to CTNS mutation.</td>
<td>No phenotype.</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Relatively easy to obtain; cystine accumulation due to CTNS mutation; established methodology for studying cell metabolism.</td>
<td>No phenotype.</td>
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<tr>
<td><strong>Renal epithelial cells</strong></td>
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<tr>
<td>Autopsy material</td>
<td>Renal phenotype; cystine accumulation due to CTNS mutation.</td>
<td>Very limited availability.</td>
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<tr>
<td>CDME-loaded control renal cells</td>
<td>Renal phenotype; cystine transport can be studied; tubular function decreased.</td>
<td>Functional CTNS transport; high toxicity when cystine levels are comparable to cystinosis levels.</td>
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<tr>
<td>Derived from urine Primary</td>
<td>Renal phenotype; cystine accumulation due to CTNS mutation; tubular function decreased.</td>
<td>Control cells should be obtained in parallel; limited proliferation.</td>
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<tr>
<td>HPV E6/E7</td>
<td>Renal phenotype; cystine accumulation due to CTNS mutation.</td>
<td>Relatively low cystine levels; difficulties in obtaining control cells using the same method.</td>
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<tr>
<td>SV40T/hTERT</td>
<td>Renal phenotype; cystine accumulation due to CTNS mutation; high cystine levels; functional transport.</td>
<td>Difficulties in obtaining control cells using the same method.</td>
</tr>
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CDME, cystine dimethylester.
Nonetheless, several in vitro studies have reported decreased levels of ATP in cystinotic cells, including fibroblasts, PMN leukocytes (76), and renal epithelial cells (71). In most studies, however, a significant overlap in ATP levels was observed between control and cystinotic cells. Recently, abnormal mitochondrial ATP production due to impaired complex I activity has been reported in cystinotic cells (115), a finding that we were not able to replicate using cystinotic fibroblasts (76). Specifically, we observed unaltered activities of the respiratory chain complexes I, II, III, and IV, which generate the proton gradient in the inner mitochondrial membrane that allows for ATP synthesis by complex V (76). Moreover, the recent development of an assay to measure complex V activity in cell homogenates (86) allowed us to compare complex V-mediated ATP production in control and cystinotic fibroblasts. In line with the observation of intact mitochondrial ATP production, we observed normal expression and activity of complex V in cystinotic fibroblasts (143). Because reduced activity of the Na-K-ATPase has not been documented in cystinotic cells (76), we suggest that altered ATP metabolism is unlikely to represent a valuable explanation for the impaired sodium-dependent reabsorption that characterizes Fanconi syndrome.

It should be noted that ATP metabolism in vitro is mainly driven by cell glycolytic activity, while the majority of ATP in vivo is derived from mitochondrial oxidative phosphorylation. Furthermore, in vivo altered tubular transport could limit substrate availability for the citric acid cycle, thus limiting mitochondrial ATP production. This represents a clear limitation of cell models for the study of Fanconi syndrome in vitro.

**Apoptosis in cystinosis.** High apoptotic rates have been reported in cystinotic fibroblasts, in proximal tubular cells, and in CDME-loaded renal tubular cells, after triggering cell death with proapoptotic stimuli such as TNF-α and UV light (71, 96). Hypothetically, cystine accumulation alters lysosomes, causing leakage of the lysosomal membrane and the release of cystine in the cytosol, where it binds the proapoptotic protein kinase PKC-δ, stimulating apoptosis (97). Apoptotic cell death may explain the “swan-neck” deformity, which has been described in cystinotic proximal renal tubules (19, 79). Progressive development of “atubular” glomeruli could lead to progressive renal failure. This hypothesis is further substantiated by the observation of increased caspase-4 expression in areas of cystinotic renal tissues with a reduced number of proximal tubules (114). Caspase-4 is a member of the cysteine proteases that play an important role in programmed cell death. Moreover, an increased number of autophagosomes and autophagic vacuoles have been observed in cystinotic fibroblasts and renal epithelial cells, suggesting that altered autophagy also plays a role in cystinosis (115). These observations were associated with structural mitochondrial abnormalities and with increased production of reactive oxygen species.

**Glutathione metabolism in cystinosis.** In recent years, cystinosis research has focused on abnormalities in the metabolism of GSH after the observation of elevated urinary levels of 5-oxoproline (pyroglutamatic acid) in cystinotic patients by Rizzo et al. (110). Oxoprolinuria is not specific for cystinosis as it has been observed in other genetic disorders affecting glutathione metabolism, such as a deficiency of 5-oxoprolinase (MIM260005) or glutathione synthetase (MIM 266130) (93, 109). GSH is the main intracellular antioxidant, protecting cells against oxidative stresses (65). Theoretically, sequestration of cystine in lysosomes may deplete the cytosolic pool of cysteine, which is the primary limiting factor for the synthesis of GSH (Fig. 2). An alternative explanation of oxoprolinuria in cystinosis can be an impairment of the Na+/H+ coupled monocarboxylate transporter SLC5A8, which is predominantly responsible for the reabsorption of 5-oxoproline in renal proximal tubules (83). SLC5A8 dysfunction can cause lactaturnia as it has an obligatory role in renal lactate reabsorption (41).

GSH has been measured in several cell models, yielding inconsistent results. Decreased levels of GSH have been reported in cystinotic fibroblasts and proximal tubular cells (20, 71). Another study performed using human fibroblasts showed no differences in basal GSH levels but documented decreased GSH levels in cystinotic cells upon inhibition of ATP synthesis and after exposure to oxidative stimuli (80), suggesting that the activity of the ATP-dependent γ-glutamyl cycle is compromised in cystinosis. Contradicting these results, one study has recently demonstrated a normal GSH/GSSG ratio in a limited number of cystinotic cell lines, suggesting intact redox status (137).

GSH levels are normal both in cystinotic HPV 16 E6/E7 immortalized proximal tubular cells (145) and in cystinotic ciPTEC lines (75). GSH levels in ciPTEC are also comparable to levels measured in tubular cells in vivo (~5 mM) (70). However, in both cell models, GSSG concentrations are increased, suggesting an increased oxidative state. Alterations in the redox state of cystinotic ciPTECs had no effect on the oxidation of proteins or fatty acids, indicating that, at least in vitro, the total cell reduction capacity was sufficient to protect against oxidative damage. Higher metabolic activity, coupled with a nearly exclusive ATP generation from mitochondrial oxidative phosphorylation, may result in higher reactive oxygen species generation in vivo. Therefore, cystinotic tissues may be more vulnerable to oxidative stimuli than estimated in vitro.

**Complexities of cell models of cystinosis.** Altered cell GSH metabolism may promote apoptotic cell death and may cause mitochondrial oxidative damage (40, 96); similarly, mitochondrial abnormalities can predispose cells to apoptosis and may lead to decreased ATP production, which further decreases GSH synthesis. Therefore, the various hypotheses that have been formulated may represent different facets of a unique cascade of events that lead from impaired lysosomal cystine efflux to disruption of cell function and decreased renal tubular reabsorption. Inconsistencies between studies may reflect differences in experimental conditions, stages of cell dysfunction, and metabolic differences between fibroblasts and renal proximal tubules used for studying the pathogenesis of the disease.

For example, recent reports indicate that intracellular cysteine levels are not necessarily decreased in cystinotic cells as a consequence of cystine trapping into lysosomes (7). Consequently, it is unlikely that alterations in GSH metabolism are primarily caused by lack of substrate for the γ-glutamyl cycle. Other factors may contribute to altered GSH metabolism. The activity of the GSH transporters OAT1/3 and MRP2/4, for example, can modulate the apoptotic cascade (40), while proximal tubular cells can internalize GSH from their basolateral aspects via sodium-dependent SDCT-2 and OAT1/3 transporters (70). Moreover, GSH is filtered through the glomerulus and is degraded to cysteinyl-glycine by the ectoenzyme γ-glutamyl transferase in the BBM of proximal tubular epithelia (Fig. 2). Cysteinyl-glycine can then be reabsorbed via the PEPT2 apical transporter and contributes to the intracellular cysteine pool (42).
To add to the complexity of this model, cysteine levels and levels of expression of CTNS mRNA and cystinosin are mutually related, as recently reported by Bellomo et al. (7). Cell treatment with small interfering RNA against CTNS, for example, results in an increase in cell cystine and cysteine levels, while changing the cysteine/cystine redox state modifies CTNS mRNA levels. These findings indicate that CTNS expression is actively regulated and suggest that the CTNS gene plays a pivotal role in the regulation of intracellular thiols. Cysteamine treatment allows the depletion of intracellular cystine but, interestingly, also increases the intracellular GSH pool in both control and cystinotic ciPTEC, and thereby increases the cell capacity to deal with oxidative stress (75). Noteworthy, this effect might also be beneficial in other chronic kidney diseases in which increased oxidation is involved in the progression of renal failure and cardiovascular morbidity (95, 116, 122).

Overall, these data highlight the complex metabolic interconnections that occur in vivo, in particular in reabsorptive epithelia, where vectorial solute transport is dependent on cell energy and oxidative state and concurrently provides vital substrates that allow cells to generate energy and to maintain their redox state. Most likely, the complete sequence of events leading to Fanconi syndrome in nephropathic cystinosis cannot be elucidated using solely in vitro models and will require disposal of an in vivo model, such as ctns-/- mice, that can reproduce the human disease.

Directions for Future Research

Although cystine accumulation is the hallmark of cystinosis and is regarded as the primary defect due to mutations in the CTNS gene, increasing evidence indicates that cystine accumulation itself is not responsible (or insufficient) for metabolic alterations in cystinosis. This idea is substantiated by the persistence of renal Fanconi syndrome after cystine depletion with cysteamine in patients with cystinosis and by the absence of significant Fanconi syndrome in ctns-/- mice despite high levels of cystine accumulation in the kidney (89). Emerging evidence indicates that lysosomes are not merely recycling factories at the end of the endocytosis pathway but that they are dynamic organelles, which are involved in various physiological processes. Newly recognized functions of lysosomes include cell signaling, plasma membrane repair, phagocytosis, bone and tissue remodeling, cholesterol homeostasis, autophagy, apoptosis, and cell necrosis, which have been excellently summarized in recent reviews (78, 112). Interactions between lysosomes and other organelles via so-called “kiss-and-run” events or via fusion put them at the center of the cell machinery (112). Recent observations support the hypothesis that other lysosomal functions are impaired in cystinosis, in addition to transmembrane cystine transport. Accumulation of autophagosomes in cystinotic cells, as demonstrated by Sansanwal et al. (103), may indicate altered lysosome-autophagosome fusion. Although autophagy is generally considered a cell-protective mechanism, both excessive or defective autophagy can induce or contribute to cell death (101). Similarly to the other lysosomal disorders such as Gaucher disease (MIM 230800), plasma chitotriosidase activity was reported to be increased in a child with cystinosis (146). Chitotriosidase is associated with activated phagocytes; its expression and release are induced by lysosomal stress (57). Thus lysosomal stress can be present in cystinotic cells, which should also be further explored.

In conclusion, exploring cellular pathways beyond cystine accumulation in cystinosis is a major challenge for future research. In-depth studies of lysosomal biogenesis and intracellular trafficking may reveal new mechanisms involved in cystinotic cell dysfunction. Identifying proteins that interact with the lysosomal and with the LKG isoforms of cystinosin will probably also help to clarify these pathways. This will be of great importance not only for elucidating the pathogenesis of cystinosis but also for expanding our knowledge of lysosomal function in general.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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