Pathogenesis of renal disease in nephropathic cystinosis
3rd Progress Report, August 2010

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Lysosomal cystine accumulation might lead 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 a cytokine 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). The influence of different mutations in *CTNS*, leading to lysosomal cystine accumulation, and the additional deletion of the *CARKL* gene - which is mutually deleted in the common 57kb deletion - are subject of this research project. Additionally, we aim to develop podocytes with the cystinotic genotype to evaluate morphological or metabolic changes.
This 3rd progress report describes the findings of the Fellowship Grant funded by the Cystinosis Research Foundation in the first part of the second year. Currently, a scientific paper, concerning the data on glutathione and oxidation status, is submitted.
The second paper on albumin-induced cytokine and chemokines production is in preparation for submission.

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

a) Transfection of ciPTEC
Cell lines generated and used in this project are listed in table 1.

	cell line	age[1] (months)	sex	Mutation
PT1	Cys1	108	m	c.922_923insG
PT2	Cys2	78	m	hom 57kb del
PT4	Cys3	152	m	c.198_218del
PT13	Cys4	129	m	hom57kb del
PT23	Cys5	82	f	[57kb del]+[c.665A>G]
PT24	Cys6	82	f	[57kb del]+[c.665A>G]
PT25	Cys7	134	f	ND
PT41	Cys8	50	m	hom57kb del
PT46	Cys9	47	m	hom57kb del
PT47	cys10		m	[57kb del]+[c.696insC]
PT48	cys11	11	f	[57kb del]+[c.927_928insG]
PT53	cys12	78	f	[57kb del]+[c.del18_21GACT]
PT54	cys13	96	f	[57kb del]+[c.665A>G]
PT55	cys14	209	m	hom57kb del

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. ND: mutations in CTNS are not detected.

To address the influence of *CTNS* and *CTNS-LKG* (a recently described cystinosin isoform) expression, we have succeeded in the transfection of both *CTNS* and *CTNS-LKG* isoforms in one cystinosis cell line (PT46.2) carrying the homozygous 57kb deletion. Cystine levels were measured using HPLC (Figure 1). Although transfection was confirmed by rtPCR (shown in progress report I, June 2009), cystine levels were only moderately decreased in those cells transfected with *CTNS* or *CTNS-LKG*.

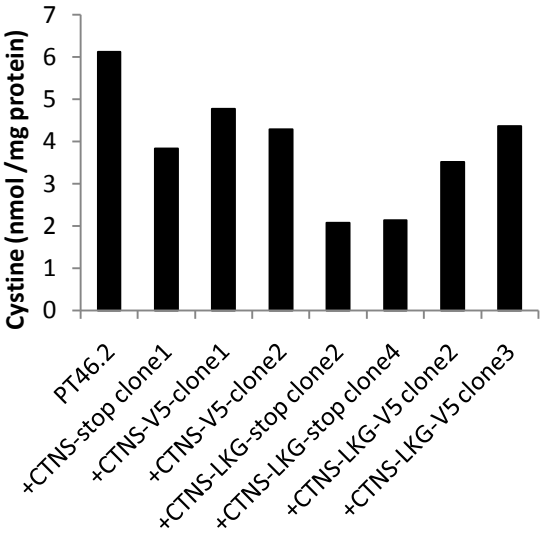

Figure 1. Intracellular cystine levels in ciPTEC after transfection with *CTNS* or *CTNS-LKG,* measured using HPLC. The vectors used contained either a normal stop codon (stop) or V5-tag (V5). Several clones of each construct were obtained and analyzed.

Since after transient transfection cystine levels are moderately decreased and 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 cells (Figure 2). Cystine levels in transduced cell lines will be measured in August/September 2010. In case of proved functionality, transduced cell lines will be further used for measuring the oxidative status and albumin-mediated cytokine/chemokines production. A lentiviral construct carrying the *CARKL* gene is currently under construction.

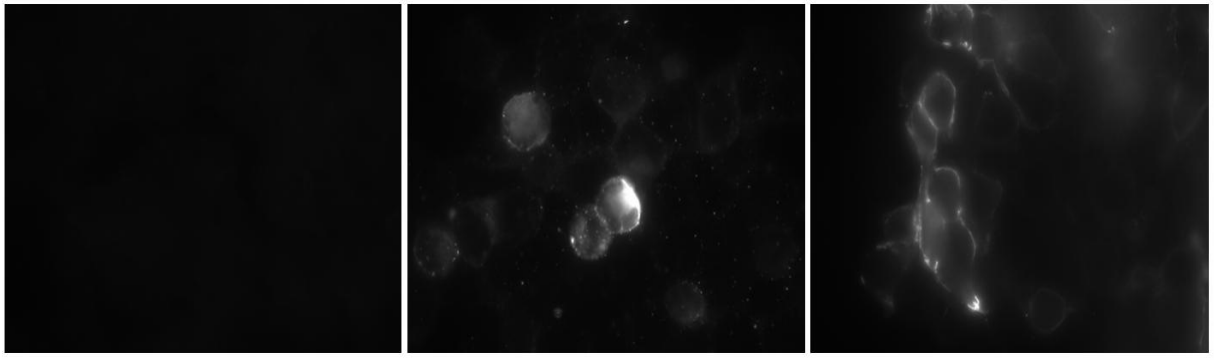

Figure 2. Cells transduced with Flag-tagged CTNS (middle panel), CTNS-LKG (right panel) or not transduced (left panel). CTNS and CTNS-LKG were visualized with rabbit-anti-Flag/anti-rabbit-Alexa488 antibodies and a digital imager under a fluorescence microscope.

b) Measuring albumin-induced production of pro-inflammatory and profibrotic substances in ciPTEC

Both cystinotic (n=8) and control (n=4) cells demonstrated uptake of bovine serum albumin (BSA), which could be inhibited by receptor associated protein (RAP) and by excess of BSA (see figure 3). No difference in BSA uptake was observed between cystinotic and control cells (data not shown).

The effect of albumin 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 in conditioned tissue culture medium (obtained after cellular albumin uptake) 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 concentrations in tubular lumen of cystinotic patients. For all measured substances, an albumin-dependent response was observed in both control and cystinotic ciPTEC (Figure 3). Cystinotic cells demonstrated a higher production of IL-8 and MCP-1, and lower production of TGF-β1 than control cells.

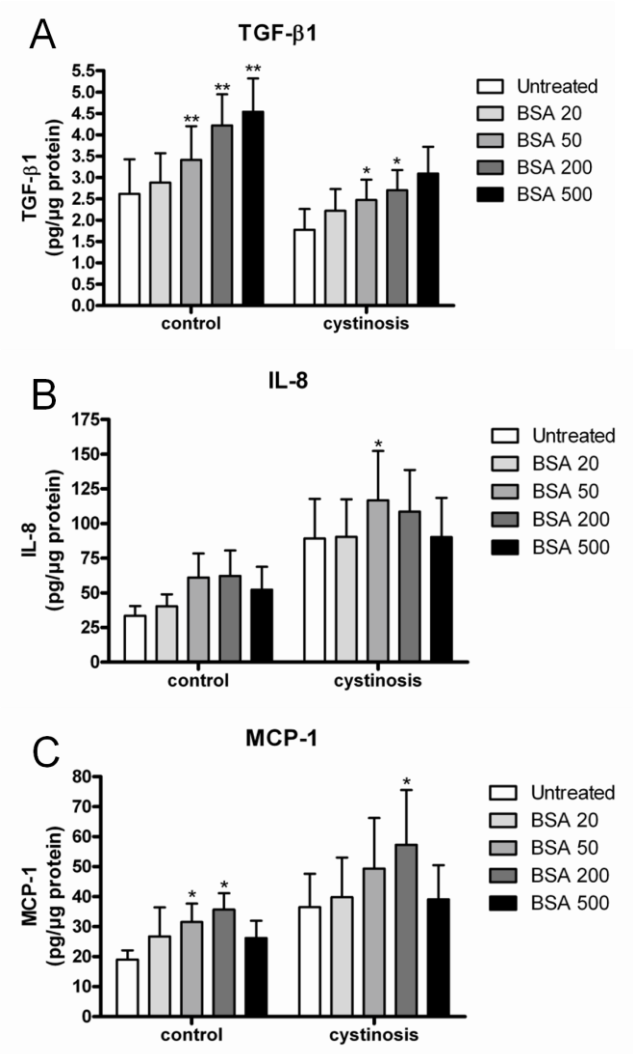

Figure 3. Chemokine and cytokine production in ciPTEC increased after induction by albumin. (A) TGF-β1, (B) IL-8 and (C) MCP-1 production is stimulated using BSA range (20, 50, 200 or 500 µg/ml), resulting in increased production of chemokines and cytokines. * $p<0.05$; ** $p<0.01$ compared to untreated sample.

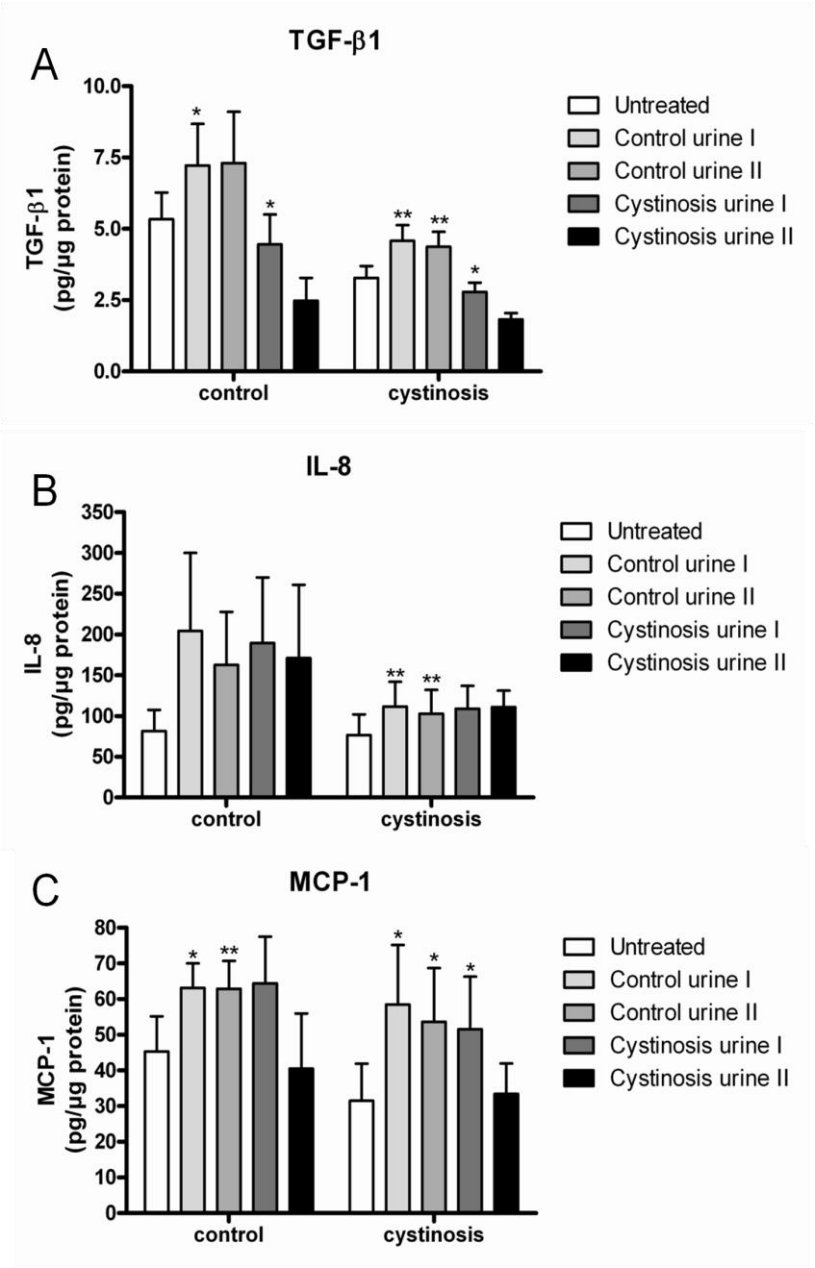

Figure 4. Chemokine and cytokine production in ciPTEC are increased due to incubation with urine. (A) TGF-β1, (B) IL-8 and (C) MCP-1 production is stimulated after exposure of ciPTEC to urine, which was diluted 1:15, corresponding to the dilution factor comprising the physiological tubular concentration. * $p<0.05$; ** $p<0.01$ compared to untreated sample.

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.

Next to measuring cytokine/chemokines concentrations in the supernatants, we are now evaluating the influence of BSA and cysteamine on cytokine/chemokines production at mRNA level using real-time PCR. The results of the first experiments demonstrate positive effect of cysteamine on the IL-8 production, however, these data needs confirmation.

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). As demonstrated in Figure 4, both cystinotic and control urine could induce the production of TGF- β1, IL-8 and MCP-1, however, no difference was observed between the effects on cells exposed to control versus cystinotic urine.

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

This part of the project was extensively described in the 12 months report. The manuscript describing this work is submitted and is currently under revision.

Aim III Evaluation of morphological and metabolic changes in cystinotic podocytes

The number of podocyte cell lines is currently being extended. Morphological studies are in progress. This part of the project will be continued by Dr. J. Schoeber, in his fellowship project, supported by CRF.