Pathogenesis of renal disease in nephropathic cystinosis

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Lysosomal cystine accumulation leads to alterations in the metabolism of antioxidant glutathione (GSH) resulting in decreased cellular capacity to deal with oxidative stress. In response to oxidative stress, cystinotic cells might have increased production of cytokines and chemokines. These mechanisms can contribute to the development of interstitial fibrosis. In this project, we aim to study the oxidative status and albumin-mediated production of pro-inflammatory and pro-fibrotic substances in a conditionally immortalized proximal tubular cell model (ciPTEC). Additionally, we aim to develop podocytes with the cystinotic genotype to evaluate morphological or metabolic changes.

This end report describes the findings of the Fellowship Grant funded by the Cystinosis Research Foundation. Currently, a scientific paper, concerning the data on glutathione and oxidation status, is published (see annex 1).

The second paper on albumin-induced cytokine and chemokines production is now under review by coauthors and will be submitted shortly.

Aim I Evaluation of albumin-mediated cytokine and chemokine production in ciPTEC

a) Cell lines used in this project

Cell lines generated and used in this project are listed in table 1.

<table>
<thead>
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<th>cell line</th>
<th>age (months)</th>
<th>sex (f/m)</th>
<th>Mutation</th>
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<td>m</td>
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Table 1: Overview of the ciPTEC cell lines developed from urine of patients with cystinosis. Patients with hom 57kb del have both dysfunctional CTNS and CARKL. \(^1\) age in months at collection of urine

To address the influence of CTNS and CTNS-LKG (a recently described cystinosin isoform) expression, we have succeeded in the transient transfection of both CTNS and CTNS-LKG isoforms in one cystinosis cell line (PT46.2) carrying the homozygous 57kb deletion using pcDNA3.1 expression vector and Lipofectamine (Invitrogen Life Technologies). Cystine levels were measured using HPLC (figure 1). Although transfection was confirmed by rtPCR, cystine levels were only moderately decreased in those cells transfected with CTNS or CTNS-LKG.

Figure 1. Intracellular cystine levels in ciPTEC after transfection with CTNS or CTNS-LKG, measured using HPLC. The vectors used contained either a normal stop codon (stop) or V5-tag (V5). Several clones of each construct were obtained and analyzed.

Since transient transfection resulted in cystine levels that were only moderately decreased and were not in the range of levels observed in heterozygous mutated subjects, we have developed lentiviral constructs containing CTNS and CTNS-LKG genes. These constructs were successfully used for stable transduction of control and cystinotic ciPTEC (Figure 2).
Figure 2. Cells transduced with 3F-tagged CTNS (upper 2), 3F-tagged CTNS-LKG (lower 2). CTNS and CTNS-LKG were visualized with rabbit-anti-Flag/anti-rabbit-Alexa488 antibodies (green) and a digital imager under a fluorescence microscope. These pictures demonstrate the successful stable transduction of control and cystinosis ciPTEC with constructs 3F CTNS and 3F CTNS-LKG.

Cystine levels in these lentiviral transduced cell lines were substantially better rescued by lentiviral constructs compared to the transient transfected cell lines (Figure 3).

Figure 3: Cystine levels measured in transduced control and cystinotic ciPTEC. Prior to harvesting, cells were differentiated for 10 days at 37°C. Transduction of CTNS into the cystinotic ciPTEC clearly results in decreased of cystine levels.

b) Albumin uptake is normal in cystinosis cells.

We have previously shown abundant proximal tubular megalin and cubilin expression and the presence of megalin and cubilin ligands in endocytotic vesicles in a kidney specimen of a cystinosis patient (Wilmer et al. Am J Kid Disease 2008).

Recently, we have demonstrated the presence of megalin expression in cell membrane fractions of cystinotic and control ciPTEC on Western blot (data not shown).

Both cystinotic and control ciPTEC demonstrated uptake of bovine serum albumin (BSA), which could be inhibited by receptor associated protein (RAP) and by excess of BSA (figure 4), suggesting megalin mediated uptake. No difference in BSA uptake was observed between cystinotic and control cells.
Figure 4: Albumin uptake in ciPTEC. (A) Albumin uptake in both control and cystinotic ciPTEC was decreased by megalin-ligand RAP in a concentration dependent manner. (B) Flow cytometry analysis of BSA-FITC uptake in control cell lines (n=4) and cystinotic cell lines (n=7) demonstrated that albumin uptake in cystinotic cell lines was normal compared to control ciPTEC (p = 0.75). Gray dots represent cystinotic patients with a homozygous 57 kb deletion. No difference in albumin uptake was observed between cells carrying a homozygous 57 kb deletion compared to cells having other mutations.

Interestingly, using the same methodology, decreased BSA-FITC uptake was observed in ciPTEC obtained from a patient with Dent’s disease (data not shown).

c) Effect of albumin on cytokines and chemokines production by cystinotic and control ciPTEC.

The effect of BSA reabsorption on the production of the cytokines TGF-β1 and interleukin 8 (IL-8) and the chemokine monocyte chemoattractant protein 1 (MCP-1) was tested using an ELISA based assay. The range of albumin concentrations were calculated from urine albumin content and renal function in cystinotic patients, hence, correspond to the concentrations in the tubular lumen of cystinotic patients. For all measured substances, an albumin-dependent response was observed in both control and cystinotic ciPTEC (figure 5). After correction for protein levels, cystinotic cells demonstrated a higher production of IL-8 (p=0.001) and a trend of higher production of MCP-1 (p=0.09), and lower production of TGF-β1 than control cells (p=0.01).
Figure 5. Chemokine and cytokine production in ciPTEC increased after induction by BSA (range 20, 50, 200 or 500 µg/ml). (A) TGF-β1, cystinosis versus control p=0.01 (B) IL-8, cystinosis versus control p=0.001 and (C) MCP-1 production, cystinosis versus control p = 0.09. Paired t-test: * p<0.05; ** p<0.01 compared to untreated sample.

As demonstrated in the 12 months progress report, the influence of cysteamine on albumin-induced cytokine/chemokines production could not be studied at the protein level as at least 24 hours incubation with albumin were required for obtaining measurable cytokine/chemokines concentrations in the supernatant of ciPTEC. This, because the medium for cysteamine treatment has to be replaced every 6 hours (due to instability of cysteamine), the evaluation of cysteamine effect was not feasible.
d) Effect of cystinotic and control urine on cytokines and chemokines production by cystinotic and control ciPTEC.

To study whether other substances in cystinotic urine (next to excessive amounts of albumin) could induce production of pro-inflammatory and pro-fibrotic substances, we incubated cystinotic and control ciPTEC with diluted control and cystinotic urine (derived from patients with overt Fanconi syndrome). Dilutions were calculated to obtain similar concentrations as estimated in the proximal tubular lumen. As demonstrated in figure 6, both cystinotic and control urine could induce the production of IL-8 and MCP-1, however, no difference was observed between the effects on cells exposed to control versus cystinotic urine. Interestingly, TGF-β1 production was induced by control urine, but decreased after incubation with cystinotic urine in both cystinotic and control cells.
Figure 6: Chemokine and cytokine production in ciPTEC are increased due to incubation with urine. (A) TGF-β1, (B) IL-8 and (C) MCP-1 production is stimulated after exposure of ciPTEC to urine, which was diluted 1:15, corresponding to the dilution factor comprising the physiological tubular concentration. * p<0.05; ** p<0.01 compared to untreated sample.

e) Vimentin expression in cystinotic versus control ciPTEC

Staining of ciPTEC for the intermediate filament protein vimentin, an important marker of epithelial-to-mesenchymal transformation (EMT), revealed a trend towards increased vimentin staining in cystinotic ciPTEC compared to control ciPTEC (figure 7).

![Vimentin expression in cystinotic versus control ciPTEC](image)

Figure 7: Control and cystinotic ciPTEC were stained for vimentin and analyzed using FACS. A trend was observed towards more cystinotic vimentin positive ciPTEC (p>0.05).

These experiments will be repeated followed BSA incubation.

f) Urinary excretion of cytokines and chemokines.

We measured the quantity of MCP-1 present in control vs. cystinotic urine. A significant increased amount of MCP-1, corrected for creatinin, was found in cystinotic urine (figure 8). Once more, this is in accordance with our hypothesis that proteinuria may contribute to the development of fibrosis and renal disease due to the production of pro-inflammatory and pro-fibrotic substances.

![Urinary excretion of cytokines and chemokines](image)

Figure 8: Detection of MCP-1 in urine, corrected for creatinin. Cystinotic urine contains elevated MCP-1 levels compared to control urine (p<0.001).
Urinary concentration of TGF-β1 and IL-8 could not be detected in urine because of their instability.

**Aim II Evaluation of ROS production and apoptosis in cystinotic ciPTEC.**

This part of the project was extensively described in the 12 months report and has already been published in an international peer reviewed scientific journal. For this part, we refer to the publication entitled “Cysteamine restores glutathione redox status in cultured cystinotic proximal tubular epithelial cells” by Wilmer et al., Biochim Biophys Acta. 2011; 1812:643-651 (see annex 1).

In summary, we observed disturbed oxidative status in cystinotic ciPTEC, which could be restored by cysteamine. This beneficial effect points to a potential role of cysteamine as anti-oxidant for other renal disorders associated with enhanced oxidative stress.

**Aim III Evaluation of morphological and metabolic changes in cystinotic podocytes**

We have successfully isolated cells from urine sediments from 2 healthy volunteers and 5 cystinosis patients up till now. Noteworthy, less than 10% of the collected urine fractions resulted in outgrowth of viable cell populations. All cells were immortalized and subcloned and characterized by Western blotting (nephrin and podocin), RT-PCR (synaptopodin, CD2AP) and cystine content. The ciPodocytes are ready to be used in experiments determining the redox state (glutathione status). Transmission electron microscopy will be performed to compare ultrastructural morphology.

The lentiviral shRNA construct has been made and will be used to knock-out the CTNS gene in the control ciPodocytes in order to establish a cystinotic podocyte cell line including cystine accumulation.

**Summary/conclusions of the entire project**

1. Cystinotic cells demonstrate comparable to controls uptake of albumin (BSA).
2. In response to physiologically relevant concentrations of BSA, cystinotic cells produce higher amounts of pro-inflammatory chemokine IL-8 and cytokine MCP-1.
3. Urinary excretion of MCP-1 is increased in cystinosis patients.
4. Cysteamine treatment results in restoration of altered GSH redox status in cystinotic cells.

Taken together, these results point to higher pro-inflammatory response to albuminuria in cystinotic proximal tubular cells, which might underlie the development of the interstitial inflammation and fibrosis leading to end-stage renal disease. Use of ACE inhibitors reduces proteinuria in cystinosis and should be recommended to slow the progression of renal damage.

Further studies are focused on studying function of glomerular podocytes in cystinosis and on the effect of gene repair on protein uptake and cytokine/chemokines production in cystinotic PTEC.