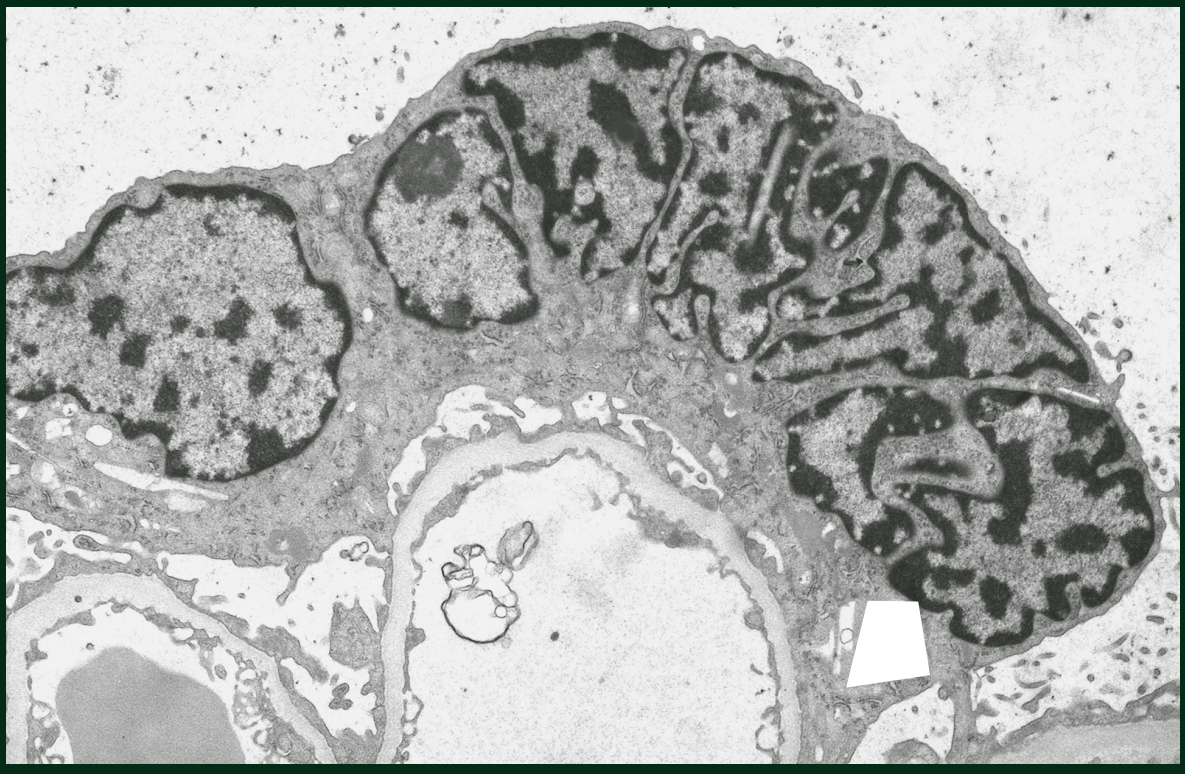
CYSTINOSIS

IMPROVED MEDICAL CARE
AND
NEW INSIGHTS INTO PATHOGENESIS



Elena Levtchenko

Cystinosis: improved medical care and new insights into pathogenesis

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Electron microscopy of the cystinotic renal tissue, showing the multinucleation of the podocyte, which is pathognomonic for cystinosis, and the contours of a cytoplasmic cystine crystal dissolved by the routine tissue processing.

Cystinosis: improved medical care and new insights into pathogenesis

een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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To the memory of my father
To my mother and Ivan
To Aimé, Anja and Philippe

Contents

New insights into pathogenesis of cystinosis

CHAPTER 1

Introduction and outline of the thesis

Introduction

Cystinosis (MIM 219800) is an autosomal recessive defect of lysosomal cystine carrier leading to the accumulation of amino acid cystine in the lysosomes. The estimated incidence of cystinosis is 1 in 100,000-200,000 life births [Gahl et al. 2001] with clustering reported in France, French-speaking population of Quebec, Germany and UK [Bickel et al. 1952, Bois et al. 1976, Manz et al. 1985]. Although the disease has also been observed in non-Caucasian patients, there are no epidemiological data available in these populations [Gahl et al. 2001].

Cystinosis was first described as a clinical entity by Abderhalden in 1903 [Abderhalden 1903] and recognized as a main cause of generalized proximal tubulopathy, called De Toni-Debré-Fanconi syndrome, in the late 1940's [Fanconi et al. 1949]. Real progress in the investigation of cystinosis started when amino acid chromatographic analysis allowed measuring elevated cystine concentrations in tissues of cystinotic patients [Schneider, Bradley et al. 1967, Schneider, Rosenbloom et al. 1967]. In the 1980's it was demonstrated that cystine accumulated within the lysosomes due to the impairment of cystine transport across the lysosomal membrane [Gahl,Bashan et al. 1982, Jonas,Smith et al. 1982]. Treatment with amino thiol cysteamine and the availability of renal transplantation dramatically improved the prognosis of patients with cystinosis allowing them to survive into adulthood [Gahl et al. 1987, Malekzadeh et al. 1977]. Cysteamine, however, does not reverse Fanconi syndrome in the majority of patients and multi-organ impairment continues after renal transplantation.

Clinical presentation and course of cystinosis

Three clinical forms of cystinosis varying in severity and age of onset are distinguished: the most frequent and most severe infantile form, the intermediate late-onset or juvenile form and ocular cystinosis [Gahl et al. 2001, Gahl et al. 2002].

Renal involvement

Infantile nephropathic cystinosis is the most frequent cause of inherited Fanconi syndrome in childhood. Fanconi syndrome is characterized by excessive urinary excretion of amino acids, phosphate, bicarbonate, glucose, sodium, potassium, low molecular weight proteins and other solutes, handled in renal proximal tubules [Foreman et al. 1998]. Patients

are usually asymptomatic at birth and develop normally until 3-6 months, when they manifest with failure to thrive, vomiting, constipation, polyuria and excessive thirst, periods of dehydration and sometimes rickets [Broyer et al. 1981, Niaudet et al. 1999, Schneider et al. 1990]. Growth retardation in case of late diagnosis may reach -4 SD [Niaudet et al. 1999]. Renal loss of sodium and potassium results in hyponatremia and hypokalemia, which may be life threatening. Hypouricemia, decreased plasma carnitine and medullary nephrocalcinosis related to increased calcium excretion are also observed [Saleem et al. 1995, Theodoropoulos et al. 1995]. Total protein excretion can reach several grams per day and contains both albumin and low molecular weight proteins. In untreated patients glomerular filtration declines gradually and progresses towards renal failure before the age of 10 years [Gretz et al. 1983, Manz et al. 1994]. Renal graft survival is generally better in patients with cystinosis compared to overall children and the disease does not recur in the transplanted organ [Ehrich et al. 1991, Kashtan et al. 1995].

Renal pathology

The age at which the first morphological changes appear in the kidneys of patients with cystinosis is unknown. No significant renal changes were observed in the fetus [Jackson et al. 1962]. Serial renal biopsies in 2 cystinotic patients demonstrated that the typical "swan-neck" deformity of proximal convoluted tubules appeared only after 6 months of life [Mahoney et al. 2000]. In a large series of kidney specimens, the most striking feature was the marked irregularities of renal tubular cells with the presence of flat cells with focal disappearance of the brush border and very large cells with a prominent and hyperchromatic cytoplasm [Gubler et al. 1999]. Glomeruli could appear normal, but most contained peculiar giant multinucleated podocytes, specific for cystinosis (see the illustration on the cover). Cystine crystals located in the lysosomes or in cytoplasm were seen mostly within interstitial cells and rarely within podocytes [Gubler et al. 1999]. Swollen mitochondria in the renal tubular cells seen by Jackson in two children of 2 and 3 years [Gubler et al. 1999], were not described in other series [Gubler et al. 1999, Mahoney et al. 2000, Spear et al. 1971].

The deterioration of renal disease is accompanied by progressive tubulo-interstitial lesions, including interstitial fibrosis, tubular atrophy and marked arteriolar thickening. The progressive glomerular damage, leading to increasing albuminuria and hematuria, consists of segmental or global collapse of the capillary tuft, accumulation of mesangial matrix material, and, observed by electron microscopy, irregular thickening of glomerular basement membrane and the effacement of podocyte foot processes [Gubler et al. 1999].

In transplanted kidneys cystine crystals, seen at graft biopsy, have no clinical relevance as they are present in the host mononuclear cells [Spear et al. 1989].

Extra-renal involvement

Ocular impairment

Ocular manifestations can be classified according to their localization and the type of treatment in those, affecting the anterior segment of the eye, treated by topical cysteamine drops, and the retinopathy, treated by systemic cysteamine.

Corneal cystine crystals, pathognomonic for the disorder, are absent at birth and generally can be seen by an experienced ophthalmologist at the age of 1 year. These crystals, causing reflections of light, result in photophobia with substantial discomfort. At 10-20 years, untreated patients may develop painful corneal erosions, punctate, filamentous or band keratopathy, iris crystals and peripheral corneal neovascularization [Kaiser-Kupfer et al. 1986, Tsilou et al. 2002]. Posterior synechiae leading to glaucoma, are rarely reported. The prevalence of the anterior segment complications increases with age [Tsilou et al. 2002].

The degeneration of the retinal pigment epithelium, resulting in patchy depigmentation of the retina starting in the periphery and extending in time, may cause visual impairment starting from the second decade of life [Broyer et al. 1981].

Endocrine impairment

The continuing multi-organ accumulation of cystine crystals leads to the impairment of endocrine organs. Hypothyroidism is found in up to 70% of untreated patients with cystinosis older than 10 years [Chan et al. 1970]. Impaired insulin production can be exacerbated by steroid therapy after renal transplantation and results in insulin-dependent diabetes mellitus [Fivush et al. 1987]. While the puberty generally proceeds normally in females, it is delayed in males, who may have primary hypogonadism and do not always complete puberal development [Winkler et al. 1993]. No male patient with cystinosis has been reported so far to have an offspring, while several females after renal transplantation have successful given birth [Reiss et al. 1988].

Central nervous system and muscle impairment

The prolonged survivals of patients with cystinosis after renal transplantation also revealed the impairment of central nervous and muscular systems, which previously were considered to be spared in these patients.

Two major types of cystinotic encephalopathy, developing mostly during the 3rd decade, are distinguished: the first, presenting with cerebellar and pyramidal signs, mental deterioration and pseudo-bulbar palsy and the second, associated with stroke-like episodes [Broyer et al. 1996]. The most common imaging finding in these patients is cere-

bral cortical atrophy seen on CT scan [Nichols et al. 1990], however, non-absorptive hydrocephalus, demyelinisation of the internal capsule and brachium pontis, mineralisation of hemispheres and basal ganglia have also been reported [Vogel et al. 1990].

Recently unexplained idiopathic intracranial hypertension with normal neuroimaging of the brain was described in 8 patients with cystinosis, warranting regular ophthalmic examination to rule out papilla edema [Dogulu et al. 2004].

The vacuolar myopathy, resulting from excessive cystine accumulation in the muscle, manifests generally after the 10th birthday [Gahl, Dalakas et al. 1988]. The patients suffer from progressive muscle wasting and reduced strength with restrictive ventilatory defects [Anikster et al. 2001]. High prevalence of swallowing difficulty (>50%) has been recently reported in 101 patients with cystinosis aged 6 to 45 years [Sonies et al. 2005]. On barium swallow examination the oral, pharyngeal and esophageal phases of swallowing were abnormal in 24%, 51% and 73% of the patients respectively. The severity of swallowing dysfunction had a direct relation with the severity of muscular disease and decreased with the number of years on cysteamine therapy [Sonies et al. 2005].

Intelligence and school performance

Although IQ values of children with cystinosis are in the normal range, they exhibit deficits in tactile recognition, spelling, mathematics and short-term visual memory, requiring adapted school tasks [Ballantyne et al. 2000, Williams et al. 1994]. Compared to healthy controls and to the chronic-disease control group, patients with cystinosis have significantly more social difficulties [Delgado et al. 2005].

Other clinical features

Diminished skin and hair pigmentation, liver and spleen enlargement, portal hypertension and hypersplenism are also observed [Broyer et al. 1987, Gahl et al. 2001, Scotto et al. 1977, DiDomenico et al. 2004]. Cytopenia, related to hypersplenism, is actually rarely seen in patients treated with cysteamine [Garnadoux et al. 1999]. Despite the fact that cystine crystals are found in the bone marrow of the cystinotic patients, there is no evidence that it has consequences for the hematopoiesis. Increased risk of bone fractures in transplated cystinosis patients can be related to cystine depositions in the bone, hypogonadism, diabetes mellitus and continued urinary phosphate wasting in some patients. Notably, dual-energy X-ray absorptiometry (DEXA scan) does not predict the risk of fractures in cystinotic patients possibly due to the false elevation of bone mineral composition by bone cystine crystals [Zimakas et al 2003].

Cystinosis in adults

Renal transplantation has transformed cystinosis from a fatal pediatric disease into a treatable one with which patients surviving into adulthood. The evaluation of 36 adult American cystinosis patients aged 17-34 years, revealed a high rate of mortality and morbidity. Seven patients died at the age 18-34 years from aspiration, pseudo-bulbar palsy, and uremia or had unexplained sudden death. Twenty two percent of the patients were blind or had severely impaired vision, 86% required thyroid hormone replacement, 30% had a distal myopathy and more than 60% had swallowing difficulties. Only 11/36 patients received adequate cysteamine treatment [Theodoropoulus et al. 1993]. The long-term prognosis of pediatric patients with cystinosis is probably better than those reported above due to the current early and continuous administration of cysteamine.

Transition from pediatric to adult care providers remains an area of concern as internists-nephrologists usually treating adult cystinosis patients are not trained to get experienced with this rare previously untreatable disorder. Good communication between paediatric and internists-nephrologists is required to guarantee the continuation of the adequate medical care [Kleta et al. 2005].

Other clinical forms of cystinosis

Less severe clinical forms of cystinosis account for less than 5% of all patients. Generally 2 variants are distinguished: nephropathic late-onset or juvenile cystinosis and non-nephropathic ocular cystinosis [Gahl et al. 2001].

Patients with nephropathic late-onset form (MIM 219900) manifest with a spectrum of symptoms, varying from a milder, compared to the infantile form, proximal tubulopathy to apparent nephrotic syndrome and generally have slower rate of renal disease progression [Gahl et al. 2001, Hauglustaine et al. 1976, Langman et al. 1985]. Cystine crystals accumulate also in the cornea and can be diagnostic. In term of the age of presentation there is probably a continium between the infantile and the late-onset form, however, most of the described patients were older than 10 years old.

Ocular non-nephropathic cystinosis (MIM 219750) affects cornea with cystine crystal deposits causing photophobia. The kidneys, retina and other organs are spared in these patients, but they do have elevated cystine leukocyte content and cystine crystals in bone marrow [Anikster et al. 2000, Schneider et al. 1968].

Genetic aspects

Cystinosis is caused by mutations in cystinosis gene (*CTNS*), which has been identified by positional cloning strategy in 1998 [Town et al.1998]. *CTNS* maps to 17p13, spans 23 kb of the genomic sequence and is composed of 12 exons, the first two of which are non-coding [Town et al.1998]. *CTNS* encodes a 367 amino acid protein, cystinosin, having 7 transmembrane domains and is highly glycosylated at the N-terminal (Figure 1). In contrast to other lysosomal membrane proteins, cystinosin contains 2 lysosomal-targeting sequences, one situated in C-terminal tail and the second – in the fifth inter-transmembrane domain (TM) loop [Cherqui et al. 2001].

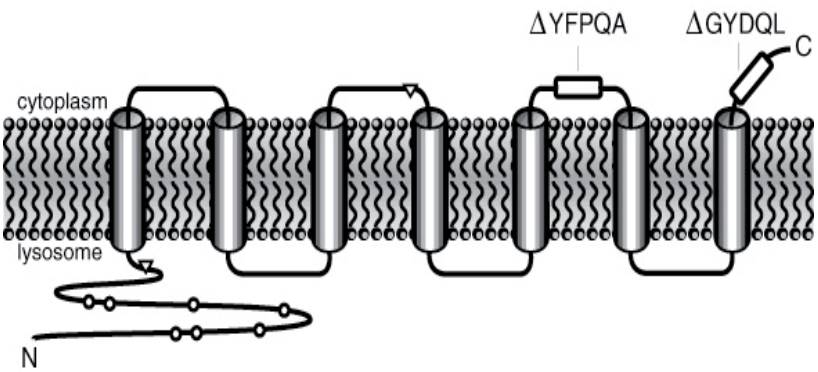

Figure 1 Schematic presentation of cystinosin in the lysosomal membrane with the two targeting motifs indicated (white bars).

Mutations of *CTNS* have been detected in the individuals with all 3 clinical variants of cystinosis, demonstrating that these forms are allelic [Anikster et al. 2000, Attard et al. 1999, Town et al. 1998]. The most common mutation found in the homozygous state in more than 50% of Caucasian patients is a large 57 kb deletion spanning exons 1 to 10 plus a large amount of the upstream sequence [Shotelersuk et al. 1998, Town et al. 1998]. FISH diagnosis of the 57 kb deletion has been recently developed [Bendavid et al. 2004]. Over 50 other reported mutations include small deletions, insertions, nonsense, missense, splicing mutations or mutations in the promoter region [Attard et al. 1999, Kalatzis et al. 2002, Kiehntopf et al. 2002, Phornphutkul et al. 2001, Town et al. 1998]. Patients with the infantile form of cystinosis generally have two severe truncating mutations, with a loss of functional protein, while patients with the

juvenile cystinosis have mutations affecting functionally less important regions of cystinosin - inter-transmembrane loops or the N-terminal region, which account for their milder phenotype [Attard et al. 1999, Thoene et al. 1999]. In patients with ocular non-nephropathic cystinosis one severe mutation and one mild mutation, allowing significant residual cystinosin activity were reported [Anikster et al. 2000]. It has been suggested that the lack of kidney involvement in ocular cystinosis can be also explained at least in some patients by the presence of tissue specific factors that allow a splicing of a normal *CTNS* transcript [Anikster et al. 2000].

Studies with an antibody against cystinosin will allow the distinction between an abnormal intracellular localization and lowered cystinosin activity at the normal site [Haq et al. 2002].

The regulation of *CTNS* has not yet been elucidated. *CTNS* promoter can bind the transcriptional factor Sp-1 and has sequence homology to AP-2, AP-4 and NF-1 regulatory elements. It shares the promoter sequence with *CARKL* (carbohydrate kinase-like gene) with unknown function, which is also deleted in patients with 57 kb deletion [Phornphutkul et al. 2001].

Recently the first animal model of cystinosis has been generated, using a promoter trap approach [Cherqui et al. 2002]. *Ctns* knockout mice, lacking the last 4 exons of the murine *ctns* gene, accumulated cystine in all organs tested, with cystine levels rising with age. Cystine concentrations in liver, kidney and muscle were increased by factor 1350, 413 and 120 respectively in mice of 1 year old. The *ctns* $^{-/-}$ mice presented with ocular abnormalities, reduced motility and myopathy in some animals. Interestingly, despite the high level of cystine within the kidney, *ctns* $^{-/-}$ mice do not develop Fanconi syndrome. The comparison of mice and man may elucidate the origin of proximal tubulopathy in humans. Although the phenotype observed in *ctns* $^{-/-}$ mouse model was less severe than that observed in human, this animal model is an important tool for studying the pathogenesis of the disease [Cherqui et al. 2002]. Regarding the therapy, the mice model might be used to investigate cystine depletion under cysteamine administration in the different tissues, including the central nervous system.

The mutations of cystinosis gene, leading to premature termination codon (PTC), can be at least partially corrected *in vitro* by an aminoglycoside gentamicin, inducing PTC readthrough and enhancing full-length protein production [Helip-Wooley et al. 2002]. Whether gentamicin has therapeutical potentials in patients carrying this specific type of mutations merits further investigation.

Function of cystinosin

Although lysosomal localization of cystinosin was predicted in the 1980s [Jonas,Smith et al. 1982], only almost 20 years later it could be confirmed by the construction of a cystinosin-green fluorescent fusion protein and the colocalization of this fusion protein with an antibody directed against another lysosomal membrane protein, LAMP-2 in transfected cells [Cherqui et al. 2001]. Immunocytochemical experiments with recently generated anti-cystinosin antisera, also confirmed the lysosomal localization of cystinosin [Haq et al. 2002].

How cystinosin transports cystine across the lysosomal membrane is not yet completely understood.

Stimulation of the cystine exodus from lysosomes loaded with cystine dimethylester was associated with increase in lysosomal transmembrane pH gradients and decrease in the potential difference across the lysosomal membrane [Smith, Greene et al. 1987].

The role of transmembrane proton gradient for cystine transport was demonstrated by Kalatzis et al. [2001], who partially redirected cystinosin to the plasma membrane in COS cells by deletion of it's C-terminal targeting motif. When these cells were placed into an acid extracellular medium, a clear increase in cystine uptake occurred. This effect was strongly inhibited after disruption of the transmembrane pH gradient by nigericin, indicating that cystinosin operates as a H^+ symporter, i.e. that it couples a translocation of cystine to a translocation of protons in the same direction (Figure 2). Thus, the H^+ ATPase, acidifying the lysosomal matrix, will actively stimulate cystinosin-mediated cystine export from the lysosomes [Kalatzis et al. 2001]. A similar mechanism for export of neutral amino acids [Sagne et al. 2001] and of sialic acid and glucuronic acid [Mancini et al. 2000] has been described.

The relationship between the transport activity of cystinosin and associated clinical phenotype has been studied by deleting C-terminal sorting motif, resulting in expression of mutated protein on the plasma membrane of COS-cells and studying the uptake of [^{35}S]L-cystine from the extracellular medium by these cells compared to the cells expressing wild-type cystinosin [Kalatzis et al. 2004]. Interestingly, the majority of mutants allowing synthesis and lysosomal membrane localization of cystinosin, identified in patients with infantile form of cystinosis (16 from 19), demonstrated completely abolished cystine uptake. However, 3/19 "infantile" mutants demonstrated well detectable residual cystinosin activity. Two of four mutants from patients with milder juvenile form of cystinosis showed no cystine uptake and two of four had the residual activity of cystinosin. The reasons of this incomplete correlation of cystinosin transport activity and the severity of the clinical phenotype are unexplained and require further study [Kalatzis et al. 2004].

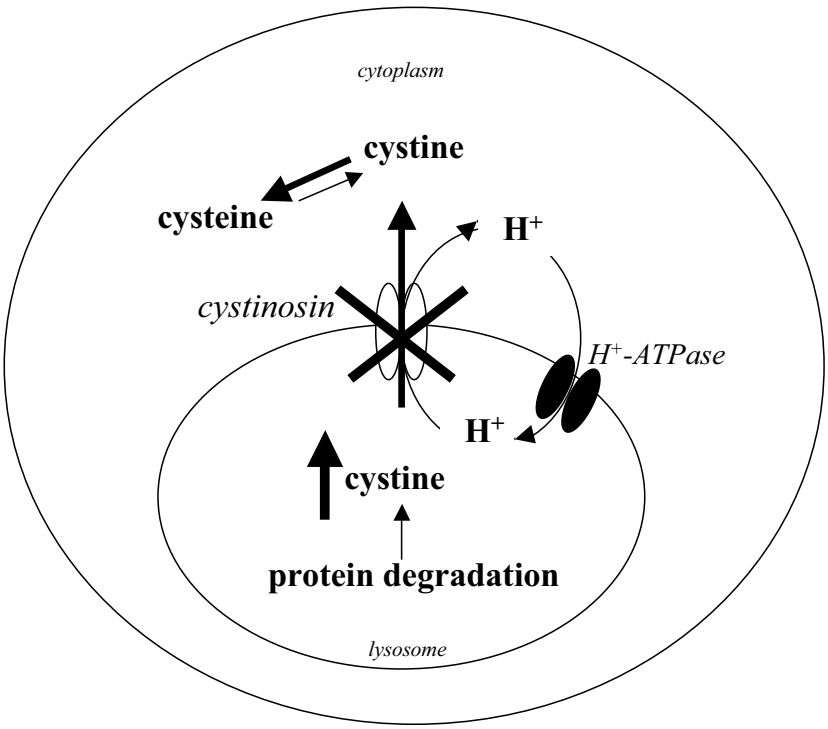

Figure 2 Cystine and protons are transported out of the lysosome by cystinosin. In cytoplasm cystine is converted to cysteine. The protons in the interior of the lysosome are provided by H^+ - ATPase.

Recently a functional homologue of cystinosin has been identified in Saccaromyces Cerevisiae (28% identity/46% similarity to cystinosin). Ers1 protein encoded by *ERS1* gene is localized on the vacuolar and endosomal membrane of the yeast and is responsible for the resistance to an aminoglycoside hydromycin B. An expression of wild type cystinosin, but not of particular cystinosin mutants can complement loss of function of Ers1 protein in the yeast. Ers1 function is modified by a novel Meh1 protein, regulating the acidification of the yeast vacuole. A homologue of Meh1 protein is not yet identified in humans. Yeast model system might be usefull for studying cystinosin function and for identifying other genes which are involved in the regulation of cystinosin [Gao et al. 2005].

Surprisingly, in I-cell disease, caused by a misrouting of lysosomal hydrolases due to a defect in the synthesis of the mannose-6-phosphate recognition marker, cystine is stored in the lysosomes to the same degree as in cystinosis [Tietze et al. 1986]. The defective function of cystinosin in this disorder may be attributed to inadequate processing by proteolytic enzymes of an inactive precursor.

Possible pathogenetic mechanisms of cystinosis

Despite current good understanding of the genetic basis of the disease, the mystery of how cystine accumulation causes clinical signs of cystinosis is not yet solved.

Before the existence of the animal model of cystinosis, several studies have been done using cystine-loaded proximal tubules. Foreman et al. [1987] have first demonstrated that the incubation of proximal tubules with cystine dimethyl ester (CDME) led to increased intracellular cystine concentrations comparable to those measured in patients with cystinosis. Lipid soluble CDME passes across cell membranes and is cleaved by intracellular esterases liberating cystine. The vast majority of cystine cleaved from CDME localizes in the lysosomes [Sakarcan et al. 1994]. Cellular cystine accumulation decreased transepithelial potential difference and inhibited volume absorption, active glucose and phosphate transport in isolated perfused rabbit proximal convoluted tubules and amino acid transport in rats after CDME treatment [Foreman et al. 1987, Salmon et al. 1990].

ATP depletion

The proposed mechanism of the inhibited active transport in proximal tubules after CDME loading was intracellular ATP depletion because a dramatic decrease of ATP was measured after CDME loading [Ben-Nun et al. 1993, Coor et al. 1991, Foreman et al. 1995]. Later it was shown that cystine loading caused a reduction of intracellular phosphate. The maintenance of intracellular phosphate prevented ATP depletion due to CDME loading and preserved proximal tubular transport [Bajaj et al. 1996]. As most proximal tubular transport is sodium coupled, less fuel for Na-K ATPase on the basolateral membrane, resulting in lower electrochemical gradient for sodium, would inhibit the transport across the apical membrane [Baum 1998]. Consistent with this theory, cystine-loaded LLC-PK1 cells, a cell line with characteristics of proximal tubular cells, had increased intracellular sodium and decreased intracellular potassium concentrations compared to the control cells, indicating decreased activity of Na-K ATPase [Ben-Nun et al. 1992]. Recently, electrophysiological studies on immortalized human proximal tubular epithelial cell line (IHKE-1), loaded with CDME, demonstrated that CDME acutely altered basal membrane voltage of IHKE-1 cells and inhibited at least 3 Na^+-dependent transporters: Na^+-alanine cotransporter, Na^+/H^+ exchanger and NaPi transporter [Cetinkaya et al. 2002]. As NaPi transporter was inhibited to the highest extent, it was suggested, consistent with the animal studies, that intracellular phosphate and ATP depletion play the key role in subsequent inhibition of

active transport [Cetinkaya et al. 2002]. The presence of Fanconi syndrome in patients with mitochondrial disorders also favors the role of intracellular ATP depletion in proximal tubular cell dysfunction in patients with cystinosis. Thus far this mechanism has not been demonstrated in human cystinotic material.

The role of intracellular phosphate depletion remains uncertain, as patients with hypophosphatemic rickets, having pronounced decrease in serum phosphate, develop no other symptoms of Fanconi syndrome.

One should realize that CDME loading does not exactly mimic the situation in cystinotic patients. In this experimental situation, cystine, accumulating in the lysosomes, is normally transported into the cytoplasm by cystinosin. Furthermore, it is not excluded that fast increase in intracellular cystine in cells due to CDME loading causes a decrease of the intracellular ATP, while gradual cystine accumulation in patient's tissues alters intracellular functions in a different fashion. Whether *ctns*-/- mice exhibit ATP depletion remains to be investigated.

Increased apoptosis

Another pathologic mechanism possibly involved in cystinosis is an altered regulation of cell survival and death due to enhanced apoptosis.

A recent study has demonstrated that the rate of apoptosis was increased in fibroblasts of patients with nephropathic cystinosis and in renal proximal tubular epithelial cells loaded with CDME compared to normal cells [Park et al. 2002]. The mechanism linking cystine accumulation and enhanced apoptosis remains speculative and has to be elucidated [Park et al. 2005]. Interestingly, increased apoptotic cell death has been demonstrated in some other lysosomal storage diseases and in a mouse model of tyrosinemia developing Fanconi syndrome [Finn et al. 2000, Huang et al. 1997, Jatana et al. 2002, Simonaro et al. 2001, Sun et al. 2000]. Whether apoptotic or necrotic cell death is enhanced in *ctns*-/- mice has not yet been investigated and studies of apoptosis in biopsies of patients with cystinosis are lacking.

Altered glutathione metabolism

Glutathione (GSH) is the most abundant cellular thiol, functioning as an important redox buffer. Cysteine together with glutamate and glycine are needed for GSH synthe-

sis. Theoretically, defective cystine exodus out of the lysosomes in cystinosis can lead to cysteine deficiency in the cytosole, where cystine is reduced to cysteine [Gahl et al. 2001]. Cysteine deficiency could be a rate-limiting factor for GSH synthesis. GSH serves as a cofactor for the GSH peroxidase family of enzymes, which metabolize H_2O_2 and lipid peroxides, defending cells against reactive oxygen metabolites [Uhlig et al. 1992]. Increased reactive oxygen damage due to altered glutathione defense would make cells prone to apoptotic cell death. Furthermore, glutathione depletion results in alteration of mitochondrial ATP synthesis [Meister et al.1995, Han et al. 2003]. Therefore, disturbances in glutathione metabolism could be the missing link between ATP depletion and increased apoptosis as a basis of cellular dysfunction in cystinosis.

Rizzo et al.[1999] made an interesting observation in three cystinotic patients, who had an increased excretion of pyroglutamic acid (5-oxoproline), that normalized with cysteamine therapy, pointing to the disturbances of glutathione metabolism in cystinosis. This abnormality was not present in patients with an idiopathic Fanconi syndrome. The fact that in fibroblasts of patients with glutathione synthetase deficiency the concentration of cysteine is increased, illustrates the importance of the pathway from cysteine to glutathione [Ristoff et al. 2002]. As 5-oxoprolinuria was also demonstrated in patients without defects in γ-glutamyl cycle, but having another inborn error of metabolism [Mayatepek et al. 1999], the meaning of this finding in cystinotic patients needs further study.

Mechanism of chronic interstitial damage

Not only low molecular weight proteins but also albumin excretion is increased in cystinotic patients. The reabsorption of the excessively filtered proteins may contribute to the renal interstitial injury observed in patients by the release of profibrogenic factors [Abbate et al. 1999, Hirschberg et al. 2005, Zandi-Nejad et al. 2004]. Search for the presence of bioactive peptides in urine of cystinotic patients, as has been done in Dent's disease and Lowe syndrome, could contribute to the explanation of chronic interstitial damage [Cutillas et al. 2003, Norden et al. 2004].

Diagnosis of cystinosis

Cystinosis should be suspected in all patients with failure to thrive and signs of renal Fanconi syndrome as it is the most common cause of inherited Fanconi syndrome in children. Some patients may present with a Bartter-like phenotype (hypokalemic metabolic alkalosis, hyponatremia, high plasma renin and aldosteron) [Pennesi et al. 2005]. However, the presence of symptoms of proximal tubular dysfunction such as

aminoaciduria, glucosuria and phosphaturia should not allow missing the diagnosis of cystinosis. The detection of elevated intracellular cystine content is the corner stone for the diagnosis of cystinosis as pathognomonic cystine crystals in the cornea become apparent only after the age of 1 year. Bone marrow aspiration was used in the past for the early diagnosis of cystinosis showing hexagonal cystine crystals which were obviously present under crossed polarizing prisms [Schneider et al. 1969]. However, this invasive method is not used anymore as cystine determination in blood cells allows the accurate and early diagnosis in most of the patients. The methods for cystine determination differ depending on the cell type: mixed leukocyte preparation or polymorphonuclear leukocytes and the method used for cystine measurement: either cystine-binding assay, amino acid chromatography or high performance liquid chromatography, making it difficult to compare the results of the different laboratories [de Graaf-Hess et al. 1999, Kamoun et al. 1999, Oshima et al. 1974]. The addition of N-ethylmaleimide to isolated cells prior to thawing and lysis is essential in order to prevent the oxidation of intracellular cysteine leading to a false increase of cystine concentration [de Graaf-Hess et al. 1999]. Cystine concentrations expressed as nmol of cystine per mg protein in polymorphonuclear leukocytes of normal controls, obligate heterozygotes and patients at diagnosis, measured by HPLC are presented in table 1. The advantages of HPLC method for cystine determination are: its lower price and no need for radioactive materials such as in cystine-binding assay and its higher sensitivity, compared to amino acid chromatography (detection limit cystine 0.15 μmol/l). Cystine binding assay remains the most sensitive method for cystine determination, requiring lowest amount of blood (3 ml compared to 5-10 ml needed for HPLC determination), however, it's high price and the use of radioactive cystine-binding protein, derived form E.Coli, limits it's use in some laboratories [Smith, Furlong et al. 1987]. Tandem mass spectrometry is a new sensitive method and might be used in the future for cystine determination in cystinosis [Wear et al. 2005].

Molecular analysis of the cystinosis gene allows an early diagnosis and can be used for prenatal diagnosis on cultured amniocytes or chorionic villi. Previously prenatal diagnosis of cystinosis was performed by measurement of [^{35}S] cystine accumulation in amniocytes or chorionic villi cells [Dumoulin et al. 1999].

Table 1 Intracellular cystine content of polymorphonuclear cells, measured by HPLC.

	Cystine content (median, range) nmol cystine/mg protein
Healthy controls (n=8)	0.1 (0.04-0.12)
Obligate heterozygotes (n=15)	0.2 (0.09-0.63)
Untreated patients with infantile cystinosis (n=5)	1.47, 1.68, 2.79, 3.79, 4.89

Treatment

Symptomatic therapy

The aim of symptomatic therapy in patients presenting with Fanconi syndrome is the maintenance of fluid and electrolyte balance, good nutrition and prevention of rickets. The dose of potassium, sodium, bicarbonate and phosphate varies substantially and shall be regularly adapted according to serum values. 1,25-dihydroxycholecalciferol should be used beginning in early childhood [Gahl et al. 2001, Loirat et al. 1999]. The excessive administration of phosphate, 1,25-dihydroxycholecalciferol and bicarbonate may result in severe nephrocalcinosis or renal stone formation [Loirat et al. 1999, Theodoropoulos et al. 1995]. Calcium supplementation is generally not indicated. Carnitine replacement normalizes plasma and muscular carnitine levels, however, it is not established whether carnitine administration results in a clinical improvement [Gahl, Bernardini et al. 1988, Gahl et al. 1993]. No cases of cardiomyopathy related to carnitine deficiency in patients with cystinosis have been reported so far.

Poor appetite, vomiting and oral motor dysfunction often require nasogastric tube or gastrostomy feeding especially in young children [Elenberg et al. 1998, Trauner et al. 2001]. Treatment with growth hormone improves the growth in children with cystinosis, allowing them to catch-up and to maintain normal growth [Wuhl et al. 1998]. As cystine-depleting agent cysteamine improves growth in patients with cystinosis, it is possible that patients treated with cysteamine starting from the early age would not need exogenous growth hormone at all.

Levothyroxin is indicated in patients with hypothyroidism, insulin in case of diabetes and testosterone may be considered in male patients with hypogonadism.

Whether albuminuria in cystinosis is responsive to the administration of angiotensin-converting enzyme (ACE) inhibitors has not been investigated. If it would be the case, ACE inhibitors could postpone the development of chronic interstitial damage as it has been demonstrated in diverse proteinuric disorders.

Specific treatment with cysteamine

The amino thiol cysteamine depletes lysosomal cystine content by a disulfide exchange reaction with cystine resulting in the formation of cysteine-cysteamine mixed disulfide and cysteine. Cysteine-cysteamine mixed disulfide exits lysosomes via a lysine carrier and cysteine via a cysteine carrier [Gahl et al. 1985]. The administration of cysteamine at 40-90 mg per kg (1.3-1.9 g/m^2) in 4 daily doses drastically lowers cystine content of the lysosomes, postpones or even prevents the deterioration of renal function and the development of extra-renal complications [Kimonis et al. 1995, Markello et al. 1993, Kleta et al. 2004]. Furthermore, cysteamine improves growth of cystinotic patients [Gahl et al. 1987].
The side effects of cysteamine are mostly restricted to gastrointestinal discomfort due to the stimulation of H^+ secretion in the stomach, mediated by cysteamine-induced gastrin release [Shiratori et al. 1997] and bad breath and sweat odor. Allergic reactions, fever, seizures and neutropenia are also reported, especially when the dose of the drug is abruptly increased [Corden et al. 1981]. Gastric acid hypersecretion and ulcerogenity of cysteamine can be improved by the administration of proton pump inhibitors [Dohil et al. 2003]. As the target tissue cystine levels necessary to prevent the progression of renal disease and the occurrence of extra-renal complications is unknown, the 0.9 percentile of heterozygote values in the polymorphonuclear cells is recommended as an upper cystine limit before the next dose of cysteamine is given (<0.5 nmol cystine per mg protein) [Middleton et al. 2003]. The regular measurements of cystine in polymorphonuclear leukocytes are required in order to adjust cysteamine dose.

Topical 0.5 % cysteamine eye drops are indicated, as systemic cysteamine therapy has no effect on corneal cystine crystals. These drops are highly effective and when administered 6 to 12 times daily are able to dissolve completely corneal cystine crystals within 8 to 41 months [Gahl et al. 2000]. The attempts to develop a new topical cysteamine formulation, stable at room temperature, until now were not successful as they were less effective compared to the standard formulation [Tsilou et al. 2003].

Unfortunately, in the majority of the patients cysteamine can not reverse Fanconi syndrome and only postpones the development of renal failure. Urinary loss of cysteamine

in patients with Fanconi syndrome might be responsible for the irreversibility of renal Fanconi syndrome.

As leukocyte cystine content returns to the initial high levels 6 hours after cysteamine administration [Belldina et al. 2003], the drug should be taken every 6 hours including the night. Non-compliance with the strict cysteamine dose regimen is another plausible explanation of the inefficiency of cysteamine treatment and development of renal failure and extra-renal complications in the majority of cystinosis patients.

Aim and outline of the thesis

The aims of this thesis concern two main directions:

1 Improving medical care of cystinosis patients

2 Obtaining new insights into the pathogenesis of cystinosis.

Improving medical care

Diagnosis of cystinosis

The diagnosis of cystinosis is based on the measurement of elevated cystine content in white blood cells in a suspected patient, most often presenting with renal Fanconi syndrome. Because in blood cystine preferentially accumulates in polymorphonuclear leukocytes and not in the lymphocytes, it is logic that polymorphonuclear leukocytes should be used for cystine determination instead of mixed leukocyte preparations. However, worldwide most of the laboratories use mixed leukocyte preparations for cystine determination because mixed leukocytes are easier to isolate from whole blood compared to polymorphonuclear leukocytes. In **chapter 2** we explored whether cystine should be measured in polymorphonuclear leukocytes and not in mixed leukocytes.

To our opinion cystinosis should be confirmed in all patients as this diagnosis has a great impact on the patient and the family and requires life-long treatment with cysteamine. Before the cystinosis gene *CTNS* was cloned, we used cultured primary skin fibroblasts for the determination of the elevated cystine content for the confirmation of the diagnosis. In **chapter 3**, we describe a simplified PCR method for the detection of a common European 57kb deletion, which was present in 60% of tested Dutch alleles. Molecular analysis of *CTNS* gene is currently used in The Netherlands for the confirmation of diagnosis in all patients with cystinosis.

Treatment of cystinosis

Longer survival of cystinosis patients due to successful renal replacement therapy, allows these patients to reach an adult age. Growing into adulthood cystinosis patients experience the common problem of patients suffering from rare disorders. Children with cystinosis are mainly treated in a limited number of university pediatric nephrology units. After the transition to the adult care providers, adult cystinosis patients are spread between numerous adult nephrology departments, which due to a small amount of cystinosis patients are generally not experienced with treatment of this rare disorder. In **chap-**

ter 4 we evaluated clinical care of adult Dutch cystinosis patients and provided the recommendations for the adult nephrologists.

Treatment with ACE inhibitors of cystinosis patients with proteinuria could possibly slow the deterioration of the renal function in these patients. The effectiveness of ACE inhibitors in cystinosis is described in **chapter 5.**

Irreversibility of Fanconi syndrome and deterioration of the renal function in the majority of the patients despite cysteamine therapy, might be explained by urinary cysteamine loss in patients with Fanconi syndrome. This hypothesis was tested in the study described **in chapter 6**. Another possible explanation of incomplete efficiency of cysteamine therapy is a non-compliance with a strict dose regimen. This was investigated in a study described in **chapter 7.**

New insights into the pathogenesis of cystinosis

Cystine accumulation in cystinosis starts already prenatally and continues after birth. Interestingly, full-blown Fanconi syndrome develops starting from the age of 6 months.

Understanding of gradual appearance of signs of proximal tubular dysfunction, demonstrated in **chapter 8**, would provide new insights into the pathogenesis of cystinosis.

Based on CDME loading model of cystinosis, the alteration of mitochondrial ATP synthesis, resulting in the inhibition of Na,K ATP-ase was proposed to be an underlying pathogenetic mechanism of the disease. In **chapter 9** we describe an extensive study of mitochondrial energy generative capacity and the activity of Na,K ATP-ase in cultured human cystinotic fibroblasts.

Searching for a possible link between lysosomal cystine accumulation and inhibited mitochondrial ATP generating capacity, we hypothesized that altered glutathione metabolism could play a role. In a study described in **chapter 10** we investigated glutathione metabolism in cultured cystinotic fibroblasts and polymorphonuclear cells.

Study of renal disease in cystinosis is hampered by unavailability of human renal cystinotic material. This problem has been overcome by developing of proximal tubular cell lines derived from urine of cystinosis patients. Study of intracellular ATP content and glutathione status in cystinotic proximal tubular cells is described in **chapter 11**.

A general discussion and summary of this thesis is provided in **chapter 12**.

CHAPTER 2

Comparison of cystine determination in mixed leukocytes vs polymorphonuclear leukocytes for diagnosis of cystinosis and monitoring of cysteamine therapy

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Abstract

Background: Cystinosis is a rare inborn error of cystine transport, leading to accumulation of cystine within the lysosomes. To diagnose cystinosis and to monitor treatment with cysteamine, adequate measurements of intracellular cystine content are required. Historically, cystine has been measured in mixed leukocyte preparations (ML), although it preferentially accumulates in phagocytic blood cells (polymorphonuclear leukocytes (PMN) and monocytes). Therefore we have switched from cystine determinations in ML to PMN cells and compared the differences in intracellular cystine content in these two cell preparations.

Methods: ML and PNM were isolated form freshly drawn blood. Cystine was measured as cysteine after reduction with sodium borohydride and derivatization with monobromobimane, followed by separation with automated HPLC.

Results: Mean intracellular cystine content (nmol cystine/mg protein) was lower in ML compared to PMN cells in obligate heterozygotes (0.07 versus 0.27, $p<0.001$) and patients treated with cysteamine (0.15 versus 0.94, $p<0.001$). At the time of diagnosis cystine values of ML in two patients were within the reference interval, while it was clearly elevated in PMN and in cultured fibroblasts. After the switch from ML to PMN the dose of cysteamine had to be increased in 80% of patients under cysteamine therapy.

Conclusions: We demonstrate that cystine must be measured in PMN leukocytes and not in ML preparation because using ML can lead to dismiss or delay of the diagnosis of cystinosis and inadequate adjustment of cysteamine dose.

Introduction

Cystinosis is a rare autosomal recessive disorder caused by mutations of cystinosis gene (*CTNS*; chromosome 17p13), which encodes the lysosomal cystine carrier. The continuous accumulation of cystine in the lysosomes leads to intracellular crystal formation throughout the body. Patients with the common infantile form of cystinosis develop renal Fanconi syndrome 3–6 months after birth and end-stage renal failure before the age of 10 years. Treatment with the aminothiol cysteamine depletes intralysosomal cystine via a disulfide exchange reaction with formation of cysteine-cysteamine mixed-disulfides and cysteine; these exit the lysosomes via lysosomal carriers for lysine and cysteine, respectively [Gahl et al. 2001]. When started at an early age, cysteamine treatment prevents or postpones the deterioration of renal function and the occurrence of extrarenal complications such as hypothyroidism, diabetes mellitus, retinopathy, encephalopathy, and myopathy [Gahl et al. 2001].

Accurate measurement of intracellular cystine content is obligatory for the diagnosis of cystinosis as well as for the monitoring of treatment with cysteamine. Historically, cystine has been measured in mixed leukocyte (ML) preparations, despite the fact that it preferentially accumulates in polymorphonuclear leukocytes (PMN) and monocytes [Schulman et al. 1970]. We therefore compared intracellular cystine content in ML preparations and in PMN cells of healthy controls, obligate heterozygotes, and patients at diagnosis and under cysteamine therapy. Because the isolation of PMN may pose practical problems in some laboratories, we also investigated whether preservation of whole blood at room temperature influenced intracellular cystine content. If the cystine concentration remains constant, it would allow the shipping of whole-blood samples.

Materials and methods

MLs were isolated exactly as described by de Graaf-Hess et al. [1999]. All solutions were kept at 4 °C. PMN cells were isolated from 10 mL of blood by addition of 2 mL of dextran solution (50 g/L dextran T500, 15 g/L EDTA, 7g/L NaCl, pH 7.4) in a 15-mL glass tube. After gentle mixing and ~ 1 h on ice, the clear upper solution was divided between two 15-mL screw-cap polypropylene tubes, brought up to a total volume of 8 mL with phosphate-buffered saline (PBS), and mixed gently. To the bottom of the tubes we carefully added 7 mL of Ficoll (Ficoll-Paque 1077; Amersham-Pharmacia) via a syringe with a 15-cm long needle; the tubes were then centrifuged at 500*g* for 20 min at 4 °C in a swing

out rotor with no braking. After centrifugation, the interphase containing the lymphocytes and the two liquid layers were completely removed. The pellet, containing PMN cells and some erythrocytes, was resuspended in 1 mL of PBS, and 3 mL of cold water was added for hypotonic lysis of the erythrocytes. After exactly 1.5 min on ice, 1 mL of 36 g/L NaCl solution was added; the solution was then mixed and centrifuged at 600*g* for 10 min at 4 °C. The cells were washed with 5 mL of PBS and centrifuged again. Finally, the pellet was resuspended in 0.5 mL of PBS, transferred to a 1.5-mL screw-cap Eppendorf tube, and centrifuged at 1000*g* for 5 min at 4 °C. The pellet was then frozen immediately in liquid N_2 and stored at - 80 °C until the cystine was measured. The differential counts of ML and PMN preparations were determined automatically (Advia; Bayer®).

To determine whether blood samples can be preserved at room temperature, we collected fresh whole blood (2 x 10 mL per patient) in tubes containing 1.5 mL of acid-citrate-dextrose solution (ACD). PMN cells were isolated immediately and after 24 h of preservation at room temperature.

Cystine was measured by HPLC as described previously [de Graaf-Hess et al. 1999].

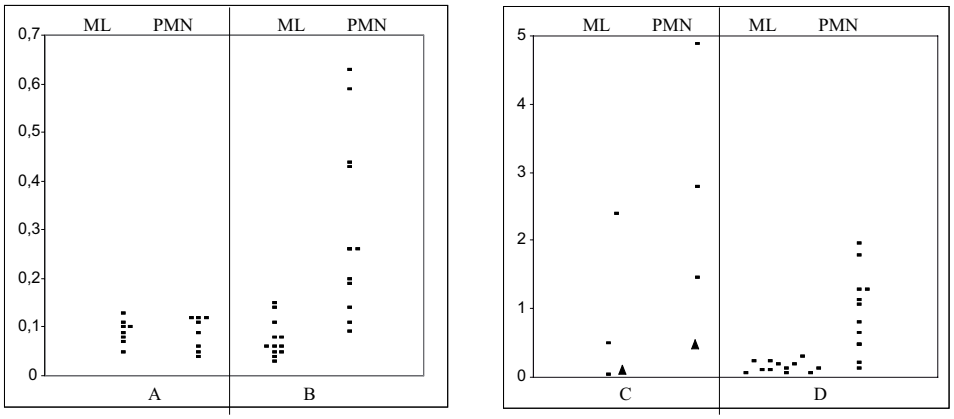
Results

ML preparations (n = 17) contained, mean (SD), 37 (15)% PMN cells, 39 (11)% lymphocytes, and 5 (7)% monocytes.

The mean (SD) intracellular cystine content (nmol/mg of protein) was lower in ML compared with PMN cells in obligate heterozygotes (n = 15; 0.07 (0.03) vs 0.27 (0.17) nmol/mg of protein; $p < 0.001$) and in patients treated with cysteamine (n = 12; 0.15 (0.08) vs 0.94 (0.58) nmol/mg of protein; $p < 0.001$; Figure 1). In two patients at the time of diagnosis (one with the infantile form and one with the late-onset form), the ML cystine content was within the reference interval, whereas it was increased in PMN cells (0.49 and 1.47 nmol/mg of protein, respectively) and in cultured fibroblasts (1.6 and 1.44 nmol/mg of protein, respectively).

Because of the switch from ML to PMN preparations, the dose of cysteamine had to be increased in 12 of 15 of patients under cysteamine therapy because their cystine concentrations measured in PMN cells were clearly above the desired concentration of 0.5 nmol cystine/mg of protein (90th percentile value of cystine concentration in PMNs of heterozygotes in our laboratory).

The mean (SD) cystine content of ML in obligate heterozygotes (n = 15) was undistinguishable from that of healthy controls (n = 8) but was clearly increased in PNM cells



Figurie 1 Cystine (nmol/mg of protein) in ML preparations and PMN cells.
(*A*), healthy controls (n = 8); (*B*), obligate heterozygotes (n = 15); (*C*), patients at diagnosis (n = 4); (*D*), patients undergoing cysteamine therapy (n = 12).▲ patient with late-onset cystinosis.

compared with the control values ((0.27 (0.17) vs 0.09 (0.03) nmol cystine/mg of protein; $p < 0.05$; Figure 1)).

PMN cells of blood samples stored in ACD tubes for 24 h at room temperature (n = 7) had increased mean (SD) cystine (0.95 (0.50) vs 0.59 (0.46) nmol/mg of protein; $p < 0.05$; Figure 2).

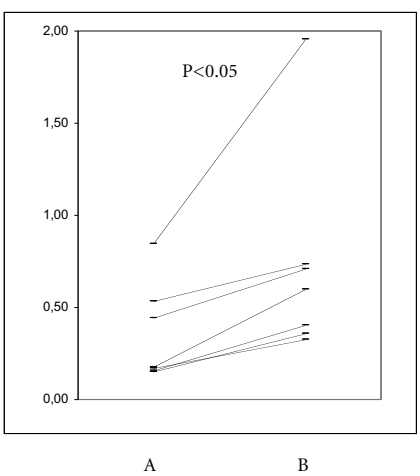


Figure 2 Cystine content (nmol/mg protein) in PMN (n=7).
(A), isolated immediately; (B), after 24 hours storage at room temperature.

Discussion

Detection of increased intracellular cystine is required for the diagnosis of cystinosis in patients presenting with Fanconi syndrome. After the diagnosis of cystinosis, intracellular cystine should be measured regularly to evaluate the cysteamine dose. Estimation of the optimum leukocyte cystine concentration to be achieved under therapy is conjectural because it is not known whether cystine accumulation in blood cells is representative of the storage in other tissues. Generally, it is recommended to strive for a ML cystine concentration < 0.5 nmol/mg of protein [Gahl et al. 2001]. This is also the upper limit of cystine seen in heterozygotes, who do not develop nephropathy [Middleton et al. 2003].

Because we observed a clear difference between cystine content in ML preparations and PMN cells, we suggest that each laboratory produces its own reference values based on the upper cystine values found in heterozygotes.

In 1970 Schulman et al. [1970], showed that cystine accumulation in cystinotic leukocytes is located primarily in phagocytic blood cells rather than in lymphocytes. Cystine measurement in purified PMN preparations improved the sensitivity of the method. Our results were comparable to those of Smolin et al. [1987], who used a cystine-binding assay for cystine determination. They also found lower cystine in MLs compared with PMNs in heterozygotes, in one untreated patient, and in patients undergoing cysteamine therapy. In our laboratory, the mean intracellular cystine content of healthy controls did not differ between MLs and PMNs, possibly because the low cystine concentrations in healthy persons are close to the detection limit of the HPLC method.

To our knowledge, no missed diagnosis of cystinosis as a result of low cystine concentrations in ML preparations has been reported previously. In our laboratory, the diagnosis of cystinosis could have been missed in two patients if cystine had been measured only in MLs. In one patient, treatment with cysteamine was delayed for 6 months because the cystine concentration in the MLs remained within the reference interval. Later the diagnosis of cystinosis in both patients was confirmed by clearly increased cystine in fibroblasts and mutational analysis of the *CTNS* gene.

The possible reasons for falsely low cystine in MLs, especially in young children, could be the overrepresentation of lymphocytes in ML preparations, typical for the first year of life. Furthermore, variations in the differential count of MLs in individual patients can lead to unreliable variations in measured cystine because it is expressed per milligram of protein in the total cell preparation.

As described by Kamoun et al. [1999], for storage experiments, we also used ACD tubes

for blood collection. In our laboratory, the storage of blood at room temperature for 24 h led to increases in intracellular cystine content. Thus, the shipping of whole-blood samples for cystine determinations is not advisable.

In summary, we recommend measurement of cystine in PMNs and not in ML preparations because the recommended approach increases the sensitivity of cystine detection for the diagnosis of cystinosis and provides a better target concentration during the monitoring of cysteamine treatment.

CHAPTER 3

The molecular basis of Dutch infantile nephropathic cystinosis

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Abstract

Infantile nephropathic cystinosis, an inborn error of metabolism with an autosomal recessive inheritance pattern, is characterized by lysosomal storage of the amino acid cystine due to an impaired transport of cystine out of the lysosomes. Initial clinical features consist of the renal Fanconi syndrome and crystals in the cornea. Oral therapy with cysteamine lowers the intracellular cystine content. Recently, the gene coding for the integral membrane protein cystinosin, which is responsible for membrane transport of cystine (*CTNS*), was cloned. Mutation analysis of the *CTNS* gene of Caucasian patients revealed a common 57-kb deletion, and several other mutations spread throughout the entire gene. In the present study, we developed an improved screening method for the detection of the common 57-kb deletion. By use of this method we detected the 57-kb deletion in 59% of the examined Dutch alleles. The remaining alleles were screened for other mutations by genomic sequencing of the different exons, revealing three previously described mutations. Furthermore, we studied a possible genotype- phenotype relation of the homozygous deleted patients, which could not be demonstrated in our study population. Next to biochemical determination of cystine in leukocytes or fibroblasts, molecular genetic analysis enables prenatal diagnosis and facilitates identification of carriers.

Introduction

Infantile nephropathic cystinosis, a rare inborn error of metabolism with an autosomal recessive inheritance pattern, is characterized by lysosomal accumulation of the amino acid cystine due to a defect in the transport of cystine across the lysosomal membrane. Infantile nephropathic cystinosis is characterized in particular by renal tubular dysfunction and the presence of crystals in the cornea [Gahl et al. 1986]. At birth, almost no symptoms are present. In the first year of life, the renal Fanconi syndrome becomes manifest, which is characterized by failure to thrive, dehydration, anorexia, polyuria, polydipsia and hypophosphataemic rickets [Gahl et al. 1986]. Even with treatment renal function progressively decreases and renal replacement therapy has to start at an age of approximately 10 years, usually followed by renal transplantation. After transplantation of the kidney, cystine continues to accumulate in other organs, resulting in a multisystem disease [Gahl et al. 1986]. Other characteristics are decreased pigmentation of the hair, eyes and skin, hypothyroidism [Chan et al. 1970], and impaired ability to sweat [Gahl et al. 1986].

Oral therapy with cysteamine lowers the intralysosomal cystine content by converting cystine to cysteine and cysteine-cysteamine mixed disulphide, which are transported across the lysosomal membrane by a mechanism independent of the defective cystine carrier [Gahl et al. 1987, Gahl et al. 1985]. Biochemical diagnosis is made by the measurement of the accumulated cystine in leukocytes or fibroblasts by HPLC [de Graaf-Hess et al. 1999].

Recently, the gene coding for the lysosomal cystine transporter cystinosin (*CTNS*) was cloned [Town et al. 1998]. This *CTNS* gene is mapped to the short arm of chromosome 17 (17p13) and consists of 12 exons. The mRNA of the *CTNS* gene is 2.6 kb and codes for a 367 amino acid cystine transporter with 7 transmembrane domains [Town et al. 1998]. Mutation analysis of the *CTNS* gene of cystinosis patients revealed a common 57-kb deletion in Caucasian patients, and several other mutations spread throughout the entire gene [Town et al. 1998, Shotelersuk et al. 1998, Attard et al. 1999, Forrestier et al. 1999, Thoene et al. 1999].

In the present study, we developed an improved screening method for the common 57-kb deletion, which enables accurate discrimination between the different genotypes. By this method we examined the prevalence of the common 57-kb deletion in the Dutch population. Alleles not carrying the 57-kb deletion were sequenced to reveal other mutations present in the coding region (including the intron-exon boundaries) of the *CTNS* gene.

Furthermore, we studied a possible genotype-phenotype correlation by comparing the phenotype of homozygous deleted patients with the phenotype of other genotypes.

Material and Methods

Patients

We studied 11 Dutch patients with early-onset nephropathic cystinosis. Biochemical diagnosis was made by the measurement of the intracellular cystine levels of leukocytes or fibroblasts [de Graaf-Hess et al. 1999]. The severity of the phenotype was determined on the basis of three parameters.

First, age of diagnosis was used, which correlates with the severity of the phenotype. Secondly, age of thyroid-stimulating hormone (TSH) production was measured, showing the increase of TSH as a response to the decline of thyroid function [Chan et al. 1970]. The third parameter used was reduction of creatinine clearance, reflecting the deterioration of kidney function.

Mutation Analysis

DNA was extracted from whole blood according to Miller et al. [1988]. The obtained DNA samples were subjected to mutation analysis of the *CTNS* gene.

57-kb Deletion Screening; an Improved Method. The common 57-kb deletion was screened for in 11 Dutch cystinosis patients, by PCR amplification across the deletion, using the LDM1 forward and reverse primers [Anikster, Lucero et al. 1999]. To discriminate between heterozygous and homozygous deleted patients, we also screened the patients for the presence of the D17S829 marker, which is situated in intron 3 of the *CTNS* gene (located in the deletion interval) [Town et al. 1998]. As an internal control we used primers for the housekeeping gene ß-actin, amplifying a product of 635 bp. Two separate multiplex PCRs were performed, one with primers for the deletion and for ß-actin and the second PCR with the primers for the D17S829 marker and for ß-actin (Figure 1a, b).

Multiplex PCR was carried out in a total volume of 50 µl on a Perkin- Elmer 9600 thermocycler (PE Biosystems, The Netherlands) containing 5 ng ß-actin primers and 50 ng of the other forward and reverse primers (LDM1 or D17S829 primers), 200 µ*M* dNTPs, 10 m*M* Tris-HCl buffer (pH 8.3), 1.0 m*M* $MgCl_2$ and 0.5 unit *Taq* polymerase (all from Life Technologies, The Netherlands). PCR parameters were as follows: 92°C/120 s (initial denaturation) followed by 35 cycles of 92°C/30 s (denaturation), 54°C/30 s (annealing), 72 ° C/30 s (elongation) followed by a final elongation step of 7 min by 72° C. The obtained PCR products of both PCRs, one to detect the presence of the dele-

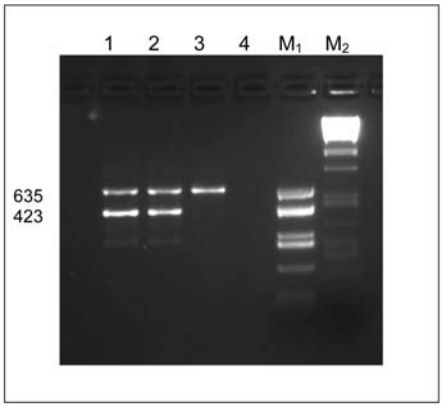

Figure 1a

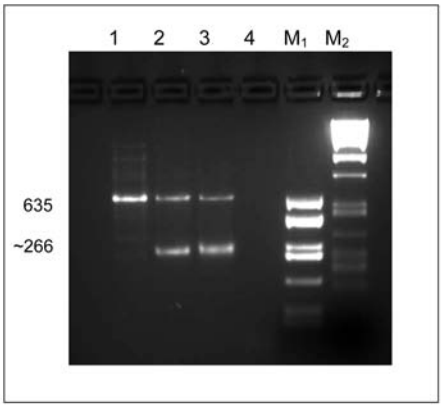

Figure 1b

Figure 1 Multiplex PCR to screen for the 57-kb deletion. **a** Gel electrophoresis pattern of multiplex PCR with primers for ß-actin and the 57-kb deletion (LDM1 primers). Each lane shows a band of 635 bp of the internal control fragment of ß-actin. The LDM1 primers only generated a fragment of 423 bp when the deletion was present (lanes 1 and 2). **b** Gel electrophoresis pattern of multiplex PCR for marker D17S829 and ß-actin. Again the product of 635 bp of ß-actin is present in each lane. The D17S829 primers, which are located in the deletion interval, only created a fragment of 266 bp when the deletion is not present (lanes 2 and 3). Samples in both **a** and **b** are from a homozygous deleted patient (lane 1), a heterozygous deleted patient (lane 2) and a nondeleted patient (lane 3). Lane 4 shows a negative control. M1 and M2 represent the size markers of respectively pUC*Hae*III and λ-*Pst*I.

tion (LDM primers) and the other one to detect the absence of the deletion (D17S829 marker), were separated on a 2% agarose gel. In presence of the deletion, next to the internal control fragment ß-actin of 635 bp, a fragment of 423 bp is amplified with the LDM1 primers (Figure 1a). In the other PCR reaction also the internal control fragment ß-actin must be present, next to a band of 266 bp of the D17S829 primers, when the deletion is not present (Figure 1b).

Sequencing of the CTNS Gene. The remaining alleles that did not carry the 57-kb deletion were sequenced to reveal possible other mutations in the *CTNS* gene. Therefore, intronic primers were designed based on the published sequences (Genbank accession Nos Y15922–Y15933, and table 1). PCR was performed according to the above-described conditions using 50 ng of forward and reverse primers.

Table 1 Primer sequences used to sequence the different exons of the *CTNS* gene.

Exon number	Primer sequence (5'→ 3')	Annealing temperature (°C)
3 for	ACGAGATTCAACATTCCCCTG	48
3 rev	TAGCCACCATTTCCCTCTTTAC	
4 for	TGTCATTGATTTGGGTCCTTCC	44
4 rev [1]	TAGGGCTTGTCTTACAGGTA	
5 for	GATCTCACTGTCCAGCTTCT	48
5 rev	TCCCTACCCATCCGTTAAG	
6 for	GCGGGGTCCTCGGTAACTG	48
6 rev	GGGCCCCTTCTTGTCACG	
7 for	CTTCATAAGCCCAGCCTCAGC	60
7 rev	CGAGAGAGCCTGCACATACG	
8 for [1]	CCCTGCCCTGTCTTGTCC	58
8rev [1]	CAGAGATGTAGGGCAGGCAA	
9 for	CCTCACCACCCAGCTTCTCC	56
9rev	GTGGCGGGTGTTGGCTG	
10 for[1]	GGCCTCTGTGTGGGTCC	54
10 rev[1]	GGCCATGTAGCTCTCACCTC	
11 for	GCCCTCCGTCTGTATGTCCG	52
11 rev	GCCCGATGCCCCAGCCGC	
12 for	GCCAACCTAACACCAGCTTC	54
12 rev	AGAGGCTGGGTACACTGGGT	

[1] According to Town et al. [1998].

After PCR, the products were automatically sequenced on the ABI Prism 377 automated sequencer using the ABI Prism BigDye Terminator cycle sequencing kit according to the instructions of the manufacturer (PE Biosystems, The Netherlands).

Results

Mutation Analysis

To reveal the molecular basis of Dutch cystinosis patients, we performed mutation analysis of the *CTNS* gene. First, we screened 11 Dutch patients for the presence of the common 57-kb deletion. For this purpose, we developed an improved method to examine the presence of the 57-kb deletion on different alleles. In this way, we were able to discriminate between homozygous or heterozygous deleted patients. Figure 1a shows the results of the PCR detecting the presence of the 57-kb deletion and figure 1b shows the results of the PCR for the D17S829 marker, which is situated in the deletion interval. In both fig-

ure 1a and b, lanes 1, 2 and 3 represent different cystinosis patients, lane 4 is a negative control and M1 and M2 represent respectively pUC-*Hae*III and λ-*Pst*I molecular weight markers. In both figure 1a and b the control fragment of ß-actin of 635 bp is present in each lane.

Figure 1a shows that in lanes 1 and 2 the deletion is present on one or two alleles and in lane 3 the deletion is not present. In figure 1b it is seen that lane 1 contains no band for the D17S829 marker (situated in the deletion interval) and lanes 2 and 3 do show a band for this marker, meaning that the deletion is not homozygously present in lanes 2 and 3 (Figure 1b). Combining the results of both PCRs gives the following results, lane 1 is a patient who is homozygous for the 57-kb deletion, lane 2 represents a patient who is heterozygously deleted and lane 3 is a patient who does not carry the deletion at all.

Of the 22 Dutch cystinosis alleles, the 57-kb deletion was detected in 13 alleles (59%), 5 patients were homozygous and 3 were heterozygous for the deletion (table 2).

Table 2 *CTNS* mutations of Dutch cystinosis patients

Patient	Exon(s)	Allele 1	Allele 2	Amino acid change
1[2]	1-10	57 Kb del	57 Kb del	
2[2]	1-10	57 Kb del	57 Kb del	
3	1-10	57 Kb del	57 Kb del	
4	1-10	57 Kb del	57 Kb del	
5	1-10	57 Kb del	57 Kb del	
6[3]	1-10	57 Kb del	?	
7[3]	1-10	57 Kb del	?	
8	5	57 Kb del	198 del 21 bp	del 67-73 (in frame)[1]
9	11	922 ins G	922 ins G	S310Q (stop AA 364)
10	11	922 ins G	922 ins G	S310Q (stop AA 364)
11	3	18 del GACT	?	T7F (stop AA 13)

[1] amino acid change due to allele 2

[2, 3] siblings

Patients without the deletion or those who carried the 57-kb deletion on one allele, were submitted to genomic sequencing of the different exons to reveal possible other mutations in the *CTNS* gene. In 4 of the remaining 6 patients, another previously reported mutation was found [Anikster, Shotelersuk et al. 1999]. Two of them are homozygous for the 922insG, 1 patient is heterozygous for the 198del21 bp and carries the 57-kb deletion on

the other allele. The fourth patient carries the 18delGACT in heterozygous state and so far no second mutation has been detected in this patient. In summary, all patients show mutations in the *CTNS* gene, although in 3 patients only one heterozygous mutation has been found (table 2).

Genotype-Phenotype Correlation

Next to mutation analysis, we studied a possible genotype- phenotype correlation, comparing the phenotype of the homozygous deleted patients with the phenotype of the other genotypes. The age of diagnosis seems to be earlier in homozygotes for the 57-kb deletion compared to the other genotypes (17.4 vs. 32.0 months respectively) but significance was not reached ($p = 0.10$, Student's t test). Furthermore, a comparison between the genotype and the age of reduction in creatinine clearance (GFR < 50 ml/min) was made (51.3 vs. 58.5 months for 57-kb homozygotes vs. others), and no significant difference was observed ($p = 0.61$, Student's t test). Increase of TSH is only detected in patients 2 and 9, again not indicating a genotype-phenotype relation (data not shown).

Discussion

Infantile nephropathic cystinosis is a rare inborn error of metabolism, which causes renal failure due to the accumulation of cystine in the lysosomes. Mutation detection in the Dutch population revealed the presence of the common 57-kb deletion in 59% of the alleles. The allele frequency of the Dutch population is somewhat lower than the European average (76%) but is still in line with the hypothesis that this deletion arose in Europe and that it is probably due to a founder effect [Shotelersuk et al. 1998].

Performing a multiplex PCR to detect both the 57-kb deletion and the D17S829 marker in one tube gave in some cases false negative results, which is also reported by others [Anikster, Lucero et al. 1999]. Performing both PCRs separately (not in one tube) may also give false negative results, due to failing of the PCR by inhibition or manual mistakes. Therefore, we developed an improved screening method for accurate detection of the common 57-kb deletion. In contrast to previously reported PCR-based detection methods [Forestier et al. 1999], we chose to perform two separate multiplex PCRs for the detection of the 57-kb deletion or the D17S829 marker. In both PCRs we applied primers for an internal control fragment of ß-actin, which amplify a larger fragment than the frag-

ments of 423 and 266 bp of the LDM1 and D17S829 primers respectively. When the band of ß-actin of 635 bp is present, the other fragment (LDM1 or D17S829) must also be amplified by PCR, because smaller fragments are in general better amplified than larger ones. In this way, we are certain that every genotype is correctly determined, and that no false negative results could be obtained. Hereby, we developed an improved, accurate and reproducible procedure for the detection of the common 57-kb deletion, which enables proper discrimination between the different genotypes of the 57-kb deletion.

In 3 patients only a mutation was found on one allele in the coding region (including intron-exon boundaries) of the *CTNS* gene, indicating that a second mutation must be present. The presence of the second mutation may be located in the 3´-UTR or intronic sequences of the *CTNS* gene causing instability of the RNA or interruption of the splicing mechanism. Another possibility is the presence of mutations in the 5´-UTR or the promoter region of the *CTNS* gene, which can alter the expression of the gene.

Recently, Attard et al. [1999] showed a correlation between the form of cystinosis and the genotype. The infantile form, which is the most severe form of cystinosis, is associated with major DNA changes (deletions, insertions) or changes in important parts of the protein, whereas the other forms of cystinosis (late-onset, nonclassical) are associated with minor DNA changes in less important parts of the *CTNS* protein [Thoene et al 1999, Anikster, Shotelersuk et al 1999, Anikster et al 2000]. In this study, we investigated the infantile form of cystinosis and found next to the 57-kb deletion, three other mutations present in the *CTNS* gene. The first one is an insertion of guanine at DNA position 922, present in homozygous state in 2 patients. This insertion causes a frameshift, resulting in a premature stop codon at amino acid position 364, thereby disrupting the lysosomal target motif [Hunziker et al. 1996]. This motif regulates the transport of the protein to the lysosomal membrane.

The second mutation found is a heterozygous deletion of GACT at DNA position 18, which results in a premature stop codon at amino acid position 13. The last mutation found is a deletion of amino acids 67–73, which results in an in-frame deletion of seven amino acids of the protein. This deletion is located in the part of cystinosin that is present in the lysosomal lumen and results in loss of an N-glycosylation site [Attard et al. 1999]. This latter mutation may not completely destroy the transport function of cystinosin and is therefore expected to cause a milder form of cystinosis [Attard et al. 1999]. On the other hand, this study shows that presence of the 57-kb deletion next to the deletion of amino acids 67–73 likely causes the infantile form of cystinosis.

In summary, all mutations found in the Dutch infantile cystinosis patients are major DNA changes located in functionally important parts of the protein, or are mutations

that contribute to the severity of the phenotype next to a truncating mutation. These findings support recent published data on genotype-phenotype correlations of cystinosis patients [Attard et al. 1999, Anikster, Shotelersuk et al. 1999].

Several other genes seem to be located in the 57-kb deletion interval [Touchman et al. 2000]. Absence of these genes next to the *CTNS* gene could contribute to the phenotype of cystinosis.

Furthermore, the presence of the 57-kb deletion results in no protein expression at all, which may result in a more severe phenotype compared to other mutations present in the *CTNS* gene. Based on this hypothesis, we compared the phenotype of the homozygous form of this 57-kb deletion with other genotypes. This relatively small study shows that patients who are carrying the 57-kb deletion on both alleles did not have statistically different parameters compared to the other patients, although, the age of diagnosis seems earlier in patients who are homozygous for the 57-kb deletion. However, the alertness of the physician is an important factor that might influence the age of diagnosis. To study this relation, larger patient groups are necessary. The results from this study show that it is not presumable that other genes located in the 57-kb deletion region contribute extensively to the phenotype of cystinosis.

Screening for the 57-kb deletion will resolve about 45% (5 out of 11 patients) of the molecular basis of cystinosis in Dutch Caucasian patients. To reveal mutations present on the remaining alleles of cystinosis patients, it is necessary to perform sequencing analysis of the whole *CTNS* gene.

Early treatment is of vital importance for cystinosis patients. The sooner the diagnosis can be made, the earlier treatment can be initiated and the better the life expectancy of these children becomes. Molecular analysis facilitates prenatal diagnosis and identification of carriers and thereby genetic counselling.

CHAPTER 4

Follow-up and treatment of adults with cystinosis in the Netherlands

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Abstract

Background: Cystinosis is a rare autosomal recessive disease, caused by intracellular cystine accumulation due to a defect in the lysosomal cystine carrier.
Treatment with cysteamine favours the transport of cystine out of the lysosomes, diminishes organ damage, and postpones the progression of renal failure. The extra-renal deposition of cystine continues after renal transplantation, leading to later complications. The objective of this study was to evaluate the follow-up, the occurrence of late complications, the social status, and the adequacy of cysteamine treatment in adult patients with cystinosis.
Methods: The medical histories of 10 adult cystinosis patients aged 19–36 years were studied. The impairment of thyroid function, central nervous system, endocrine pancreas, and ocular manifestations, as well as treatment with cysteamine were evaluated.
Results: Eight patients received in total 12 renal grafts, one patient was dialysed and one received conservative treatment for chronic renal failure. Extra-renal complications were noted in six patients, loss of visual acuity in four, hypothyroidism in three, diabetes mellitus in one, cerebral atrophy and epilepsy in one, and swallowing difficulties in two patients. Ophthalmic control was not performed in two patients, thyroid function was not controlled in two and glycaemia not controlled in two patients. Seven patients received 2100–4000 mg cysteamine per day in 2 (n=2), 3 (n=1), 4 (n=3), or 6 (n=1) doses. Cystine concentration in leukocytes was measured once or twice a year in eight patients and was within the recommended range only in three patients.
Conclusion: A high rate of extra-renal complications in adults with nephropathic cystinosis was found. Optimizing the cysteamine therapy may attenuate these complications. Better communication between paediatric and 'adult's' nephrologists is needed to improve follow-up and treatment of grown-up cystinosis patients.

Introduction

Cystinosis, a rare autosomal recessive disease, caused by intracellular cystine accumulation due to a defect in the lysosomal cystine carrier, occurs in approximately 1 in 100,000–200,000 live births [Gahl et al. 2001]. The cystinosis gene, *CTNS*, identified by positional cloning strategy, has been mapped to the short arm of chromosome 17.

This gene encodes a protein, named cystinosin, with features of a lysosomal membrane protein [Town et al. 1998]. Renal Fanconi syndrome, an early clinical manifestation of the infantile form of nephropathic cystinosis, generally becomes apparent 3–6 months after birth. Untreated patients usually develop end-stage renal disease (ESRD) before the age of 10 years, with a wide range up to 20 years [Niaudet et al. 1999]. Late-onset form of cystinosis generally appears at the age of 12–14 years, often does not presents with complete Fanconi syndrome, but may progress to ESRD within a few years of diagnosis [Gahl et al. 2001]. An aminothiol, cysteamine, depletes intralysosomal cystine content by reacting with cystine to form cysteine–cysteamine mixed disulphide and cysteine, which can leave lysosomes via lysosomal lysine and cysteine carriers respectively. However, cysteamine does not reverse Fanconi syndrome and only postpones the commencement of renal-replacement therapy. After renal transplantation, the ongoing accumulation of cystine causes multi-organ damage: photophobia and loss of visual acuity due to corneal cystine crystals and retinopathy, bradykinesia, dementia, convulsions or spasticity due to cerebral atrophy, basal ganglia and periventricular calcifications or ischaemic lesions, muscle weakness and swallowing difficulties due to vacuolar myopathy, hypothyroidism, exocrine pancreas deficiency and diabetes mellitus [Kaiser-Kupfer et al. 1986, Broyer et al. 1996, Sonies et al. 1990, Kimoniset al. 1995, Fivush et al. 1988, Gahl et al. 1987]. Growth retardation, delayed puberty, hypogonadism and male infertility are frequent [Winkler et al. 1993, Tête et al. 1999].

These serious late complications require the continuation of cysteamine treatment after renal transplantation in order to diminish extra-renal cystine accumulation [Almond et al. 1993]. Regular measurement of intracellular cystine is indicated to control the efficacy of cysteamine treatment in pre- and post-transplant patients [Kamoun et al. 1999].

The aim of the study was to evaluate the follow-up, the occurrence of extra-renal complications, the social status, and the adequacy of cysteamine treatment in adult patients with nephropathic cystinosis in the Netherlands.

Subjects and methods

A retrospective analysis of case histories of 10 adult cystinosis patients who were followed in five Dutch university hospitals was performed. The diagnosis of cystinosis was based on the evidence of cystine corneal crystals and/or elevated leukocyte cystine level. The impairment of thyroid function, central nervous and muscular system, endocrine pancreas and ocular manifestations, the social status of the patients, and treatment with cysteamine (dose, frequency, and cystine measurements in leukocytes) were evaluated. Cystine leukocyte content was measured by HPLC in the same laboratory [de Graaf et al. 1999].

Results

Characteristics of the patients

The patient's age range was 19–36 years, there were six male and four female patients. Cystinosis was diagnosed during the first decade of life in all patients. At presentation, all patients had renal Fanconi syndrome and cystine corneal crystals. The median age at diagnosis was 3 years.

Renal replacement therapy (RRT) was initiated at median age of 12 years. Eight patients received in total 12 renal grafts, one patient dialysed and one had chronic renal failure and received conservative treatment.

Among the transplanted patients, six had well functioning renal grafts and two had pre-terminal graft failure due to chronic rejection (Table 1). Renal graft loss was not related to the cystinosis and was caused by arterial bleeding after the graft biopsy in patient no. 2, acute rejection (first graft), renal-artery stenosis and chronic rejection (second graft) in patient no. 5, and membranous glomerulopathy and chronic rejection in patient no. 10.

Table 1 Clinical characteristics of adult cystinosis patients.

Patients No	Age	Sex	Height cm	Age at start dialysis	Age at renal transplants	Most recent creatinine value µmol/l	Professional status	Visual acuity	Thyroid function	Glycemia	Neurological and muscle dysfunction
1	19	F	161	18	-	600	health care studies	decreased	normal	normal	no
2	21	F	160.1	10	12/14	160	shop assistant	normal	normal	normal	swallow dysfunction
3	21	M	158.5	12	14	100	low professional studies	normal	normal	*	no
4	23	F	150	11	12	79	secretary	*	normal	normal	no
5	24	M	150	7	7/9/19	58	does not work	decreased	decreased	**IDDM type 1	epilepsy
6	25	M	168.8	17	19	655	does not work	normal	decreased	*	no
7	29	M	163	13	16	800	driver	normal	*	normal	no
8	30	M	175.0	not yet started	-	571	mechanician	*	normal	normal	no
9	33	F	154.7	11	22	96	nurse	decreased	*	normal	no
10	36	M	166	22	19/25	120	does not work	decreased	decreased	normal	swallow dysfunction, dyspnoea

* not controlled/unknown
** IDDM - insulin-dependent diabetes mellitus

Extra-renal organ involvement (Table 1)

No patient died. Six patients suffered from major extra-renal complications. Two patients had multiple extra-renal organ involvement.

Visual acuity

Four patients had loss of visual acuity due to excessive cystine corneal accumulation, retinopathy at fundus examination in patients nos 1 and 5, and band keratopathy in patient 10. Two patients received no ophthalmic control.

Endocrine complications

One patient had insulin dependent diabetes mellitus, three had hypothyroidism and required thyroid hormone treatment. The thyroid gland function was not controlled in two patients. The glycaemic control was not performed in three patients and only occasionally in three other patients. Gonadal function was not assessed in any patient.

Central nervous system and muscle involvement.

Patient no. 8 had epilepsy with evidence of periventricular calcifications and cerebral atrophy on computer tomography of the brain. Two patients had swallowing difficulties due to myopathy. Patient no. 1 had mild dysphagia. In patient no. 10, swallowing problems appeared at the age of 22 years, predominantly during the passage of food through the pharynx, and were not progressive. At the age of 31 years he presented with complaints of dyspnoea. The evaluation revealed no heart involvement, but decreased force of respiratory muscles. Lung function examination showed a restrictive respiratory dysfunction. He was advised to stop smoking, which resulted in short reduction in complaints. Later, dyspnoea increased and was a cause of severe disability.

Growth

The median length of male patients was 164.50 cm (–3.70 SD) and of female patients 157.50 cm (–2.50 SD).

Table 2 Cysteamine treatment of adult cystinosis patients.

Patients No	Age at start cysteamine therapy years	Duration of cysteamine therapy years	Daily cysteamine dose mg/kg	Cysteamine daily doses times per day	Frequency of cystine measurement yearly	Last cystine leukocyte content nmol/mg protein	Instillation of cysteamine eye drops
1	8	11	60	2	2	0.45	yes
2	11	10	40	2	2	0.35	yes
3	3	18	70	4	2	0.11	yes
4	14	9	40	4	1	0.83	yes
5	*	-	-	-	**	-	no
6	*	-	-	-	2	0.23	yes
7	*	-	-	-	**	-	no
8	17	13	40	6	1	0.072	no
9	15	18	55	3	1	0.12	yes
10	25	11	40	4	2	3.00	no

* not treated, ** not measured

Social status, education, and integration

Three patients did not complete any professional study, two were still occupied with low and intermediate professional education, and five received low or intermediate education and were working (Table 1).

Patients nos 8 and 10 have a stable relationship.

Cysteamine treatment (Table 2)

Median age at start of cysteamine therapy was 14 years (range 3–25). At the time of evaluation, seven patients were receiving oral cysteamine treatment. The dose varied from 40 to 70 mg/kg cysteamine base per day in 2 (n=2), 3 (n=1), 4 (n=3) or 6 (n=1) doses. Three patients were not treated with cysteamine: one because of the patient's refusal (no. 5), and two patients did not receive a prescription from their physicians. Patient 4 was obviously not compliant with the prescribed medication.

In two patients no measurements of cystine levels in the leukocytes were done. In eight patients cysteamine was measured at least once a year; at last measurement median cystine content was 0.35 with a range of 0.072–3.00 nmol/mg protein. Only three patients had leukocyte cystine content within the recommended range (< 0.2 nmol/mg protein). No information about the interval between the last cysteamine dose and the time of blood examination was available.

Cysteamine eye drops were not administered in four patients.

Relation between cysteamine treatment and the occurrence of late complications

Among the three untreated patients, one (no. 5) had a severe course of the disease with multiple complications, including diabetes mellitus, epilepsy, and retinopathy. Patient no. 6 had impaired thyroid function, but patient no. 7 has not yet any extra-renal organ involvement.

Two patients treated according to the guidelines (nos 3 and 8) had no extra-renal complications.

Discussion

Renal transplantation and the availability of cysteamine treatment have transformed cystinosis from a fatal paediatric disease into a treatable one with which patients can survive into adulthood.

The evaluation of 36 adult American cystinosis patients, aged 17–34 years revealed a high rate of mortality and morbidity [Theodoropoulus et al. 1993]. Seven patients died at ages between 18 and 34 years from aspiration, pseudobulbar palsy, uraemia, or unexplained sudden death. Twenty-two per-cent of the patients were blind or had severely impaired vision, 86% required thyroid hormone replacement, 30% had distal myopathy, and more than 60% had swallowing difficulties. Only 11 of 36 patients received an adequate cysteamine treatment [Theodoropoulus et al. 1993].

Adult cystinosis patients are generally followed up by nephrologists, who pay major attention to the renal function, risks of renal osteodystrophy, hypertension, and other symptoms shared by all nephrological patients. The purpose of this study was to evaluate the adequacy of the follow-up and treatment of adult cystinosis patients, as they suffer from additional complications related to cystinosis and require specific cysteamine treatment.

Case histories of all Dutch adult cystinosis patients, followed in five University hospitals, were studied.

Despite the fact that the diagnosis of cystinosis was made before the age of 10 years and all patients had Fanconi syndrome at presentation, this series of patients was not homogenous. Patients 1, 6 and 10 reached ESRD at the age of 17–18 years, which is later than classically described in patients with an infantile form of cystinosis. Cysteamine treatment was not likely to explain the late onset of ESRD as it was either not administered or was administered late. Surprisingly, patients 1 and 2, who are siblings, developed terminal renal failure at the ages of 10 and 18 years respectively. The data from European Dialysis and Transplant Association Registry showed the median age at the start of renal replacement therapy to be 9.5 years, with a wide range of 1–20 years. No information on cysteamine therapy or clinical presentation was available in this database [Rigden et al. 1999].

Patient no. 8 presented at the age of 9 years with Fanconi syndrome and cystine crystals in the cornea.

Cysteamine was administered only at the age of 17 years. However, at the age of 30 he still did not require renal replacement therapy, which prevents him being classified as having an infantile form of cystinosis.

Attard et al. [1999] also described two patients, presenting at early age with classical infantile cystinosis, who did not develop renal failure at the age of 18 and 22 years. DNA analysis showed that these patients had at least one mutation, presumably permitting the production of some functional protein, which could account for their milder phenotype [Attard et al. 1999]. We did not perform mutation analysis in our studied patients, but believe that the diversity of genotype could explain their different clinical courses.

The morbidity of Dutch cystinosis patients, with an exception of visual impairment, was lower than in the American series, possibly due to the amelioration of cystinosis treatment during the last decade. A milder course of the disease in some patients could be another explanation for this lower morbidity

Seven of 10 Dutch adult patients were treated with cysteamine compared to 30% Americans; however, only three of 10 patients had the recommended leukocyte cystine level. Cystine was measured in the mixed leukocyte preparation by the HPLC method in the same biochemical laboratory, which excluded methodological differences in the determination.

According to the laboratory guidelines, blood for cystine determination has to be taken before the next cysteamine dose. However, we were not able to determine whether this recommendation was always followed. Measured by the HPLC method, cystine value in healthy controls (n=15) was 0.04–0.013 and in the obligate heterozygotes (n=15) 0.03–0.2 nmol/mg protein. As the cystine leukocyte level necessary to prevent the progression of renal disease and the occurrence of extra-renal complications is unknown, the upper heterozygote value was recommended as an upper limit of cystine before the next dose of cysteamine is given. We have recently changed from measuring cystine in mixed leukocytes to the granulocyte preparations, as cystine preferentially accumulates in the granulocytes. Heterozygote value was significantly higher when measured in the granulocytes (0.11–0.63). However, as all previous measurements have been done in the mixed leukocyte preparations, we used the last cystine leukocyte value to evaluate the current status of the patients.

No uniform follow-up and treatment strategy was applied by Dutch nephrologists. While renal function and arterial hypertension were assessed at each ambulatory visit, the extra-renal organ involvement relating to cystinosis was evaluated only occasionally or not at all. Two nephrologists did not prescribe any cysteamine treatment. Even when the measured cystine leukocyte content was above the recommended level, the dosage of cysteamine was not systematically adapted. Three patients received cysteamine in 2–3 doses, which is insufficient as the leukocyte cystine content returns to its original levels as early as 4–6 h after the cysteamine dose [Schneider et al. 1995].

Cysteamine eye drops are effective in reducing photophobia and density of corneal crystals in cystinosis patients; however, four patients did not receive this treatment. The effectiveness depends on the concentration and the number of daily instillations: a dose of 0.5% at 5–6 times a day is recommended [Dureau et al. 1999].

We have no information on compliance with the cysteamine therapy, except in patient no. 4, who was obviously non-compliant. Non-compliance also could be suggested in patient no. 10, who had a high leukocyte cystine content despite adequate cysteamine prescription. Non-compliance with cysteamine treatment is a difficult problem, as for patients the potential later benefits of cysteamine do not always outweigh the more immediate inconveniences such as an unpleasant odour or gastrointestinal discomfort.

In summary, the finding of a high incidence of extrarenal complications in adult Netherlands cystinosis patients, and a rather unsystematic follow-up, indicate the necessity of optimizing the care of these patients.

Better communication between paediatric and 'adults' nephrologists is required in order to continue adequate follow-up and treatment of cystinosis patients growing into adulthood. A minimum requirement would be an annual neurological examination, including the evaluation of the strength of oropharyngeal and hand muscles, and an ophthalmologic examination, regular determination of plasma TSH, T3 and glycaemic control, as well as measurement of intracellular cystine content at least twice a year.

CHAPTER 5

ACE inhibitor enalapril diminishes albuminuria in patients with cystinosis

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Abstract

Background/Aims: Cystinosis, a rare autosomal recessive disease, manifests with renal Fanconi syndrome during the first year of life. Interstitial damage is a major cause of renal failure in patients with cystinosis. We presume that albuminuria contributes to the development of renal failure in these patients. The aim of this study was to examine whether the administration of ACE-inhibitor enalapril diminishes albuminuria in patients with cystinosis.

Methods: Five patients with cystinosis aged 4-9 years were studied. All patients had Fanconi syndrome and were treated with cysteamine. Median creatinine clearance was 48 ml/min/1.73 m^2 (range 21-61). The excretion of albumin and α-1 microglobulin as well as arterial blood pressure and serum creatinine were evaluated before and at 3 months on oral administration of enalapril (0.15 mg/kg once daily).

Results: At three months on enalapril, albuminuria decreased in all patients (1042 vs 629 mg per 24 hrs, $p<0.05$). The median reduction of albuminuria was 43% (range: 4-72%, $p<0.05$). Urinary excretion of α-1 microglobulin remained constant.

Systolic blood pressure decreased from median 110 to 100 mm Hg ($p<0.05$), while diastolic blood pressure remained stable (median 60 mm Hg). Creatinine clearance decreased from median 48 to 45 ml/min/1.73 m^2 ($p<0.05$) and returned to previous values after the discontinuation of enalapril.

Conclusion: ACE-inhibitor enalapril diminishes albuminuria in patients with cystinosis and might be used in these patients in order to slow the progression of renal insufficiency attributed to proteinuria.

Introduction

Cystinosis, a rare autosomal recessive deficiency of the lysosomal cystine carrier, resulting in lysosomal cystine accumulation, occurs in approximately 1 in 100,000-200,000 live births [Gahl et al. 2001]. The cystinosis gene *CTNS*, encoding the lysosomal cystine carrier protein, named cystinosin, has been mapped to the short arm of chromosome 17 [Town et al. 1998].

Cystinosis is the most common cause of Fanconi syndrome in children, which generally becomes apparent 3-6 months after birth [Niaudet et al. 1999]. The excessive urinary loss of low molecular weight proteins such as α–1 microglobulin in patients with cystinosis is attributable to Fanconi syndrome. Albuminuria might occur due to increased glomerular filtration of albumin or diminished tubular re-absorption [Norden et al. 2001]. Untreated patients develop end stage renal failure due to chronic interstitial damage before the age of 10 years [Gretz et al. 1983, Gahl et al. 2002]. The aminothiol cysteamine depletes intra-lysosomal cystine content via a disulfide exchange reaction with cystine to form cysteine-cysteamine mixed disulfide and cysteine, which exit the lysosomes via lysosomal lysine and cysteine carriers respectively [Gahl et al. 1987, Schneider et al. 1995]. However, cysteamine does not reverse Fanconi syndrome and only postpones the start of renal replacement therapy [Markello et al. 1993].

The exposure of renal proximal tubules to an excessive amount of albumin changes gene expression profile of proximal tubular cells and leads to interstitial inflammation and fibrosis [Nakajima et al. 2002, Abbate et al. 1998]. The therapy with angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptors blockers diminishes albuminuria and slows the decline of renal function in different chronic nephropathies [Ruggenenti, Perna et al. 1998, Lewis et al. 1993, Ruggenenti, Mosconi et al. 1998]. As albuminuria has been shown to be an important factor in renal disease progression, we hypothesised that it might also contribute to the development of renal failure in patients with cystinosis and can be treated by ACE inhibitors.

The aim of this study was to examine whether the administration of the ACE-inhibitor enalapril could diminish albuminuria in patients with cystinosis by reducing the glomerular leakage.

Patients and methods

Five patients with Fanconi syndrome due to cystinosis, aged 4-9 years, were studied. All patients had homozygous mutations of *CTNS* gene: four - homozygous 57 kb deletion and one - homozygous 922insG. All of them were on regular cysteamine treatment (50-100 mg/kg divided in 4-5 daily doses), started at median age of 14 months (range 7-22 months). Median creatinine clearance was 48 ml/min/1.73 m^2 (range 21-61). Urinary excretion of albumin and α-1 microglobulin (α-1 MG) as well as arterial blood pressure, serum creatinine and creatinine clearance were evaluated before and at 3 months on oral administration of enalapril (0.15 mg/kg once daily). Creatinine clearance was also controlled 3 months after the discontinuation of enalapril. Blood pressure was measured in supine position by Dinamap (Criticon, Tampa, FL). The lowest value of 3 consecutive readings was used for further analysis. Wilcoxon signed-rank test was applied for statistical analysis. Differences were considered statistically significant at $p<0.05$.

Results

Table 1 summarises patient's data before and at 3 months on enalapril administration.

Table 1 Influence of enalapril on blood pressure, the excretion of albumin, α-1 microglobulin and creatinine clearance of cystinotic patients with Fanconi syndrome.

Patients	1	2	3	4	5
Age [years]	8	7	7	5	4
Mutation of *CTNS*	hom 57 kb del	hom 922insG	hom 57 kb del	hom 57 kb del	hom 57 kb del
Blood pressure [mm Hg]					
before	110/60	110/60	110/60	110/70	130/60
at 3 months	100/60	100/60	95/50	100/60	120/60
Albuminuria [mg/24 hours]					
before	1695	2704	2211	602	173
at 3 months	959	1998	629	308	166
α-1 MG [mg/24 hours]					
before	219	234	234	172	107
at 3 months	364	279	526	164	114
Creatinine clearance [ml/min/1.73 m^2]					
before	21	24	48	57	61
at 3 months	15	17	45	53	55
3 months after discontinuation of enalapril	21	22	51	54	68

Albuminuria diminished in all patients. The median reduction of albuminuria was 43% (range: 4-72%, $p<0.05$) (Figure 1a). The urinary excretion of α-1 MG statistically remained unchanged (Figure 1b).

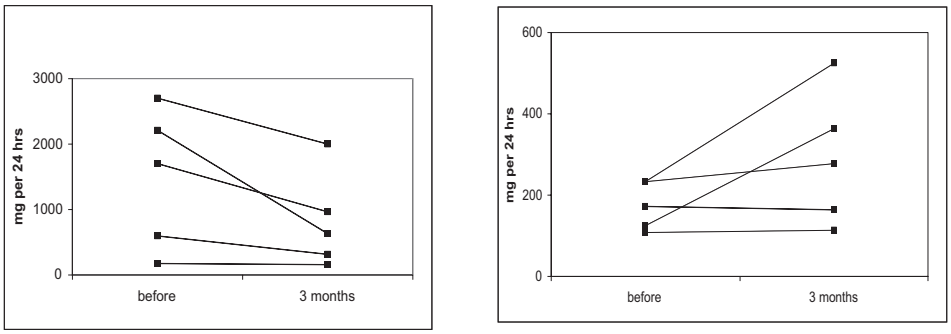

Figure 1 In patients with cystinosis, influence of enalapril on a. albuminuria excretion; b. α-1 microglobulin excretion

Systolic blood pressure decreased from median 110 to 100 mm Hg ($p<0.05$), while diastolic blood pressure remained stable (median 60 mm Hg). Two patients had complaints related to the lowed blood pressure such as fatigue and dizziness.

Creatinine clearance decreased from median 48 ml/min/1.73 m2 (range 21-61) to 45 ml/min/1.73 m2 (range 15-55) ($p<0.05$). The most apparent rise in serum creatinine occurred in patients 1 and 2 with creatinine clearance below 25 ml/min/1.73 m^2. After the discontinuation of enalapril creatinine clearance returned to the base line in all patients (median: 51, range: 21-68).

Discussion

It is established that the intervention in the renin angiotensin system reduces proteinuria and slows the progression of renal insufficiency independently of blood pressure correction [Ruggenenti et al. 2001]. Although it is presumed that anti-proteinuric effect of ACE inhibitors reflects changes in intraglomerular haemodynamics, the influence of these drugs on nephrin expression may be important for the reduction of glomerular hyperpermeability [Benigni et al. 2001, Kelly et al. 2002, Davis et al. 2003].

This issue as far as we know has never been addressed in patients with cystinosis. As albuminuria is constantly found in urine of patients with cystinosis, we hypothesised that it might contribute to the development of the chronic interstitial inflammation and fibrosis, leading to renal failure [Gubler et al. 1999], even on cysteamine therapy. Although it is unknown to which degree albuminuria contributes to the development of interstitial fibrosis in cystinotic patients, the reduction of albuminuria could mitigate the loss of kidney function in these patients.

Our study shows that albuminuria was significantly decreased in all patients 3 months on the administration of the ACE-inhibitor enalapril. Interestingly, only a slight decline (4%) was observed in patient 5 with the highest creatinine clearance (61 ml/min/1.73 m^2) and lowest albumin excretion, indicating that the contribution of glomerular albuminuria is probably low in this patient. The fact that albuminuria decreases, while the excretion of low molecular proteins remains stable, favours an effect of enalapril on the glomerular leakage of albumin.

A strong association exists between acute increase of serum creatinine of up to 30% within first months on ACE therapy and long-term preservation of renal function in patients with diabetic and non-diabetic renal disease [Bakris et al. 2000]. The most common course of this acute rise in serum creatinine level is a decreased effective arterial blood volume, obviously present in cystinotic patients, having sodium loss and polyuria. In our group, creatinine rise was most obvious in 2 patients with creatinine clearance below 25 ml/min/1.73 m^2. These changes were transient and the discontinuations of enalapril resulted in return to the base line in all patients. Even a low dose of enalapril should be used with caution in normotensive patients with cystinosis, especially in those with a severe degree of renal insufficiency, warranting frequent monitoring of serum creatinine.

We can speculate whether the administration of angiotensin II (ATII) receptor blocker would result in less hypotensive effect in this patient's group. In pre-clinical studies ACE-inhibitors and ATII receptor blocker were shown to have similar effect on glomerular capillary pressure [Lafayette et al. 1992]. In clinical settings it was suggested that ACE-inhibitors have a slightly more hypotensive effect [Gansevoort et al. 1999].

In summary, we have demonstrated that the ACE-inhibitor enalapril diminishes albuminuria in patients with cystinosis and might be used in these patients in order to slow the progression of renal insufficiency attributed to proteinuria.

CHAPTER 6

Negligible urinary cysteamine loss in cystinosis patients with Fanconi syndrome

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Clin Nephrol 57: 349-351, 2002

Abstract

Background/Aims: Cystinosis is an inborn error of lysosomal cystine transporter, resulting in cystine accumulation in lysosomes of all cells. Renal Fanconi syndrome is an early sign of kidney involvement in cystinosis patients. Cysteamine, a small amino thiol, depletes intralysosomal cystine content and reduces organ damage. However, it does not reverse renal Fanconi syndrome and only postpones the progression to renal failure. We examined whether cysteamine could be lost in urine of cystinosis patients with Fanconi syndrome, which may explain the inefficiency of treatment. Urinary cysteamine loss was studied in cystinosis patients with and without Fanconi syndrome.

Patients and methods: Urine of 6 cystinosis patients was collected during 6 hours following a morning gift of cysteamine. Four patients had renal Fanconi syndrome, 2 patients were transplanted and had no Fanconi syndrome. Each urine sample was examined for cysteamine, α-1 microglobulin and creatinine. Cysteamine was determined, after reduction of all disulfides with dithioerythriol and sodiumboriumhydrate, by HPLC.

Results: The administered morning doses of cysteamine ranged 150-1000 mg (40-150 mg/kg/day). All four patients with renal Fanconi syndrome had high alpha-1 microglobulin excretion. One transplanted patient had a normal α-1 microglobulin excretion. In all patients urine cysteamine excretion ranged 0.9-7.2 mg/portion, which was less that 1% of the ingested dose.

Conclusion: Negligible urinary cysteamine loss occurs in cystinosis patients with or without Fanconi syndrome.

Introduction

Cystinosis is an autosomal-resessive defect of the lysosomal cystine carrier, resulting in accumulation of free amino acid cystine in lysosomes [Gahl 1986]. Renal Fanconi syndrome is an early sign of kidney involvement in cystinosis. Long-term therapy with the amino-thiol cysteamine depletes intralysosomal cystine content and reduces organ damage, the rate of progression to renal failure and improves growth [Gahl et al. 1985, Schneider et al. 1995]. However, treatment with cysteamine does not reverse the renal Fanconi syndrome in cystinosis patients. Cysteamine is a small amino-thiol with a molecular weight of 77.14, which could be lost in urine of cystinosis patients with renal Fanconi syndrome, resulting in insufficient cystine depletion in proximal tubular cells.

The aim of this study was to estimate urinary cysteamine loss in cystinosis patients with and without Fanconi syndrome, treated with cysteamine.

Patients and methods

Urine of 6 cystinosis patients, 6-13 years old, was collected during 6 hours following morning gift of phosphocysteamine or cysteamine bitartrate (Cystagon®). Four patients had renal Fanconi syndrome, 2 transplanted patients had no Fanconi syndrome: one with well functioning renal graft and the other with pre-terminal renal failure. Each urine sample was examined for cysteamine, alpha-1 microglobulin and creatinine content.

Cysteamine was determined according to Fiskerstrand et al. [1993] with some modifications [de Graaf-Hess et al. 1999]. In short, all disulfides are reduced by dithioerythriol and sodiumboriumhydrate. Next all free thiol groups are derivatized by Thiolyte, resulting in fluorescent compounds which are separated by HPLC with reverse-phase column and quantified by a fluorometer. Figure 1 shows the typical HPLC elution patterns.

Creatinine was measured by a standard procedure. α-1 microglobulin was measured by a immunonephelometry.

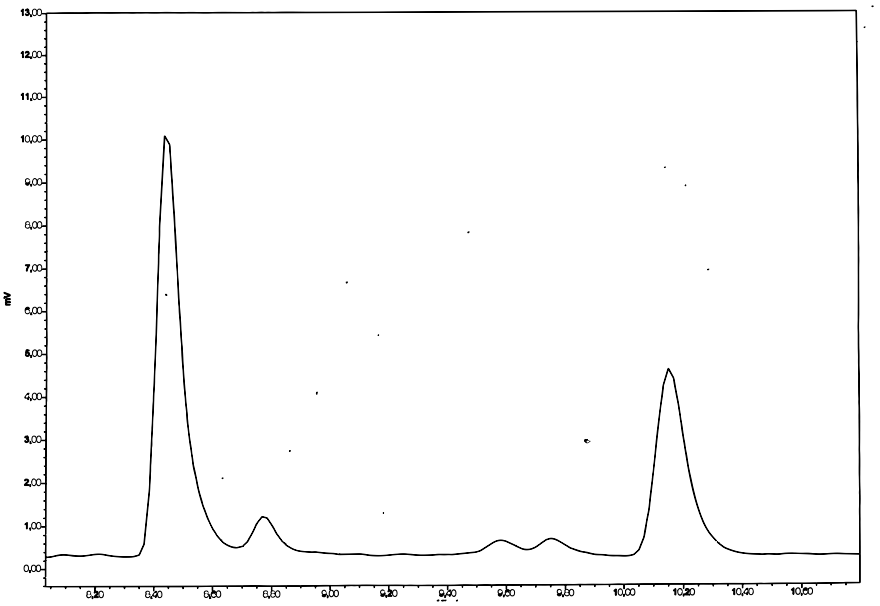

Figure 1 HPLC chromatograms of thyolyte derivatized thiols in urine of patient SE. Cysteamine peak at 10.15 min

Results

Data of the patients are presented in a table 1. The orally administered morning doses of the cysteamine ranged 150-1000 mg (40-150 mg/kg/day). All four patients with renal Fanconi syndrome had high α-1 microglobulin excretion. One transplanted patient had normal α-1 excretion. Urine cysteamine excretion ranged 12.7 - 99.5 μmol/l. Knowing the molecular weight of cysteamine and urine volume during 6 hours collection, we calculated cysteamine excretion per 6 hours, which was 0.9-7.2 mg, which was less than 1% of ingested morning doses. The highest cysteamine excretion was observed in a patient KK, who has well functioning renal graft.

Discussion

Cysteamine treatment of cystinosis patients does not restore the renal Fanconi syndrome and in most patients only postpones the development of renal failure. Theoretically, orally ingested cysteamine could be lost in urine of patients with Fanconi syndrome and resulted in insufficient cystine depletion in proximal tubular cells.

Table 1 Cysteamine and alpha-1 microglobulin excretion in 6 cystinosis patients

Patient's initials	Age	Morning cysteamine dose	Daily cysteamine dose	Urine cysteamine			Alpha-1-MG
	years	mg	mg/kg/day	mmol/l	mg/6h portion	% of ingested dose	mg/g creatinine
AK	13	500	60	7.4	0.2	0.04	990
SE	7	500	90	99.5	1.7	0.34	789
VB	6	150#	40	20.5	1	0.66	817
SA	8	700	140	12.7	1.3	0.19	564
BM*	14	1000	150	31.6	0.9	0.09	not done
KK**	14	1000	80	13.3	7.2	0.72	13,3

normal value: <30

* transplanted, preterminal renal failure
**transplanted, well functioning graft
cysteamine bitartrate

Our patients received recommended dose of cysteamine [Gahl 1999], which was adjusted to leukocyte cystine content 4 times/year. We found that less than 1% of ingested cysteamine was excreted in urine. Our data confirms those of Jonas Schneider et al. [1982], who measured 24 hours cysteamine excretion in 5 cystinosis patients, receiving an aqueous solution of cysteamine, which is less stable than phosphocysteamine or cysteamine bitartrate, administered in our patients. They applied an indirect method for cysteamine measurement, using the binding of cysteamine in patients plasma or urine with radiolabeled cystine thiosulfonate [Jonas et al. 1981].

It was shown that plasma cysteamine is less than 20 % protein bound [Smolin et al. 1988]. This fraction could be lost in urine bound to albumin as in 4 cystinosis patients with Fanconi syndrome an increased albumin excretion (0.173-2.7g/24 hour) was found (own unpublished data). However, using our method, total urine cysteamine could be determined, which excludes that protein-bound fraction was missed.

Finally, we may conclude that negligible urinary cysteamine loss occurs in cystinosis patients with and without Fanconi syndrome.

CHAPTER 7

Strict cysteamine dose regimen is required to prevent nocturnal cystine accumulation in cystinosis

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Abstract

Cystinosis is an autosomal recessive disorder, caused by mutations in the lysosomal cystine carrier cystinosin, encoded by the *CTNS* gene. The disease generally manifests with Fanconi syndrome during the first year of life and progresses towards end stage renal disease before the age of 10 years. Cysteamine depletes intralysosomal cystine content, postpones the deterioration of renal function and the occurrence of extra-renal organ damage. Based on the pharmacokinetic data, patients with cystinosis are advised to use cysteamine every 6 h. The aim of this study was (1) to evaluate the cysteamine dose regimen in Dutch patients with cystinosis and (2) to determine morning polymorphonuclear (PMN) leukocyte cystine content 6 h vs 9 h after the last evening cysteamine dose. Only 5/22 of Dutch cystinosis patients ingested cysteamine every 6 h. Morning (8 a.m.) PMN cystine content in 11 examined patients was elevated 9 h after 12.5–15 mg/kg evening cysteamine dose compared to the value 6 h after the ingestion of the same dose (0.73± 0.81 nmol vs 0.44 ± 0.52 nmol cystine/mg protein, p=0.02). In conclusion, only the minority of Dutch cystinosis patients follows the recommended strict cysteamine dose regimen. We provide evidence that cysteamine has to be administered every 6 h, including the night, as it has much better effect for maintaining low PMN cystine levels.

Introduction

Cystinosis is a rare autosomal recessive disorder caused by a defect in the lysosomal cystine carrier cystinosin, encoded by the *CTNS* gene [Kalatzis et al. 2001, Town et al.1998]. Cystine accumulation due to impaired cystine exit from the lysosomes in all tissues causes a multi-organ disease, with kidneys being clinically first affected. In general patients with cystinosis manifest with poor growth and generalized proximal tubular dysfunction (Fanconi syndrome) during the first year of life and develop end stage renal disease before the age of 10 years. Longer survival of these patients due to renal transplantation reveals extrarenal organ damage, such as hypothyroidism, diabetes mellitus, hypogonadism, peripheral and central neuropathy, distal myopathy and retinal blindness, usually becoming apparent after the first decade [Gahl et al. 2001, Gahl et al. 2002].

In 1976 Thoene et al. demonstrated that the aminothiol cysteamine lowered leukocyte cystine content in cystinosis patients [Thoene et al. 1976]. Cysteamine depletes intralysosomal cystine via disulfide exchange reaction with cystine resulting in a formation of cysteine and cysteine-cysteamine mixed disulfide, which exit the lysosomes via cysteine and lysine carriers, respectively, therefore bypassing the defective cystine carrier [Pisoni et al. 1985] (Figure 1).

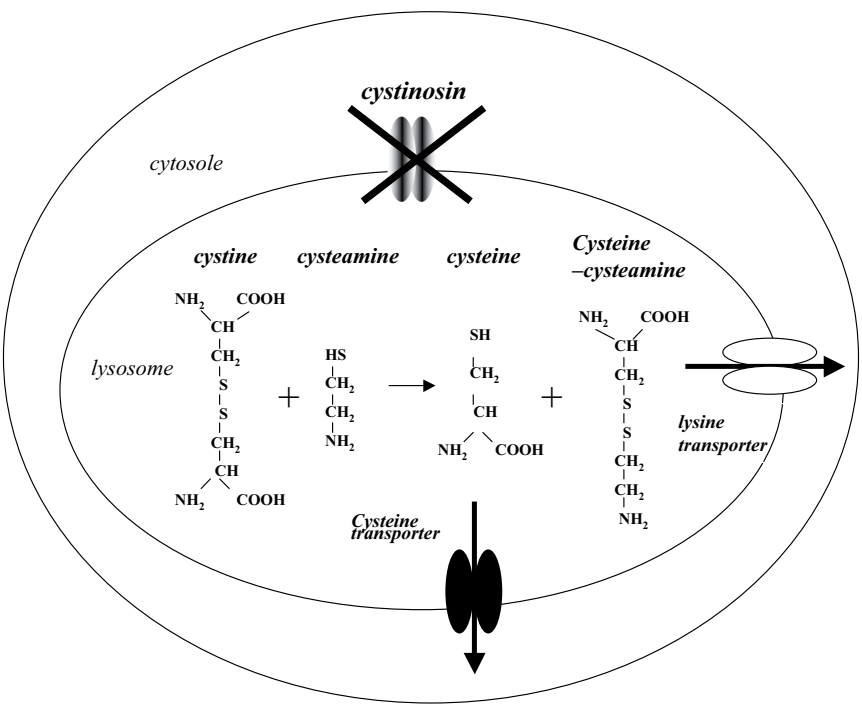

Figure 1 Disulfide exchange reaction between cysteamine and cystine, resulting in formation of free cysteine and cysteine-cysteamine mixed disulfide, exiting the lysosome via cysteine and respectively lysine carriers

To diagnose cystinosis and to monitor cysteamine treatment, cystine determination in the leukocytes is mandatory. Although cystine content of leukocytes might not completely reflect the accumulation in the other organs, the levels below 1 nmol 1/2 cystine/mg protein (= 0.5 nmol cystine/mg protein) are considered to reflect an adequate cysteamine dose [Gahl et al. 2001, Gahl et al. 2002, Kleta et al. 2005]. Cysteamine treatment slows the deterioration of renal function, improves growth and should be continued also after renal transplantation, in order to postpone the occurrence of extrarenal damage [Gahl et al. 2001, Gahl et al. 2002, Kleta et al. 2005, Markello et al. 1993, Sonies et al. 2005]. Unfortunately, renal Fanconi syndrome generally remains irreversible, and some of the patients still develop renal insufficiency and extrarenal complications, despite cysteamine treatment.

Because intracellular leukocytes' cystine content decreases to the minimal levels about 2 h after cysteamine ingestion and then returns to pre-dose levels 6 h after the drug intake, the adequate cysteamine dose regimen implies the administration of the drug every 6 h [Smolin et al. 1988, Belldina et al. 2003]. This dose regimen, however, is extremely difficult to follow, especially at night. We assumed that the nocturnal cystine accumulation might be partially responsible for the ineffectiveness of cysteamine therapy in some patients and, therefore, we evaluated the current cysteamine dose schedule in a Dutch cohort of cystinosis patients. Additionally, we compared morning polymorphonuclear (PMN) leukocyte cystine content after 6 h vs 9 h night pause following the last evening cysteamine ingestion. PMN leukocytes were used instead of mixed leukocyte preparations, as cystine preferentially accumulates in these cells and not in the lymphocytes [Smolin et al. 1987, Levtchenko et al. 2004].

Patients and methods

Questionnaire

Twenty-two patients aged 14.7 ± 9.7 years, 14 males, filled out the questionnaire concerning the schedule of cysteamine bitartrate (Cystagon®) intake. The diagnosis of cystinosis was made in all patients, presenting with renal Fanconi syndrome, by determination of elevated PMN cystine content and finding of corneal cystine crystals.

Comparison of morning polymorphonuclear cystine content 9 h vs 6 h night pause after evening cysteamine intake

In 11 compliant patients, after signing informed consent, 12.5–15 mg/kg cysteamine bitartrate was administered four times daily:

1. During the first week at 8 a.m., 2 p.m., 8 p.m., and 2 a.m.
2. During the second week at 8 a.m., 1 p.m., 6 p.m. and 11 p.m.

At the end of each week, blood samples were taken at 8 a.m. for PMN cystine dosage prior to the ingestion of the first morning cysteamine dose.

Intracellular cystine content of PMN cells was determined by high-performance liquid chromatography (HPLC) as described previously and expressed in nmol cystine/mg protein (cystine concentration x 2 = 1/2 cystine concentration) [Levtchenko et al. 2004, de Graaf et al. 1999].

Statistical analysis

Data are presented as mean ± SD. To compare data between patients following strict (every 6 h) Cystagon® dose schedules and those not taking the drug during the night, unpaired student t-test was applied. To compare morning intracellular PMN cystine values 6 h versus 9 h night pause after the last evening Cystagon® dose, paired student t-test was applied. Values were considered statistically significant at $p<0.05$.

Results

Questionnaire

Five patients (23%) followed the strict Cystagon® dose regiment (group 1). Seventeen patients (group 2) received Cystagon® only during the wake time. Patients of group 1 were younger compared to those of group 2 (7.5± 2.7 years vs 15.8± 9.2 years, p=0.004). Daily Cystagon® dose was not different between the two groups. The night pause between the last evening/night and the first morning Cystagon® ingestion was significantly longer in patients from group 2 (8.9± 2.0 h vs 6.1± 0.7 h, p=0.001). Mean PMN cystine content (the average of all determinations during 2004), determined 5–6 h after

Cystagon® ingestion, was significantly higher in patients of group 2 compared to group 1 (0.6± 0.3 nmol vs 0.37± 0.13 nmol cystine/mg protein, p=0.02) (Table 1). No relation between the age of the patients and mean PMN cystine content could be detected.

Intracellular PMN cystine content was determined four times yearly in all patients of group 1. In patients of group 2 it was determined four times yearly in eleven, three times in two and two times in four patients.

Table 1 Clinical data on Dutch patients following strict (every 6 hours) Cystagon® dose regiment (Group 1) and patients receiving Cystagon® only during the wake time (Group 2).

	Group 1 n=5	Group 2 n=17	*p*
Age (years)	7.5 ± 2.7	15.8 ± 9.2	0.004
Cystagon® dose (mg/kg/day	62 ± 16	53 ± 11	0.3
Night pause (hrs)	61 ± 0.7	8.9 ± 2.0	0.001
Average PMN cystine (nmol/mg protein)	0.37 ± 0.13	0.60 ± 0.30	0.02

Comparison of morning polymorphonuclear cystine content 9 h vs 6 h after evening cysteamine intake

In 11 examined patients, morning PMN cystine content after 9 h night pause following the evening cysteamine dose (12.5–15 mg/kg) was significantly elevated compared to the PMN content 6 h after the ingestion of the same dose (0.73 ± 0.81 nmol vs 044 ± 0.52 nmol cystine/mg protein, p=0.02) (Figure 2).

Discussion

Cysteamine is the only available cystine-depleting drug, postponing the deterioration of the renal function, improving growth and delaying the occurrence of extrarenal complications in patients with cystinosis. To achieve the maximal effectiveness of the treatment, the following recommendations have to be followed [Kleta et al. 2005, Schneider 2004]:

1. Starting cysteamine as early in life as possible (target dose 60–90 mg/kg/day or 1.3–1.95 g/m²/day) [Gahl et al. 2001, Gahl et al. 2002, Kleta et al. 2005]

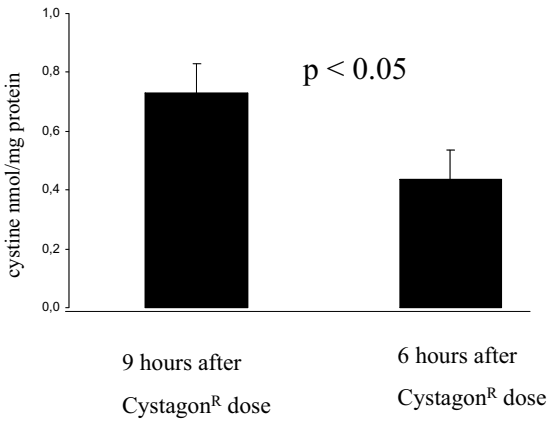

Figure 2 Morning polymorphonuclear cystine content (mean+SD) in 11 examined patients 6 h vs 9 h night pause following the last evening/night Cystagon® ingestion

2. Administering cysteamine every 6 h
3. Frequent monitoring of leukocyte cystine content
4. Obtaining blood for cystine determination 5–6 h after cysteamine ingestion

The most difficult recommendation to follow is to administer cysteamine every 6 h, which means that at least one cysteamine dose should be taken during the night. The nocturnal accumulation of cystine in patients who do not follow the strict 6 h dose regimen might be responsible for the relative ineffectiveness of the treatment.

In this study, we evaluated actual cysteamine treatment schedule in 22 Dutch patients with cystinosis. The medical advice to administer cysteamine every 6 h was respected only by the minority of the patients (23%). In patients following the strict cysteamine dose regimen, average PMN cystine content was significantly lower compared to the other patients, despite the fact that their daily cysteamine doses were comparable.

Despite the evident pharmacokinetic data [Smolin et al. 1988, Belldina et al. 2003], many patients and even many physicians do not realize that 2–3 h extra night pause may cause a significant cystine accumulation. Our study demonstrates that the administration of the same daily cysteamine dose distributed equally during 24 h results in significantly lower morning PMN cystine content, compared to the "only wake-time" regimen, applied by the majority of the patients.

Although it is unknown whether leukocyte cystine content reflects cystine accumulation in the other tissues, it can be suggested that in patients ingesting cysteamine only during the wake time, nocturnal accumulation of cystine may occur in the other organs.

Well preserved creatinine clearance in two siblings 15 years and 8 years old with infan-

tile cystinosis adequately treated with cysteamine, starting from early age, supports the idea that a dose regimen of every 6 h is necessary for the preservation of renal function [Kleta et al. 2004].

All well-treated patients from our Dutch cohort were relatively young, meaning that their parents were responsible for the compliance with therapy. During puberty, it often becomes difficult to convince the patient to continue with the strict cysteamine dose regimen, especially taking into account the annoying side effects of the drug, such as bad breath odor and gastrointestinal discomfort. The latter side effect, caused by an increased secretion of gastric acid after cysteamine ingestion, can be successfully treated by proton-pump inhibitors [Dohil et al. 2003]. Some breath odor improving drugs such as chlorophyll or essential oils (Breath Assure®) are used by some cystinosis patients, helping to improve their compliance.

Interestingly, in all patients following a strict dose regimen, PMN cystine determination was performed four times per year compared to the less frequent measurements in 7/18 of the other patients, suggesting better awareness and responsibility of the physicians treating these patients.

In conclusion, cysteamine treatment is effective in reducing cystine accumulation in cystinosis when taken on a regular basis. We have provided additional evidence that cysteamine has a much better effect in maintaining low PMN cystine levels when taken every 6 h, including during the night.

CHAPTER 8

Development of Fanconi syndrome during infancy in a patient with cystinosis

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Acta Paediatr, in press

Abstract

Cystinosis, an autosomal recessive disorder due to a defective cystine exit out of the lysosomes, is a major cause of inherited Fanconi syndrome in childhood. The causative gene *CTNS* (17p13) encodes the lysosomal cystine carrier cystinosin. The pathogenesis of cystinosis is poorly understood and ATP depletion is suggested based on the studies of proximal tubules loaded with cystine dimethyl ester.

We describe a patient with cystinosis from the age of 3 weeks who demonstrated gradual development of Fanconi syndrome during the first 6 months of life. The absence of complete Fanconi syndrome and cystine content of white blood cells within the heterozygote range led to a delay of the diagnosis of cystinosis. Gradual deterioration of proximal tubular dysfunction contrasts an immediate appearance of transport defects after cystine dimethyl ester loading and therefore in our opinion contradicts the hypothesis of ATP depletion being a central player in the pathogenesis of cystinosis. Further studies are required to explain differential sensitivity of proximal tubular transporters to cystine accumulation. Additionally, we would like to stress that treatment with cystine depleting agent cysteamine, should be started as soon as possible as full-blown Fanconi syndrome is not present at birth and might be prevented.

Introduction

Cystinosis is an autosomal recessive lysosomal storage disease due to a defect of *CTNS* gene (17p13) encoding the lysosomal membrane protein cystinosin [Gahl et al. 2002]. While the function of cystinosin has been elucidated [Kalatzis et al. 2003], the explanation of the clinical symptoms of cystinosis remains enigmatic. It is generally accepted that the lysosomal accumulation of cystine induces in some way a decrease in intracellular ATP, which would diminish sodium-coupled transport in the proximal tubular cells. Renal proximal tubules loaded with cystine dimethyl ester displayed an impaired oxidative phosphorylation and reduction of intracellular ATP [Foreman et al. 1987, Coor et al. 1991, Baum 1998]. A direct proof for ATP depletion as a keystone in the pathogenesis of cystinosis is lacking. The clinical symptoms observed in cystinotic patients do not directly underpin this hypothesis. We investigated the renal function of a patient with proven defect in *CTNS* during the first 6 months of life, demonstrating gradual development of proximal tubular dysfunction.

Case report

A male sibling of a patient with cystinosis, born after an uneventful pregnancy with a birth weight of 3720 gram, presented at the age of 3 weeks at the outpatient clinic with a weight of 4210 gram in a perfect clinical condition. Laboratory investigations showed normal values for serum creatinine, potassium, phosphate and bicarbonate (table 1) and mild aminoaciduria (table 2). The mixed leukocyte cystine content was within the heterozygote range 0.15 nmol/mg protein (normal 0.09±0.03). Cystine measurement in polymorphonuclear leucocytes, a superior method [Levtchenko et al. 2004], was not performed. At the age of 6 months he weighted 8260 gram and his serum concentrations of phosphate and bicarbonate were decreased. The cystine content in the mixed leukocyte was 0.14 nmol/mg protein. As the tubular abnormalities became more severe, a skin biopsy was performed to determine cystine content in skin fibroblasts, demonstrating clearly an elevated value: 1.6 nmol/mg protein (normal 0.10±0.07). The diagnosis of cystinosis was made and cysteamine treatment was started. Molecular analysis demonstrated a homozygous 57kb deletion of *CTNS* gene, confirming the diagnosis. Actually he is 8 years old, has a creatinine clearance 75ml/min/1.73 m^2 and a persistent Fanconi syndrome.

The clinical and laboratory data are presented in tables 1-3.

Table 1 Clinical and laboratory data of the patient.

Age	3 weeks	3 months	6 months	Reference*
Weight (gr)	4210	6850	8280	-
Serum creatinine (mmol/l)	31	35	43	3 weeks: 25-90 3 months: 20-80 6 months: 20-50
Serum phosphate (mmol/l)	2.14	1.52	0.91	1 months: 1.4-2.8 6 months: 1.2-2.2
Serum bicarbonate (mmol/l)	24.5	22.3	18.3	20-26
Serum potassium (mmol/l)	4.5	4.7	4.8	1 month: 3.5-6.0 >1 month:3.5-5.0
Tubular phosphate reabsorption (%)	90	78	43	> 80%
Urine α-1 microglobuline (mg/l)	not done	45	150	< 50 mg/l**

*References values from N. Webb & R. Postlethwaite ed. "Clinical paediatric nephrology" 3rd edition, Oxford, 2003, p. 494-503
** Reference value from Lehrnbecher et. al. Pediatr Nephrol 1998; 12: 290-292

Table 2 Amino acid excretion (mmol/mmol creatinine).

Age	3 weeks	3 months	6 months	Reference
3-methylhistidine	29	24	22	19-40
a-aminobutyrate	2	8	15	0-7
b-aminoisobutyrate	4	102	292	0-216
Alanine	276	1228	1090	72-206
Arginine	10	87	75	0-11
Asparagine	50	575	600	0-58
Citrulline	3	201	195	0-10
Cystine	70	380	346	13-48
Glutamine	235	2554	3663	63-229
Glutamic acid	6	39	26	0-29
Glycine	1758	3820	2642	210-743
Histidine	287	1030	1018	72-342
Hydroxyproline	505	583	220	0-143
Isoleucine	not done	10	8	0-5
Leucine	45	73	91	4-12
Lysine	239	1416	1167	15-199
Methionine	8	8	7	6-22
Ornithine	14	256	425	0-13
Phenylalanine	9	93	176	7-28
Proline	101	862	881	0-130
Serine	300	1610	1462	42-194
Taurine	456	482	780	6-89
Threonine	168	1613	881	17-92
Tyrosine	36	276	257	12-52
Valine	32	103	198	4-19

Table 3 Monosaccharides and polyoles excretion (mmol/mmol creatinine).

Age	3 weeks	3 months	6 months	Reference
Allose	*	7	6	0-11
Arabinose	*	46	46	8-70
Arabitol	*	66	47	51-99
Erythriol	*	852	789	89-158
Fructose	*	25	31	3-154
Fucose	*	15	10	0-30
Galactitol	*	55	44	10-63
Galactose	*	468	553	0-358
Glucose	<2300	4516	9630	4-92
Lactose	*	99	164	0-150
Mannitol	*	12	8	0-17
Myo-inositol	*	563	398	15-113
Ribitol	*	11	7	10-17
Sorbitol	*	8	8	2-13
Treitol	*	31	30	24-79
Xylitol	*	9	5	5-11
Xylose	*	23	27	0-139

* - not done

Discussion

As far as we know this is the first detailed report, demonstrating gradual appearance of Fanconi syndrome in an infant with cystinosis.

Excellent reports of Brodehl et al. previously demonstrated a disturbed phosphate reabsorption and hyperaminoaciduria already at the age of 4 1/2 months [Brodehl et al. 1965, Hagge et al. 1965]. Reznik et al. [1991] described proximal tubular dysfunction in 3 infants with cystinosis 6-8 months old both having decreased phosphate and amino acid reabsorption. These studies, however, did not provide longitudinal data on the development of Fanconi syndrome during infancy.

In our patient full-blown Fanconi syndrome developed gradually during the first 6 months of life as reflected by the lowering of serum bicarbonate, a defect in the reabsorption of phosphate and more pronounced aminoaciduria and glucosuria. Gradual deterioration of proximal tubular function can be related to progressive morphologic changes of proximal tubules in cystinotic patients. While no significant changes were observed in the fetus [Gubler et al. 1999], typical proximal tubular atrophy called "swan neck" deformity was demonstrated by serial biopsies of 2 cystinotic patients only after the age of 6 months [Mahoney et al. 2000].

Although this is a single case, our observation indicates differential sensitivity of apical proximal tubular transporters to cystine accumulation. Gradual development of

Fanconi syndrome during the first 6 months of life contrasts with the rapid appearance of disturbed tubular transport after loading of proximal tubules with cystine dimethylester (CDME). CDME loading during 10-30 minutes caused a pronounced inhibition of proximal tubular transport and a drastic inhibition of mitochondrial ATP production [Foreman et al. 1987, Coor et al. 1991], suggesting that CDME might have a direct unrelated to cystine accumulation effect on mitochondria. Interestingly, 10 minutes incubation of isolated mitochondria with CDME resulted in a significant inhibition of mitochondrial oxygen consumption with glutamate and not with succinate, possibly reflecting an inhibitory effect on the respiratory chain complex I, as succinate enters respiratory chain beyond complex I [Foreman et al. 1995]. A detailed study of energy metabolism in human cystinotic tissues is required to answer the question whether mitochondrial ATP synthesis is disturbed in cystinosis. Furthermore, recently proposed alternative pathogenetic mechanisms such as enhanced apoptosis [Park et al. 2002] and altered glutathione metabolism [Levtchenko et al. 2005] should be further investigated. A new technique of proximal tubular cell culture from urine of cystinotic patients allows obtaining these cells for metabolic research [Laube et al. 2005].

Another intriguing observation, requiring explanation, is the specific excretion pattern of sugars and polyols in this patient. While urinary concentration of glucose, galactose, myo-inositol and erythriol were elevated, the excretion of the other monosaccharides remained normal (table 3). ATP dependent, Na-coupled transport of glucose, galactose and myo-inositol should be altered in this patient, while the non-Na-coupled transport of fructose is probably not affected [Wright et al. 2004].

Probably the most important message of this report is the possibility of missing the diagnosis of cystinosis in a young infant. Naturally because our patient was a sibling of a known patient with cystinosis and had already at the age of 3 weeks signs of mild proximal tubular dysfunction, the diagnosis of cystinosis was highly suspected. However, total white blood cell cystine content being within the heterozygote range did not allow making the diagnosis. The other possible explanations of aminoaciduria such as galactosemia, vitamin D deficiency, UTI, Lowe syndrome were all excluded. Finally the diagnosis of cystinosis was made at the age of 6 months due to the determination of the elevated cystine content in cultured fibroblasts, and was confirmed later by molecular analysis of *CNTS* gene. Delayed diagnosis of cystinosis in this case motivated a study comparing cystine determination in total white blood cell preparations and polymorphonuclear cells, which demonstrated that polymorphonuclear cells should be preferentially used for the diagnosis of cystinosis and for monitoring of cysteamine therapy [Levtchenko et al. 2004]. Early diagnosing of cystinosis is extremely important for the immediate initiation of

treatment with cystine depleting agent cysteamine. Cysteamine should be started as soon as possible as it may prevent the deterioration of glomerular filtration rate, improves growth, protects the impairment of extra-renal organs [Gahl et al. 2002] and in some patients can even diminish renal tubular dysfunction [da Silva et al. 1985, Kleta et al. 2004].

In conclusion, gradual development of Fanconi syndrome in an infant with cystnosis requires further studies explaining a differential sensitivity of proximal tubular transporters to cystine accumulation. Furthermore, in suspected patients we recommend immediate cystine determination in polymorphonuclear cells.

CHAPTER 9

Decreased intracellular ATP content and intact mitochondrial energy generating capacity in human cystinotic fibroblasts

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Abstract

Cystinosis is an autosomal recessive lysosomal storage disorder caused by a defect in the lysosomal cystine carrier cystinosin. Cystinosis is the most common cause of inherited Fanconi syndrome leading to renal failure, in which the pathogenesis is still enigmatic. Based on studies of proximal tubules loaded with cystine dimethyl ester, altered mitochondrial ATP production was proposed to be an underlying pathological mechanism. Thus far, however, experimental evidence supporting this hypothesis in humans is lacking. In this study, energy metabolism was extensively investigated in primary fibroblasts derived from eight healthy subjects and eight patients with cystinosis. Patient's fibroblasts accumulated marked amounts of cystine (median, range: 4.3 (2.7-5.5) versus 0.2 (0.1-0.3) nmol/mg protein, $p<0.001$). Moreover, they displayed a significant decrease in intracellular ATP content (median, range: 37.3 (26.9-55.0) versus 51.5 (44.7-58.5) nmol/mg protein, $p<0.05$). Remarkably, however, overall energy generating capacity, activity of respiratory chain complexes, ouabain-dependent rubidium uptake, reflecting Na,K-ATPase activity, and bradykinin-stimulated mitochondrial ATP production were all normal in these cells.

In conclusion, the data presented demonstrate that mitochondrial energy generating capacity and Na,K-ATPase activity are intact in cultured cystinotic fibroblasts, thus questioning the idea of altered mitochondrial ATP synthesis as a key-stone for the pathogenesis of cystinosis.

Introduction

Cystinosis is an autosomal recessive lysosomal storage disorder caused by a defect in the lysosomal cystine carrier cystinosin, encoded by the *CTNS* gene (17p13) [Kalatzis et al. 2001]. Intralysosomal accumulation of cystine virtually in all tissues leads to a multi-organ disease with kidneys being clinically first affected. Patients with cystinosis mostly present with generalized proximal tubular dysfunction (Fanconi syndrome) during the first year of life and, when untreated with the cystine depleting agent cysteamine, develop end stage renal disease before the age of 10 years [Gahl et al. 2002].

Because proximal tubular cells are highly metabolic active [Klahr et al. 1992], the appearance of Fanconi syndrome as a first clinical sign in cystinosis points to disturbances in energy metabolism in these patients. This idea is substantiated by the occurrence of Fanconi syndrome in patients with mitochondrial disorders [Niaudet et al. 1996].

Although lysosomal accumulation of cystine in cystinosis was demonstrated already in the 60s [Schulman et al. 1969], studies on the pathogenesis of this disease were initially hampered by the absence of a proper *in vitro* model of lysosomal cystine accumulation. This issue was solved with the introduction of dimethyl esters of amino acids, which readily passed the lysosomal membrane and, once inside the organelle, were rapidly degraded by lysosomal hydrolases to yield free amino acid and methanol thus allowing amino acid loading of the lysosomes [Goldman et al. 1973, Reeves 1979]. Cystine dimethyl ester (CDME) loading of intact leukocytes, cultured lymphoblasts and fibroblasts established defective cystine exodus from the lysosomes as basic defect in cystinosis [Gahl, Tietze et al. 1982, Jonas, Smith et al. 1982, Pisoni et al. 1985].

Subsequently, CDME loading of proximal tubular cells was used for studying the pathogenesis of Fanconi syndrome in cystinosis. Foreman et al. demonstrated that incubation of rat cortical tubules with CDME increased intracellular cystine concentrations to values comparable to those measured in patients with cystinosis and inhibited active amino acid and glucose transport [Foreman et al. 1987]. CDME loading of rabbit proximal tubules perfused *in vitro* resulted in decreased transepithelial potential difference and inhibition of volume absorption, active glucose and bicarbonate transport, mimicking renal Fanconi syndrome in cystinosis [Salmon et al. 1990].

ATP depletion was postulated to be responsible for inhibition of active transport in proximal tubular cells as dramatic decreases in ATP content and mitochondrial respiration rate were demonstrated after CDME loading [Coor et al. 1991, Baum 1998]. Because most proximal tubular transport is sodium coupled, less fuel for the Na,K-ATPase on the basolateral membrane will lower the sodium electrochemical gradient thus inhibiting trans-

port across the apical membrane. Consistent with this idea, cystine-loaded porcine epithelial tubular cells (LLC-PK1) showed a diminished activity of Na,K-ATPase, demonstrated by reduced rubidium uptake, leading to increased sodium and decreased potassium concentrations inside the cells [Ben-Nun et al. 1993].

Although ATP depletion as a keystone for the pathogenesis of cystinosis is well documented in the CDME loading model, it has not yet been proved in human cystinotic tissues. Furthermore, the possible link between intralysosomal cystine accumulation and ATP depletion is still unknown.

Cultured cystinotic skin fibroblasts are known to accumulate cystine [Guillet et al. 1998] and are easily available via skin biopsy in patients with cystinosis. Because a metabolic defect caused by cystine accumulation has to be present in all body tissues, we used cystinotic fibroblasts to investigate the energy metabolism in cystinosis. The latter was done by determining total intracellular ATP content, overall mitochondrial energy generating capacity, activity of mitochondrial respiratory chain complexes I, II, III and IV , maximal bradykinin-stimulated mitochondrial ATP production and ouabain-dependent rubidium uptake in primary skin fibroblasts derived from patients with cystinosis compared to control fibroblasts.

Subjects and methods

Patients

The study was approved by the Institutional Review Board. Skin biopsies were performed after obtaining informed consent in 8 patients with cystinosis prior to starting of cysteamine therapy and in 8 healthy subjects. In all patients cystinosis manifested with renal Fanconi syndrome at the age of 6-18 months. The diagnosis of cystinosis was made by measuring an elevated cystine content in polymorphonuclear cells (>0.5 nmol cystine/mg protein) and was confirmed by molecular analysis of *CNTS* in all patients.

Fibroblast's cell culture, isolation of polymorphonuclear leukocytes and intracellular cystine measurements

Skin fibroblasts were cultured in custom made M199 medium with Tween-20 (5mg/L, Gibco) supplemented with fetal calf serum (10%), penicillin (100 U/ml, Gibco) and

streptomycin (100U/ml, Gibco). Polymorphonuclear leukocytes were isolated as described previously [Levtchenko et al. 2004]. Intracellular cystine was determined by high performance liquid chromatography (HPLC) and expressed as nmol cystine/mg protein [Levtchenko et al. 2004].

Measurement of total intracellular ATP content

Cystinotic and control fibroblasts were grown to confluence in 75 cm^2 tissue culture flasks (approximately $1.0x10^6$ cells) and detached using trypsin. After washing in PBS cell pellets were divided into two portions for duplo experiments, shock frozen in liquid N_2 and stored at -80°C until use. Prior to ATP determination, pellets were resuspended on ice in 0.5 ml cold PBS. A 25-fold diluted fraction (25µl) of the suspension was transferred to a transparent microtiter plate and ATP was measured using ATP Bioluminescence Assay Kit HSII (Roche®) according to the instructions of the manufacturer. The residue of the undiluted cell suspension was used for protein determination using the method of Lowry. Data are represented as mean of two separate experiments in nmol ATP/mg protein.

Analysis of metabolic origin of intracellular ATP

To investigate the metabolic origin of intracellular ATP in cultured fibroblasts, we measured the total intracellular ATP content after specific inhibition of glycolysis or the oxidative phosphorylation pathway. To inhibit glycolysis, fibroblasts were incubated for 5hr at 37°C with 0.3mM sodium-iodoacetate (SIA), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase or 50mM 2-deoxyglucose (2-DOG), a competitor of glucose. Glycolytic activity was determined in a 96 well plate assay by lactate production and was more then 80% decreased by SIA and 2-DOG compared to the control conditions without the inhibitors [Yang et al. 2004]. The inhibition of mitochondrial ATP production was achieved by incubating fibroblasts for 5hr at 37°C with 1µM rotenone, which is a specific inhibitor of complex I, or with 1µg/ml antimycin A, a specific inhibitor of complex III, inhibiting both succinate and NADH-dependent respiration [Dickman et al. 1990]. The viability of the cells after the inhibition of glycolysis or mitochondrial ATP production was assessed in a fluorescence assay using resazurin (0,01%) as a marker [Yang et al. 2004]. Following incubations with each of the inhibitors and with vehicle controls, intracellular ATP content was determined.

Measurement of overall oxidative phosphorylation (OXPHOS) activity in intact mitochondria

Intact mitochondria were isolated from fresh cultured fibroblasts (approximately $20x10^6$ cells) according to Bentlage [Bentlage et al. 1996] and suspended in SEF buffer (0.25 M sucrose, 10 mM KPi, 2 mM EDTA, pH 7.4). Overall energy generating capacity was determined by measuring $^{14}CO_2$ production rates from oxidation of ^{14}C labeled substrates ([1-^{14}C]-pyruvate, [U-^{14}C]-malate, [1,4-^{14}C]-succinate and [1-^{14}C 2-oxoglutarate) in combination with several co-substrates. Oxidation rates were normalized against citrate synthase (CS) activity and expressed as nmol $^{14}CO_2$/h [Trijbels et al. 2004].

Measurement of the activity of respiratory chain complexes (I-IV) in intact mitochondria

Intact mitochondria were isolated as described above, aliquoted for separate measurements of the respiratory chain complexes and stored at –80°C. With slight modifications, complex I (NADH:Q1 oxidoreductase) activity was determined spectrofotometrically by monitoring NADH oxidation at 340nm as described by Fischer [Fischer et al. 1986]. Activity of complex III (Coenzyme Q_{10}: cytochrome C oxidoreductase) was determined by measuring the reduction of cytochrome C at 550 nm with reduced decylubiquinone as an artificial substrate according to Bentlage et al. Complex II+III (succinate:cytochrome C-oxidoreductase, SCC) activity was determined spectrofotometrically at 550nm by measuring reduction of cytochrome C using succinate as a substrate in the presence of rotenone and KCN to inhibit complex I activity and eliminate re-oxidation of cytochrome C, respectively [Fischer et al. 1985]. Complex IV (cytochrome C oxidase, COX) activity was determined by the method described by Cooperstein and Lazarow [Cooperstein et al. 1951]. Mitochondrial citrate synthase (CS) activity was measured by a method described by Srere [Srere et al. 1963] and expressed as mU/mg protein. Activities of complexes I, II, III are expressed as mU/ U COX after normalization of the activity of complex IV against that of CS.

Luminescence monitoring of bradykinin stimulated mitochondrial ATP production in intact fibroblasts

To monitor mitochondrial ATP production, ~25,000 fibroblasts were seeded on a $13mm^2$ glass coverslip and cultured for 24 h. Cells were then infected with a baculovirus containing the cDNA for mitochondria targeted luciferase generated as described by Visch [Visch et al. 2004]. The cDNA was kindly provided by dr. G.A. Rutter [Ainscow et al. 2001]. After 48h of culture in the presence of 1.75 mM Na-butyrate, the coverslip was placed in the

thermostated (37°C) luminometer and perfused (3 ml/min) with a Hepes-Tris medium (132 mM NaCl, 4.2 mM KCl, 1 mM $MgCl_2$, 5.5 mM D-glucose, 10 mM Hepes, 1 mM $CaCl_2$, pH 7.4) containing beetle 5 μM luciferin (Promega). Luciferase luminescence was monitored continuously using a custom build setup that consisted of a light shielded low-noise photomultiplier tube (PMT) with a built-in H7360-1 amplifier-discriminator (Hamamatsu Photonics K. K., Shizuoka-Ken, Japan). PMT output was monitored in time using a PCI-6601 photon counting board (National Instruments, Austin, USA) coupled to an IBM-compatible computer using custom written software (Drs. S.P. Srinivas and W. van Driessche, Laboratory of Physiology, K.U. Leuven, Belgium). Light output was recorded at 2 s intervals, after which the traces were smoothed off-line by using a 3-point moving average (Origin pro 6.1, OriginLab Corporation, Northampton, MA, USA). Typically, light output from a coverslip of fibroblasts expressing mitochondrial luciferase was 500-1,500 counts/s with a background of 15 counts/s. At the indicated time, 1 μM bradykinin was included in the perfusion medium for maximal stimulation of mitochondrial ATP production. Bradykinin releases Ca^{2+} from the endoplasmic reticulum leading to a rapid increase in cytosolic and consequently intramitochondrial Ca^{2+} concentration, which, in turn, leads to an increase in activity of the pyruvate dehydrogenase system, 2-oxoglutarate dehydrogenase and isocitrate dehydrogenase [Jouaville et al. 1999]. Results are expressed as the percentage increase in luciferase luminescence.

Measurement of the activity of Na,K- ATPase by Rb^+ influx

^{86}Rubidium ($^{86}Rb^+$), a congener of K^+, uptake was measured to determine Na,K-ATPase activity in cystinotic and control fibroblasts as previously described [Munzer et al. 1994]. Briefly, cells were grown to 40-60% confluence in 24 well plates, washed 3 times with Rb^+-transport medium (140mM choline choride, 6mM NaCl, 0.5mM $MgCl_2$, 5mM glucose, 4mM Na-Tris (pH 7.4)) and pre-incubated in 180μl of this medium with or without 10mM ouabain, a specific Na,K-ATPase inhibitor, for 5 min at 37°C. Uptake was started by adding 20μl of a mixture of KCl (5mM) and 15μCi /ml $^{86}Rb^+$ (8.6μM), followed by 30 min of incubation at 37°C. Then, the medium was removed and cells were washed 3 times with ice-cold Rb-transport medium. After solubilisation in 0.5ml NaOH (0.2M) for 40 min, 0.4ml was used for liquid scintillation counting (Packerd). Ouabain sensitive $^{86}Rb^+$ uptake was expressed in nmol/mg protein/h. Control experiments with 10μM bumetanide (inhibitor of Na^+,K^+,$2Cl^-$ cotransport) showed no significant ouabain-insensitive uptake (data not shown).

Statistical analysis

Mann-Whitney U test was used for the comparison of values from cystinotic and control cells. The correlation between two variables was estimated by a Spearman rank correlation method. The differences were considered statistically significant at $p<0.05$.

Results

The intracellular cystine content was significantly elevated in cystinotic fibroblasts (median, range: 4.3 (2.7-5.5) versus 0.2 (0.1-0.3) nmol/mg protein, $p<0.001$).

Data on intracellular ATP content, activity of mitochondrial respiratory chain enzyme complexes I, II, III and IV and Na,K-ATPase activity are presented in Table 1.

Table 1 Intracellular cystine and ATP content (nmol/mg protein), activity of respiratory chain enzyme complexes I, II, III (mU/mU COX), IV (mU/U CS) and activity of Na,K- ATPase (nmol/mg protein/ h) in cultured skin fibroblasts.

	cystinosis n=8	controls n=8	P
cystine	4.3 (2.7 - 5.5)	0.2 (0.1 - 0.3)	<0.001
ATP	38.8 (26.9 - 72.1)	51.5 (44.7 - 58.5)	< 0.05
complex I	158 (91 - 223)	162 (97 - 220)	0.3
complex II	922 (685 - 1139)	982 (744 - 1230)	0.9
complex III	1513 (1179 - 2000)	1429 (1300 - 1841)	0.8
complex IV	957 (846 - 1281)	930 (764 -1151)	0.4
Na,K-ATPase	50.8 (32.1 - 89.0)	60.5 (45.6 - 95.0)	0.3

Total intracellular ATP content and metabolic origin of intracellular ATP

The total ATP content in fibroblasts derived from patients with cystinosis was decreased compared to the healthy controls (median, range: 38.8 (26.9-72.1) versus 51.5 (44.7-58.5) nmol/mg protein, $p<0.05$). No significant correlation could be detected between intracellular cystine accumulation and intracellular ATP content (Figure 1).

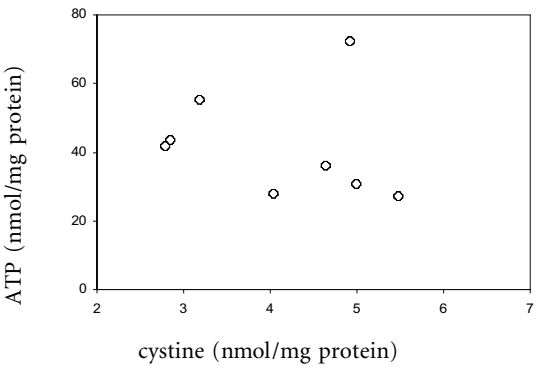

Figure 1 Intracellular cystine and ATP content in fibroblasts of patients with cystinosis (n=8, r=-0.45, p=0.3).

Additionally we determined intracellular ATP content in polymorphonuclear leukocytes isolated directly from patients with cystinosis (n=15), treated with cysteamine (40-60 mg/kg/day), and compared the values with those derived from control subjects (n=18). As in cultured cystinotic fibroblasts we detected a significant decrease of intracellular ATP content in cystinotic versus control cells (median, range: 317 (149-1141) versus 557 (207-1101) nmol/mg protein, $p<0.05$) and no correlation between the intracellular cystine and ATP content (Figure 2).

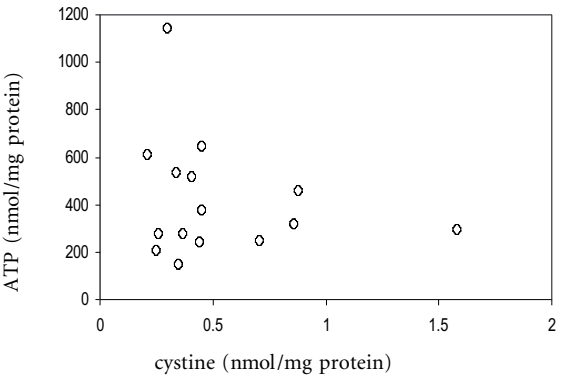

Figure 2 Intracellular cystine and ATP content in PMN cells of patients with cystinosis (n=15, r=-0.02, p=0.9).

In order to investigate the relative contribution of glycolysis and mitochondrial oxidative phosphorylation for ATP synthesis in cultured fibroblasts, we determined the intracellular ATP content after incubation of the cells with the inhibitors of glycolysis (SIA, 2-DOG) and respiratory chain complexes (rotenon and antimycin A). Prior to determination of intracellular ATP content a cell viability test was performed showing that under the used conditions the majority of cells (>90%) remained viable (data not shown).

As demonstrated in Figure 3, inhibition of the glycolysis resulted in a remarkable decrease (>80%) of intracellular ATP content compared to vehicle control in both cystinotic and control fibroblasts. After the inhibition of complex I by rotenone or complex III by antimycin, less than 30% decline of initial intracellular ATP content was observed in cystinotic and control fibroblasts. Cystinotic fibroblasts exhibited a slightly more pronounced decline of intracellular ATP content due to the inhibition of respiratory chain complexes, but the difference with controls did not reach statistical significance (Figure 3).

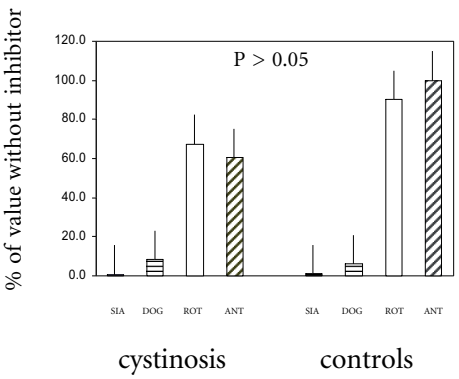

Figure 3 Decrease of intracellular ATP content (mean, SD) after inhibition of glycolysis (sodium-iodoacetate: SIA; 2-deoxyglucose: DOG) and respiratory chain complexes (I: rotenon (ROT); III: antymicin A (ANT)) in control (n=5) and cystinotic (n=4) fibroblasts cell lines ($p > 0.05$)

Overall energy generating capacity and activity of respiratory chain complexes of isolated mitochondria and ATP production rate in intact cells

As shown in Table 2, comparable overall oxidation rates of [1-^{14}C]-pyruvate, [1-^{14}C]-2-oxoglutarate, [U-^{14}C]-malate and [1,4-^{14}C]-succinate in isolated mitochondria were detected in cystinotic (n=4) and control fibroblasts (n=4).

The activity of individual mitochondrial respiratory chain complexes did not differ between cystinotic and control cells (Table 1).

Furthermore, maximal bradykinin-induced increase of mitochondrial ATP production measured in intact cells by intramitochondrial luciferase probe was not different between cystinotic (n=3) and control (n=4) fibroblasts (Figure 4).

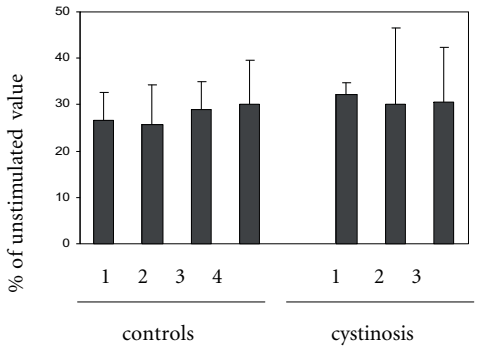

Figure 4 Maximal bradykinin-induced increase in mitochondrial ATP production (mean, SD) in control (n=4) and cystinotic (n=3) fibroblast cell lines (p>0.05).

Na,K-ATPase activity

Although the activity of Na,K-ATPase, measured by ouabain-sensitive Rb influx, was slightly decreased in cystinotic cells (50.8 (32.1-67.0) versus 60.5 (45.6-95.0) nmol/mg protein/h, this difference was not statistically significant (Table 1).

Table 2 $^{14}CO2$ production rate (nmol $^{14}CO2$/h) from [^{14}C] citric acid cycle intermediates in cultured skin fibroblasts

	cystinosis n=4	controls n=4	P
[1-^{14}C] pyruvate+malate	922 (717-1332)	706 (567-1278)	0.3
[1-^{14}C] pyruvate+ carnitine	801 (690-1252)	664 (550-1326)	0.3
[1-^{14}C] 2-oxoglutarate	454 (276-539)	291 (226-488)	0.3
[U-^{14}C] malate+pyruvate	430 (374-1517)	469 (317-1368)	0.9
[1,4-^{14}C] succinate+pyruvate	232 (191-325)	261 (216-286)	0.7

Discussion

Cystinosis is the most frequent cause of inherited Fanconi syndrome, leading to renal failure, in which the pathogenesis is still unknown. Based on the studies of rat and rabbit proximal tubular suspensions or cultured proximal tubular cells loaded with CDME, intracellular ATP depletion with subsequent decrease of Na,K-ATPase activity has been proposed to be the underlying biological mechanism of cellular dysfunction in cystinosis

[Salmon et al. 1990, Coor et al. 1991, Baum 1998, Ben-Nun et al. 1993]. Inhibited energy metabolism, however, has not yet been demonstrated in human cystinotic material. Here, for the first time, we attempted to confirm such an alteration of intracellular ATP status and oxidative phosphorylation in cystinosis by an extensive study of the energy metabolism in human cystinotic fibroblasts. Intracellular ATP content was decreased in fibroblasts derived from patients with cystinosis compared to the controls. As the metabolism of cultured cells could be influenced by *ex-vivo* growth and regarding the overlap of ATP ranges between cystinotic and control cells, we additionally determined intracellular ATP content in polymorphonuclear leukocytes immediately isolated from freshly drawn blood. Interestingly, in agreement with the fibroblast data, intracellular ATP was also decreased in PMN cells of the patients compared to the controls, despite the fact that their cystine values were decreased compared to the fibroblasts due to cysteamine treatment.

Intracellular ATP is produced by cytosolic glycolysis and mitochondrial OXPHOS. *In vivo* studies demonstrated that energy of mammalian kidneys is mostly derived from the oxidative metabolism. The activity of glycolytic enzymes in proximal tubules is minimal [Klahr et al. 1992]. In contrast, the contribution of oxidative phosphorylation for ATP production is limited in cultured skin fibroblasts [Robinson 1996], which was confirmed in this study. Cystinotic fibroblasts were, however, chosen as the subject of our study since they accumulate cystine *in vitro* and therefore will exhibit the metabolic consequences thereof. Secondly, cultured fibroblasts from patients with mitochondrial disorders have been successfully used to demonstrate reduced ATP production and deficient OXPHOS activity [Visch et al. 2004, Loeffen et al. 2000, Ugalde et al. 2004]. Additionally, an inhibited activity of Na,K-ATPase after CDME loading has been demonstrated in cultured LLC-PK1 cells [Ben-Nun et al. 1993], despite the fact that these cells are also predominantly glycolytic [Felder et al. 2002].

Several approaches were used to investigate mitochondrial ATP generating capacity of cystinotic and control fibroblasts. Initial experiments were performed in isolated mitochondria. Overall mitochondrial energy generating capacity as well as the activity of individual mitochondrial respiratory chain complexes (I, II, III, IV) was comparable between cystinotic and control cells, providing evidence that intracellular cystine accumulation did not cause a mitochondrial defect.

Assuming that the presence of high lysosomal cystine content in the cell is obligatory for the alteration of oxidative phosphorylation, additional experiments were performed in intact cells to study bradykinin-stimulated intramitochondrial ATP production. These experiments, however, did not reveal any difference between cystinotic and control

fibroblasts. In summary, altered mitochondrial ATP generating capacity was not responsible for decreased ATP content in cystinotic fibroblasts.

The slightly more pronounced decline of intracellular ATP after the inhibition of the respiratory chain complexes in cystinotic fibroblasts might indicate the inhibition of glycolytic activity or a relative higher contribution of the OXPHOS system. Elevated ATP consumption could be an alternative explanation of decreased ATP content in cystinotic cells; however, this possibility was not examined in this study.

Finally, we examined whether intracellular ATP decrease in cultured fibroblasts caused a functional inhibition of the activity of Na,K-ATPase. The conditions of rubidium uptake experiments (low sodium and physiologic potassium concentrations in the medium) were carefully selected to stimulate the maximal activity of Na,K-ATPase, which was found to be comparable in cystinotic and control cells. This might be different in proximal tubular cells, because Na,K-ATPase activity in fibroblasts is trivial compared to proximal tubular cells *in vivo* [Klahr et al. 1992].

How to reconcile our findings with the results obtained in proximal tubules loaded with CDME? It is not excluded that CDME loading can provoke effects different from those attributed to cystine accumulation in cells with a deficient lysosomal cystine carrier. In concordance with this idea, recent work on human IHKE-1 cells demonstrated that CDME itself had an acute (within 1 minute) effect on the basal membrane potential (an initial depolarization followed by a more pronounced repolarization). This effect was distinct from those on Na^+ dependent alanine transporter, which was inhibited after at least 30 minutes of incubation with CDME. As the hyperpolarization induced by CDME could be blocked by Ba^{2+}, the authors concluded that CDME activated a K^+ conductance [Cetinkaya et al. 2002].

It is also possible that CDME can directly affect the mitochondrial respiration as the incubation of isolated mitochondria with CDME resulted in reduced mitochondrial O_2 consumption with glutamate as substrate, but not with succinate, which electrons enter the electron transport chain beyond complex I at the level of Coenzyme Q_{10}, suggesting the direct inhibition of complex I by CDME [Foreman et al. 1995].

An increase in intracellular cysteine concentration was demonstrated *in vivo* in rats after 4 days of intraperitoneal CDME injections and in rabbit proximal tubules loaded with CDME *in vitro* probably as a result of reduction of cystine to cysteine in the cytosol [Foreman et al. 1987]. To determine whether cystine or its breakdown product cysteine was responsible for alterations in proximal tubular transport, the effect of cysteine dimethyl ester loading was studied. Strikingly, cysteine ester could indeed inhibit volume absorption, glucose and bicarbonate transport in rabbit proximal tubules in a concentration-

dependent manner, while methyl esters of leucine and tryptophan had no effect on these parameters [Salmon et al. 1990]. In contrast to CDME, cysteine ester loading, however, did not effect the O_2 consumption rate, despite a remarkable increase in intracellular cysteine concentration [Sakarcan et al. 1994].

More research is required for the understanding of the pathogenesis of cystinosis. This research should further explore already introduced alternative pathogenic mechanisms of the disease as enhanced apoptosis and possibly increased reactive oxygen cell damage [Park et al. 2002, Chol et al. 2004, Levtchenko et al. 2005]. New techniques such as micro arrays and proteomics applied in human *in vitro* models of cystinosis might help to create new ideas for the future studies. Human proximal tubular cells from cystinotic patients can be cultured *in vitro* [Laube et al. 2005] and possibly represent an excellent model for studying the pathogenesis of the disease. However, the metabolism of these cells can be influenced by *ex-vivo* growth. In addition, cultures from urine might contain other than proximal tubular epithelial renal cells, including glomerular podocytes, which can be exfoliated to urine. This would mean that prior to the transport and metabolic studies, the cells from urine should be cloned in order to obtain a pure proximal cell culture.

In conclusion, intracellular ATP depletion in cultured human cystinotic fibroblasts is not caused by the alteration of mitochondrial oxidative phosphorylation and does not result in a functional inhibition of Na,K-ATPase activity. Study of proximal tubular cells derived from patients with cystinosis is required to explain the discrepancy of our results with those obtained in CDME loading model of cystinosis.

Acknowledgements

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CHAPTER 10

Altered status of glutathione and its metabolites in cystinotic cells

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Abstract

Background: Cystinosis is an autosomal recessive disorder, caused by mutations of the lysosomal cystine carrier cystinosin, encoded by the *CTNS* gene (17p13).

The concomitant intralysosomal cystine accumulation leads to multi-organ damage, with kidneys being the first affected. Altered mitochondrial oxidative phosphorylation has been demonstrated in animal proximal tubules loaded with cystine dimethyl ester, mimicking cystine accumulation in cystinosis, but has not been confirmed in cells of patients with cystinosis.

Furthermore, the link between cystine accumulation and mitochondrial damage is also missing. We hypothesized that cytosolic cysteine deficiency resulting in intracellular glutathione (GSH) shortage might be involved in cellular dysfunction in cystinosis.

Methods: Components of the γ-glutamyl cycle were measured in cultured skin fibroblasts (n=9) and polymorphonuclear (PMN) leukocytes (n=15) derived from patients with cystinosis and compared with the values in cultured fibroblasts (n=9) and PMN cells (n=18) of healthy controls.

Results: Cystine content in cystinotic fibroblasts and PMN cells was significantly elevated compared with the controls, consistent with the lysosomal cystine accumulation in these cells. Although no reduction of total intracellular GSH content was found in cystinotic cells, it inversely correlated with cystine levels. Furthermore, GSH disulfide (GSSG) was elevated in cystinotic cells, resulting in an increased GSSG/total GSH (%) ratio. No relationship between intracellular cystine and GSH was found in control fibroblasts and PMN cells.

Conclusion: An elevated GSSG/total GSH (%) ratio might indicate increased oxidative stress present in cystinotic cells. Inverse correlation between cystine accumulation and intracellular GSH content indicates that under stress conditions such as intensive energy demand or increased oxidative insult, cystinotic cells may be more prone to GSH depletion.

Introduction

Cystinosis, affecting 1:200 000 newborns annually, is a lysosomal cystine storage disease caused by a defect in a lysosomal cystine carrier cystinosin, encoded by the *CTNS* gene (17p13). Patients with the most severe infantile form of cystinosis develop renal Fanconi syndrome during the first year of life and end-stage renal disease before the age of 10. Longer survival of cystinosis patients due to the success of renal transplantation reveals later extra-renal complications such as hypothyroidism, diabetes mellitus, hypogonadism, and musculo-, neuro- and retinopathy. The cystine depleting drug cysteamine postpones the deterioration of renal function, improves growth and diminishes extra-renal organ damage in cystinosis. Patients with late-onset or juvenile cystinosis generally have less pronounced proximal tubular dysfunction, but may develop severe proteinuria and renal failure. Extrarenal organs are also involved in patients with late onset form. The third very rare ocular form of cystinosis is characterized by photophobia due to accumulation of cystine crystals in the cornea and the absence of renal or extra-renal disease [Gahl et al. 2001]. Mutations in *CTNS* have been demonstrated in all three clinical forms of cystinosis [Attard et al. 1999].

Although the molecular basis of cystinosis is elucidated, the link between intralysosomal accumulation of cystine and cellular dysfunction is not yet clarified.

Altered ATP synthesis has been demonstrated in rabbit and rat proximal tubules loaded with cystine dimethylester, mimicking cystine accumulation in cystinosis [Baum et al. 1998]. However, this pathogenic mechanism has not been confirmed in cells of patients with cystinosis. Assuming that ATP deficiency is indeed the cause of cellular damage in cystinosis, it still has to be explained how cystine accumulation alters ATP synthesis. Several theoretical considerations and experimental findings indirectly point to disturbances in glutathione (GSH) metabolism. In normal cells, proteins are degraded within lysosomes to amino acids, which are transported to the cytosol. Cystine is carried by cystinosin to the cytosol where it is reduced to cysteine by cytosolic reducing systems [Gahl et al. 2001].

Theoretically, the intralysosomal cystine accumulation in cystinosis can result in the cytosolic deficit of cysteine. Cysteine together with glutamate and glycine is needed for GSH synthesis (Figure 1), requiring two successive enzymatic reactions, both consuming one ATP molecule per enzymatic cycle. The first and ratelimiting reaction couples glutamate and cysteine and is catalysed by γ-glutamylcysteine synthetase (γ-GCS), resulting in the formation of γ-glutamylcysteine (γ-GC). The second reaction couples γ-GC with glycine and is catalysed by GSH synthetase (GSH-S). Humans with deficient GSH-S

accumulate and excrete elevated amounts of 5-oxoproline, due to diminished conversion of 5-oxoproline to glutamate by 5-oxoprolinase (Figure 1) [Larsson et al. 2000]. The elevated 5-oxoproline excretion has also been demonstrated in cystinotic patients with Fanconi syndrome, untreated with cysteamine, suggesting an alteration in GSH synthesis in these patients [Rizzo et al. 1999]. Additionally, the presence of aminoaciduria and metabolic acidosis in patients with a deficiency of γ-GCS suggests that GSH deficiency might result in renal proximal tubular dysfunction [Larsson et al. 2000].

GSH is the most abundant cellular thiol, functioning as an important redox buffer. GSH serves as a cofactor for the GSH peroxidase family of enzymes, which metabolize H_2O_2 and lipid peroxides, defending cells against reactive oxygen metabolites. Glutathione reductase, using the NADPH oxidase system, is necessary to regenerate GSH [Uhlig et al. 1992].

GSH depletion results in mitochondrial dysfunction in several cellular and animal models [Meister et al. 1995, Han et al. 2003] and therefore might affect ATP synthesis in cystinosis. Interestingly, increased glutathionylation of the mitochondrial complex I due to elevated mitochondrial glutathione disulfide (GSSG) has been shown recently to increase superoxide production [Taylor et al. 2003]. An increased superoxide production has been demonstrated by chemiluminescence assay in isolated polymorphonuclear (PMN) cells and mononuclear phagocytes from cystinotic children [Pintos et al. 1985].

GSH has also a role in maintaining the intracellular pool of cysteine via the reaction catalysed by γ-glutamyl transpeptidase (γ-GT), resulting in formation of cysteinyl-glycine (Figure1).

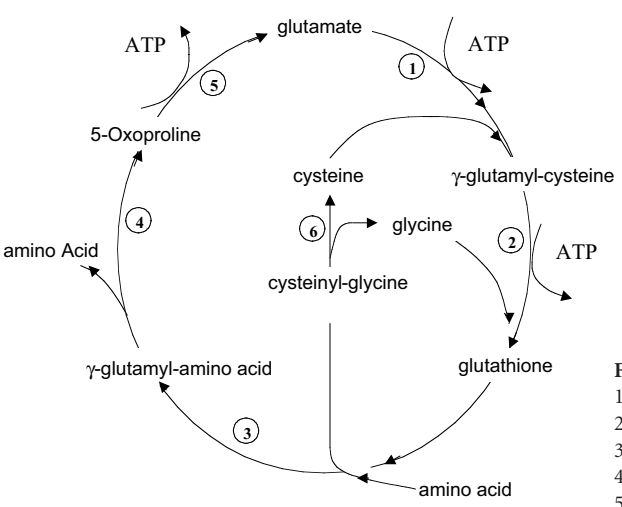

Figure 1 γ-Glutamyl cycle.
1: γ-glutamylcysteine synthetase, (γ-GCS);
2: glutathione synthetase (GSH-S);
3: γ-glutamyl transpeptidase (γ-GT),
4: γ-glutamyl cyclotransferase;
5: 5-oxoprolinase;
6: cysteinyl glycinase.

Taking these considerations together, we suggested that GSH deficiency might link intralysosomal cystine accumulation and mitochondrial dysfunction in cystinosis.

To test this hypothesis, we investigated total, reduced and oxidized intracellular GSH, cysteine and total γ-glutamyl-cysteine and cysteinyl-glycine levels in cultured fibroblasts and PMN leukocytes derived from patients with cystinosis and compared these results with control values.

Subjects and methods

Subjects

The age of the patients (n=15) at the time of the study was 5–40 years (median 17); there were nine males. The diagnosis of cystinosis was made at the age 6–28 months by demonstrating an elevated intracellular cystine content in PMN leukocytes (>0.5 nmol cystine/mg protein) and the presence of corneal cystine crystals, and was confirmed by measurements of elevated cystine content in cultured skin fibroblasts in nine patients and mutational analysis of the *CTNS* gene in 14 patients. Seven patients had renal Fanconi syndrome with a glomerular filtration rate (GFR) between 28 and 84 ml/min/1.73m^2 (median 57) and eight patients had a functioning renal graft (GFR 20–160 ml/min/1.73m^2, median 84). All patients were treated by cysteamine (Cystagon®) 40–80 mg/kg /day.

Cell preparations

Skin fibroblasts culture. Skin fibroblasts were obtained by skin biopsies after receiving informed consent from nine patients with cystinosis prior to the start of cysteamine treatment and in nine healthy controls, followed by culturing in custommade M199 medium with Tween-20 (5 mg/l, Gibco) supplemented with fetal calf serum (10%), penicillin (100 U/ml, Gibco) and streptomycin (100 U/ml, Gibco) as described previously [Levtchenko et al. 2004]. Approximately 5x10^6 cells were grown for each assay to confluency and detached using trypsin. After washing twice in phosphate-buffered saline (PBS; Gibco), cells were centrifuged and pellets were shock-frozen immediately in liquid N_2 and stored at - 80 °C.

Polymorphonuclear leukocytes.

PMN leukocytes of 15 cystinotic patients and 18 adult healthy controls were isolated immediately from 10 ml of freshly drawn blood as described previously, frozen in liquid N_2 and stored at - 80 °C [Levtchenko et al. 2004].

Determination of thiol-amino acids and small thiol-peptides

Preparation of cell extracts was performed as described previously [Levtchenko et al. 2004]. Briefly, frozen cell pellets were resuspended on ice in sodium phosphate buffer (pH 7.2) with 1mM *N*-ethylmaleimide (NEM) (resulting in determination of oxidized thiols) or without NEM (resulting in total thiols measurement) followed by sonication. NEM binds to all free -SH groups. Our method does not allow us to distinguish between cytosolic disulfides and thiols bound to intracellular proteins.

The homogenates were centrifuged at 14, 000 g for 10 min at 4 °C. The supernatants were used for measurements of protein concentrations by the Lowry method and for determination of intracellular cystine and total cysteine, GSSG and total GSH, total γ-glutamyl-cysteine and total cysteinyl-glycine by using high-performance liquid chromatography (HPLC) [de Graaf-Hess et al. 1999]. Free cysteine content was calculated as the difference between total cysteine and oxidized cysteine.

Cystine was measured as cysteine (1/2 cystine) after addition of NEM and expressed as nmol cystine/mg protein (as some centres express cystine as 1/2 cystine/mg protein, we provide a conversion factor: 1/2 cystine/mg protein = 2 x cystine/mg protein). A modification of our method was using an elution buffer with pH 3.34 instead of pH 3.88 to obtain a better separation of the components. Values were expressed as median and range.

Statistical analysis

Non-parametrical Mann–Whitney U-test was used for the comparison of values from cystinotic and control cells.

The correlations between two variables were estimated by a Spearman rank correlation method. The differences were considered statistically significant at $p<0.05$.

Results

Thiol compounds in cultured skin fibroblasts

The median intracellular cystine content in cystinotic fibroblasts was elevated compared with normal (4.3 (2.7–5.5) vs 0.2 (0.1–0.3) nmol/mg protein, $p<0.001$), consistent with lysosomal cystine accumulation in these cells. Intracellular free cysteine and total GSH values did not differ between cystinosis and controls. GSSG was elevated in cystinotic fibroblasts (0.7 (0.5–1.7) vs 0.3 (0.2–0.9) nmol/mg protein, $P<0.05$). The GSSG/total GSH ratio (%) was also significantly elevated (Table 1).

The direct precursor of GSH, γ-glutamyl-cysteine, was detectable neither in cystinotic nor in control fibroblasts, as its concentration was under the detection limit of our assay (<0.5 mmol/l).

Intracellular cysteinyl-glycine content was elevated in cystinotic fibroblasts (median 0.5 (0.1–0.6) vs 0.1 (<0.1–0.2) nmol/mg protein) (Table 1).

Table 1 Thiol compounds (median, range) in cultured skin fibroblasts (nmol/mg protein)

	cystinosis n=9	controls n=9	*p*
cystine	4.3 (2.7 - 5.5)	0.2 (0.1 - 0.3)	< 0.001
free cysteine	7.8 (3.8 - 12.3)	5.5 (3.2 - 15.7)	NS
total GSH	11.5 (4.7 - 13.2)	9.7 (4.2 - 14.3)	NS
GSSG	0.7 (0.5 - 1.7)	0.3 (0.2 - 0.9)	< 0.05
% GSSG /total GSH	9.1 (3.9 - 17.0)	4.7 (2.2 - 15.2)	<0.05
cysteinyl-glycine	0.5 (0.1 - 0.6)	0.1 (<0.1 - 0.2)	<0.05

Thiol compounds in polymorphonuclear cells

As all examined cystinosis patients were treated with the cystine-depleting drug cysteamine, the intracellular cystine content of PMN cells was elevated to a lesser extent compared with cystinotic fibroblasts, confirming the efficacy of cysteamine therapy.

However, it was still significantly increased compared with the control subjects (0.5 (0.3–0.8) vs 0.1 (0.09–0.2) nmol/mg protein, $p<0.001$). As in cystinotic fibroblasts, intracellular free cysteine and total GSH contents did not differ between patients and controls in PMN cells.

GSSG was significantly elevated (median 0.9 (0.3–1.8) vs 0.3 (0.2–0.4) nmol/mg protein, $p<0.05$), resulting in an elevated GSSG/total GSH ratio (Table 2).

Table 2 Thiol compounds (median, range) in polymorphonuclear cells (nmol/mg protein)

	cystinosis n=15	controls n=18	*p*
cystine	0.5 (0.3 - 0.8)	0.1 (0.09 - 0.2)	< 0.001
free cysteine	31.6 (19.5 - 35.7)	27.2 (9.4 - 49.5)	NS
total GSH	12.1 (6.4 - 16.5)	14.3 (7.7 - 27.1)	NS
GSSG	0.9 (0.3 - 1.8)	0.3 (0.2 - 0.4)	< 0.05
% GSSG/total GSH	7.1 (2.7 - 26.1)	2.0 (1.9 - 3.3)	<0.05
cysteinyl-glycine	2.1 (0.8 - 4.9)	1.8 (0.6 - 4.4)	NS

No relationship between intracellular GSH content and the age, GFR and the presence or absence of Fanconi syndrome in our group of patients could be demonstrated.

Theoretically, the increased GSSG in PMN cells of the patients treated with cysteamine may have been influenced by cysteamine treatment. Therefore, we have measured GSSG in two cystinotic patients untreated with cysteamine, whose PMN cystine content was 1.58 and 1.97 nmol/mg protein, respectively. In these patients, GSSG was 1.1 (within the range of treated patients) and 2.8 nmol/mg protein (above the range of treated patients), respectively, opposing a possible role for cysteamine treatment in the elevation of GSSG in PMN cells.

Relationship between intracellular cystine accumulation and glutathione content

Although total GSH content was not different between cystinotic and control cells, we observed a significant inverse correlation between cystine accumulation and intracellular GSH content in cystinotic fibroblasts. In PMN cells, this correlation was less pronounced probably due to the lower cystine content in patients treated by cysteamine (Figure 2).

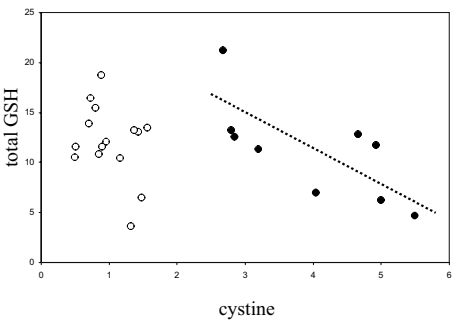

Figure 2 Intracellular cystine (nmol/mg protein) and total GSH (nmol/mg protein) in PMN cells (n=15) (open circles) (r= - 0.31, p=0.3) and in cultured skin fibroblasts (n=9) (filled circles) (r= - 0.82, p<0.01) of patients with cystinosis.

In control fibroblasts and PMN cells, no relationship between intracellular cystine and GSH could be demonstrated (data not shown).

Discussion

We found increased GSSG and GSSG/total GSH ratio in cultured skin fibroblasts and PMN leukocytes derived from patients with cystinosis. Strikingly, in fibroblasts of patients with cystinosis, intracellular cystine accumulation was inversely correlated with their total GSH content. This relationship was not present in fibroblasts and PMN cells of controls.

Cultured cystinotic fibroblasts accumulated cystine to a higher extent compared with PMN cells of the patients treated with cysteamine, and therefore fibroblasts may be a better model for studying the pathogenesis of the disease. However, the metabolism of cultured cells could be influenced by *ex vivo* growth, which is not the case for PMN cells immediately derived from patients with cystinosis or healthy controls.

Interestingly, the trends found were comparable in both cell types.

The hypothesis of alterations in intracellular glutathione and cysteine levels in cystinosis was first tested >30 years ago. Comparably with our results, these early studies demonstrate no difference in absolute glutathione and cysteine contents between cystinotic and normal fibroblasts [Schulman et al. 1972, States et al. 1977]. A direct and sensitive HPLC method with and without the use of NEM allowed us to determine not only total GSH, but also GSSG, which was elevated in cystinotic fibroblasts and PMN cells compared with the controls. Our data, however, are distinct from the results of States et al.,[1977] who found decreased GSSG in cystinotic fibroblasts. The possible reason for this discrepancyis the deproteinization of samples prior to thiol detection by States et al., which would eliminate the detection of GSH bound in disulfides to protein.

In contrast to the recent findings of Chol et al.[2004] who detected a modest decrease of GSH in three cystinotic conditionally immortalized fibroblast cell lines compared with three controls [Chol et al. 2004], we were not able to demonstrate any reduction of total intracellular GSH in a larger study population. Although in our opinion the data of Chol et al. should be interpreted with caution due to their rather small number of observations, the measurements of intracellular thiols during the different phases of the cell cycle could be an additional explanation of this difference. Chol et al. found decreased intracellular GSH content during the phase of exponential cell growth, while we determined intracellular thiol components in cells grown to confluency. This also explains the higher fibroblast cystine content in our study (4.3 vs 0.88 nmol cystine/mg protein, respectively), which is closer to cystine accumulation in tissues *in vivo* (for example in kidney: >25 nmol 1/2 cystine/mg wet weight) [Gahl et al. 2001].

Deficient cytosolic cysteine resulting in impaired intracellular glutathione synthesis, due to the altered exit of cystine out of the lysosomes, was our starting hypothesis which could not be directly confirmed in this study. Equal free cysteine content in cystinotic and control cells was found in both cultured fibroblasts and PMN cells. Comparably with our results, no difference in cysteine content between cystinotic and control fibroblasts could be demonstrated earlier by Shulman et al. [1972]. However, the intracellular cysteine content measured by their group was almost 30 times lower compared with our data. More recent studies, using direct determination by HPLC, in fibroblasts are in line with our measurements of intracellular cysteine content [Ristoff et al. 2002]. The cysteine content in the cells, however, do not necessary reflect the dynamic process of cysteine formation, consumption and transport into and out of the cells. The normal cysteine content in cystinotic cells might be maintained via increased import of this amino acid or from increased degradation of GSH.

Although we do not have a clear explanation for the increased cysteinyl-glycine in cystinotic fibroblasts, it might be speculated that these cells use GSH degradation more extensively to replenish their cytosolic cysteine pool, especially as this amino acid is essential for the growth of human fibroblasts in culture [Eagle et al. 1961]. The inverse correlation between cystine accumulation and total intracellular GSH content demonstrated in our study indicates that under stress conditions such as intensive cell proliferation or increased oxidative stress, cystinotic cells may become GSH depleted. In these situations, cysteine deficit due to a defective exit of cystine out of the lysosomes might limit GSH synthesis. As tissue cystine levels measured in patients with cystinosis are much higher compared with those detected in cultured fibroblasts [Gahl et al. 2001], GSH deficiency might occur *in vivo*. To obtain more insights into GSH synthesis in cystinotic cells, studies with stable isotopes at rest and under stress conditions are required in order to investigate the activities of individual enzymes of the γ-glutamyl cycle.

Probably the most important finding of this study is significantly increased GSSG and concordantly GSSG/total GSH ratio, present in both cystinotic fibroblasts and PMN cells, which reflects a changed redox potential in the studied cells. The redox regulation of gene expression and intracellular communication is just emerging as a vital mechanism in health and disease [Cadenas et al. 2004]. As mitochondria are important sources of intracellular reactive oxygen species (ROS), it is tempting to speculate that cystinotic cells are less protected from mitochondrial free radical production.

Mitochondrial superoxide is converted to H_2O_2 by inducible Mn-superoxide dismutase (Mn-SOD) and, when released to the cytoplasm, can change the redox state to the rest of the cell [Cadenas et al 2004]. Increased free radical production can be responsible for increased apoptosis, demonstrated in cystinotic fibroblasts and renal tubular epithelial cells [Park et al. 2002, Imai et al. 2004]. In agreement with this hypothesis, increased activity of SOD, used as an index of superoxide overproduction, has been demonstrated in cystinotic fibroblasts by Chol et al. [2004]. Increased oxidative stress might be the missing link between cystine accumulation within the lysosomes and two other proposed pathogenic mechanisms of cystinosis such as mitochondrial dysfunction and apoptosis.

In conclusion, although no depletion of absolute GSH and cysteine contents is found in cystinotic fibroblasts and PMN cells, we demonstrate an inverse correlation between cystine accumulation and intracellular GSH content. In addition, the elevated GSSG might indicate the presence of increased oxidative stress, which can be responsible for cell damage in cystinosis. An increased peroxidation in, for example, proximal tubular cells could have striking results on the signal transduction in these cells [Imai et al. 2004]. The study of the mitochondrial GSH content and ROS production in cystinotic cells in rest

conditions and in conditions of intensive energy demand and increased oxidative stress are in our opinion promising directions for future research.

CHAPTER 11

Elevated oxidized glutathione in cystinotic proximal tubular epithelial cells

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Abstract

Cystinosis, the most frequent cause of inborn Fanconi syndrome, is characterized by the lysosomal cystine accumulation, caused by mutations in the *CTNS* gene. To elucidate the pathogenesis of cystinosis, we cultured proximal tubular cells from urine of cystinotic patients (n = 9) and healthy controls (n = 9), followed by immortalization with human papilloma virus (HPV E6/E7). Obtained cell lines displayed basolateral polarization, alkaline phosphatase activity, and presence of amino

patients and nine healthy controls, and evaluated for the first time intracellular ATP levels and intracellular glutathione status in human cystinotic proximal tubular cells.

Methods

Study population

Urine was collected from nine patients with cystinosis aged 5–15 years. In all patients, cystinosis manifested with renal Fanconi syndrome at the age of 6–18 months. The diagnosis of cystinosis was made by determining elevated cystine content of polymorphonuclear cells (>0.5 nmol cystine/mg protein) and was confirmed in eight patients by molecular analysis of *CTNS* gene.

Urine of 48 healthy subjects (4–13 years old) was used for obtaining control proximal tubular cells.

Cell culture and immortalization

Urine was collected and within 5 h centrifuged (223g) for 5 min, at room temperature. After washing in phosphate-buffered saline (PBS) and a second centrifugation step, urine sediment was resuspended in 3 ml PTEC culture medium (DMEM– HAM_s F12; Cambrex Biosciences) containing FCS (10%), penicillin (100 U/ml, Gibco), streptomycin (100 U/ml, Gibco), insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), epithelial growth factor (10 ng/ml), and tri-iodothyronine (40 pg/ml) [Detrisac et al. 1984]. The suspension was transferred to 25 cm^2 tissue culture flask and placed at 37 °C in a 5% CO2 incubator. The medium was changed every 2–3 days.

To maintain proliferation, cells at passage number 4 or less were transfected with plasmid DNA, containing the HPV 16 E6/E7 genes, using the amphotropic packaging cell line PA 317 [Ryan et al. 1994].

Characterization of proximal tubular epithelial cells

As a positive control, commercially available human HK-2 HPV E6/E7 cell line (HK-2) was used (ATCC) [Ryan et al. 1994]. As a negative control undifferentiated human podocyte

cell line immortalized with Simian virus containing t-sensitive allele (SV40-ts58), kindly provided by Dr. M. Saleem [Saleem et al. 2002], was used.

Light and electron microscopy

To demonstrate the characteristic cobblestone morphology of primary cultures, phase contrast microscopy (Axiolab) was applied. Transmission electron microscopy (TEM) was used to show ultrastructural morphology of proximal tubule cells. After immortalization and growing to confluence, cells were gently scraped from flasks, fixed, dehydrated, and embedded in Epon 812 (Merck) as described previously [de Graaf et al. 2004]. Semithin (1 μm) and ultrathin sections were cut on an ultratome, Reichert Ultracut S (Leica Microsystems). The semithin slices were stained with toluidine blue and examined using light microscopy. The ultrathin specimens were contrasted with 4% uranyl acetate and lead citrate before examination by electron microscopy (Jeol 1200 EX2).

Enzymatic studies

To confirm proximal tubular origin of cultured cells, activity of BBM alkaline phosphatase was measured in cell suspensions (approximately $0.1x10^6$ cells) in at least two independent experiments using BM Chemiluminescence ELISA substrate (AP) kit (Roche) according to instructions of the manufacturer. To quantify activity, a standard curve was made with dilutions of Shrimp Alkaline Phosphatase (Amersham Biosciences). Protein levels were determined using a method of Lowry.

Immunological studies

The presence of aminopeptidase N (CD-13) on the membrane of cultured cells ($0.25x10^6$) as an indication of their proximal tubular origin was determined by flow cytometry analysis (Coulter XL) with monoclonal mouse-anti-human CD13-FITC antibody (Dako) with a 1:100 dilution in PBS. Unlabeled cell suspensions were used as negative controls to evaluate positive staining profile.

The presence of PTEC specific protein megalin was examined using monoclonal antibody 6C5, which was a kind gift of Dr. W.S. Argraves (Charleston SC, USA) [Kounnas et al. 1993]. Cells were cultured to semi-confluence on cover slides and fixed using paraformaldehyde (2%). After incubation with anti-megalin for 1 h, cells were washed and incubated for a second hour with goat-anti-mouse-FITC conjugate (Dako). Slides were examined by immunofluorescence microscopy.

Lectins staining pattern specific for proximal tubular cells was examined with Sophora japonica agglutinin (aggl) (SJA, Vector Laboratories), Lotus tetragonolobus aggl (LTA,

Sigma), and Erythrina cristagalli aggl (ECA, Vector Laboratories) by incubating cells with each of the lectins (75 μg/ml) labeled with FITC (LTA and ECA) or rhodamine (SJA) for 1 h at room temperature [Grupp et al. 2001].

Determination of thiol-amino acids and small thiol-peptides

Preparation of cell extracts and measurement of intracellular thiols (total GSH, oxidized glutathione (GSSG), cystine, and cysteine) using HPLC were performed as described previously [Levtchenko et al. 2005]. GSSG is glutathione present in disulfides.

Measurement of total intracellular ATP content

Cystinotic and control cell cultures were grown to confluence in 75 cm^2 tissue culture flasks (approximately $5.0x10^6$ cells) and detached using trypsin. After washing in PBS, cell pellets were shock frozen in liquid N_2 and stored at - 80 °C until ATP determination. Prior to ATP determination pellets were resuspended on ice in 0.5 ml cold PBS. A 25-fold diluted fraction (25 μl) of the suspension was transferred to a white microtiter plate and ATP was measured using ATP Bioluminescence Assay Kit HSII (Roche) according to the instructions of the manufacturer. The residue of the undiluted cell suspension was used for protein determination using the method of Lowry.

Statistical analysis

Data (mean, SD) are presented as a mean of two separate experiments. Unpaired t test was used for statistical analysis.

Differences were considered statistically significant at $p < 0.05$.

Results

Immortalized exfoliated cells express PTEC phenotype

While colonies with a cobblestone morphology developed in all nine collected cystinotic

urine samples within 2 weeks (Figure 1A), only nine out of 48 control urine samples showed cell proliferation. After immortalization with HPV 16 E6/E7, obtained cell lines exhibited the presence of microvilli and tight junctions, characteristic for PTEC, indicating that cultured cells develop apical and basolateral polarization, emphasized with the toluidine blue staining (Figs. 1B and C). High activity of alkaline phosphatase (AP) was detected in both cystinotic and control PTEC cultured from urine (11456 ± 7135 μU/mg protein) (Figure 2). The activity of AP in cells derived from urine was about 100-fold higher compared to commercially available HK-2 cell line (143 ± 309) and 1000-fold higher compared to the podocytes (14 ± 13). Megalin (Figure 1D) and aminopeptidase N (Figure 3) were present in all examined PTEC cultured from urine. Aminopeptidase N was not detected on the podocytes. Furthermore, the staining with SJA, ECA, and LTA lectins was all positive in obtained PTEC in concordance with their proximal origin (data not shown).

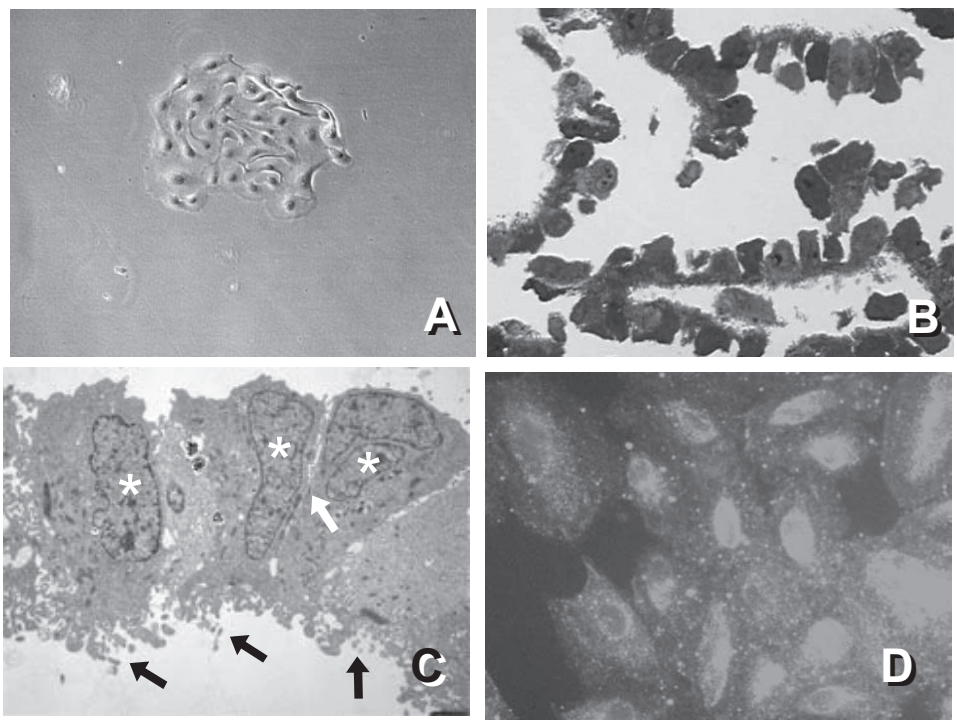

Figure 1 (A) Phase contrast image of primary cultures showing cobblestone morphology (original magnification 100x). (B) Light microscopy of cultured cells stained with methylene blue after scraping from tissue culture flask showing bipolar orientation (original magnification 400x). (C) Transmission electron microscopic image showing microvilli (black arrows), tight junctions (white arrow), and nuclei (asterisks) (original magnification 3000x). (D) Immunofluorescence image showing positive staining with monoclonal anti-megalin antibody 6C5 (original magnification 400).

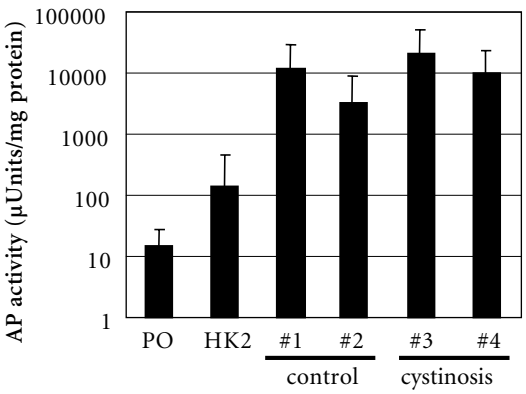

Figure 2 Alkaline phosphatase activity in cell suspensions. Activities (mean values of at least two independent experiments) quantified using shrimp alkaline phosphatase. Data of control PTEC (#1 and #2) and cystinosis PTEC (#3 and #4) are representative for total study population. PO, conditionally immortalized podocyte.

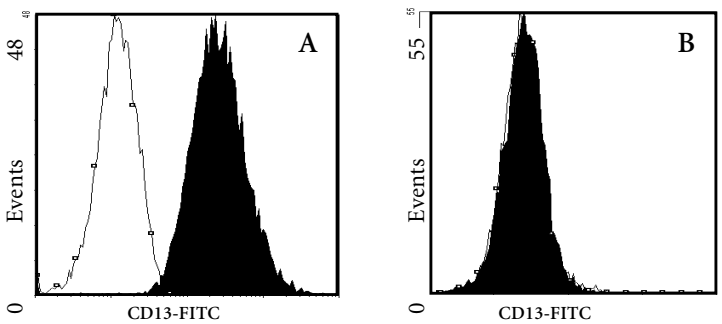

Figure 3 Fluorescence intensity of mouse-anti-human CD-13-FITC (aminopeptidase N) antibody analyzed by flow cytometry. (A) Cultured PTEC cell lines derived from urine and (B) conditionally immortalized podocytes. Unlabeled cells are presented in white, labeled cells in black.

Cystinotic cell lines accumulate cystine and contain increased oxidized glutathione

Cystine levels in cystinotic PTEC were significantly elevated compared to healthy control cells (0.86 ± 0.95 nmol/ mg versus 0.09 ± 0.01 nmol/mg protein, $p = 0.03$), confirming their cystinotic phenotype (Figure 4A).

Total GSH content was below the detection limit of 0.10 nmol/mg protein in three cystinotic PTEC and four control PTEC. In the remaining cultures, cystinotic cells demonstrated a significant increase of GSSG compared to healthy controls (1.16 ± 0.83 nmol/mg versus 0.29 ± 0.18 nmol/mg protein, $p = 0.04$) (Figure 4B). Total GSH (16.77 ± 5.48 versus 9.85 ± 9.25, $p = 0.16$) and free cysteine (11.45 ± 3.94 versus 11.38 ± 4.07, $p = 0.97$) were not different between cystinotic and control PTEC.

Total intracellular ATP contents are comparable between cystinotic and control cell lines
Total intracellular ATP content in the cultured cells did not differ between cystinotic and control PTEC (3.49 ± 1.01 versus 4.03 ± 2.62, $p = 0.558$) (Figure 5). There was no correlation between cystine and ATP contents and between ATP and glutathione contents in cystinotic and control PTEC.

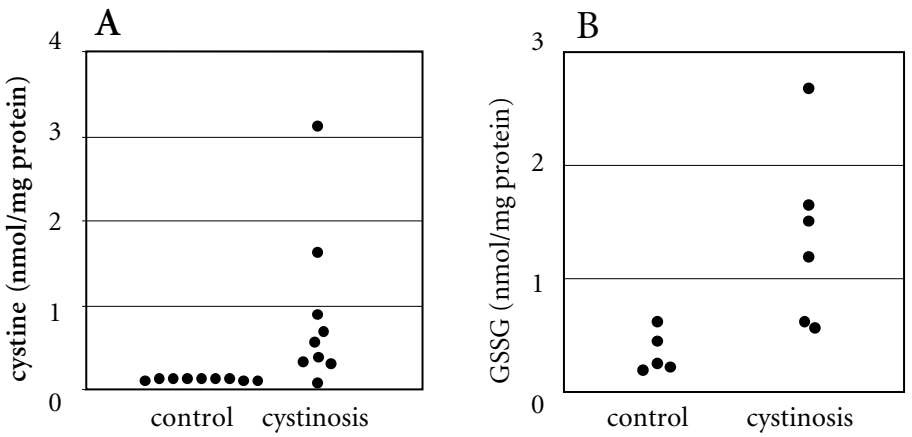

Figure 4 (A) Cystine content (nmol/mg protein) in control (n=9) versus cystinotic (n=9) PTEC cell lines, p=0.03. (B) GSSG content in control (n=5) versus cystinotic (n=6) cell lines, p=0.04.

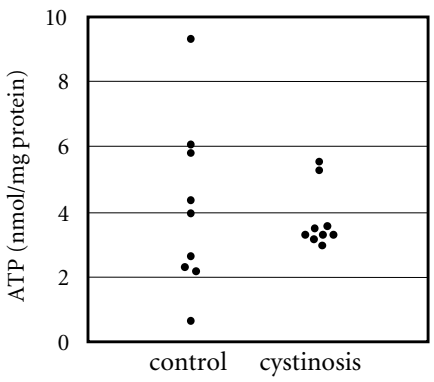

Figure 5 ATP concentration in PTEC cultured from urine of controls (n = 9) and cystinotic patients (n = 9). Values are expressed as nmol/mg protein, $p > 0.05$.

Discussion

In the present study, we developed human proximal tubular epithelial cell lines from urine of patients with cystinosis and healthy controls. This non-invasive approach is used because no other *in vitro* or *in vivo* model for studying the pathogenesis of renal disease in cystinosis is available.

In contrast to previous studies [Racusen et al. 1991, Racusen et al. 1995, Laube et al. 2005], we immortalized proximal tubular cells with HPV E6/E7, allowing us to obtain sufficient material for metabolic studies.

The presence of typical proximal tubular cell morphology, brush border enzyme AP activity, positive staining with anti-CD13 and anti-megalin antibodies, and proximal tubular specific lectin pattern confirmed proximal origin of obtained cell lines. These features were not changed after the transfection.

Human podocytes conditionally immortalized with SV-40 ts 58 were used as a negative control to demonstrate that obtained cell lines were not podocytes, which also can be exfoliated in urine [Vogelmann et al. 2003]. Anti-CD 13 antibody (anti-aminopeptidase N) appeared to be an ideal marker to discriminate podocytes from proximal tubular cells, as this marker was positive for all tested urinary PTEC cell lines but always negative for the podocytes.

Culturing PTEC from urine of healthy controls was less successful compared to patients with cystinosis (100% proliferation in cystinosis versus 19% in controls). A possible explanation could be that through increased mechanical stress in cystinotic patients more viable cells are exfoliated.

Our present *in vitro* model is superior to CDME-loading method, which only mimics lysosomal cystine accumulation, since the molecular defect in *CTNS* gene is naturally present in cells derived from urine of cystinotic patients. Strikingly, in contrast to the studies in CDME loaded proximal tubular cells [Salmon et al. 1990, Coor et al. 1991, Ben-Nun et al. 1993, Foreman et al. 1995, Baum 1998, Cetinkaya et al. 2002], we found no significant difference in intracellular ATP, questioning the hypothesis of ATP depletion being the cause of defective proximal tubular transport.

It might be suggested that higher cystine concentrations are necessary to cause ATP depletion in cystinotic cells, as cystine accumulation in our *in vitro* model was lower compared to cystinotic kidney: >15 nmol/mg wet weight [Gahl et al. 2001]. Further extensive study of mitochondrial ATP synthesis in cystinotic proximal tubular cell lines is required to answer the question whether decreased ATP production is responsible for cellular dysfunction in cystinosis.

In seven cell lines (three cystinotic and four controls), intracellular GSH was depleted and could not be quantified because it was under the detection levels of the assay. A possible explanation of this observation is that not all obtained cell lines express glutathione transporters [Lash et al. 2005].

The most important finding of this study is a significantly elevated GSSG content in cystinotic PTEC, which might point to increased oxidative stress and altered redox status, while total GSH was comparable in cystinotic and control cells. Increased GSSG was already reported by our group in primary cystinotic fibroblasts and polymorphonuclear cells, while total GSH and free cystine were within the normal range in all tested cell types [Levtchenko et al. 2005]. Concordant with our data, Chol et al. [2004] have recently demonstrated in conditionally immortalized cystinotic fibroblasts an increased activity of superoxide dismutase, converting superoxide into H_2O_2, possibly indicating increased oxidative stress. Interestingly, oxidized glutathione (GSSG) can activate pro-apoptotic PKCd [Chu et al. 2003] and might play a role in increased apoptosis demonstrated in cystinotic fibroblasts [Park et al. 2002]. Altogether these findings indicate that further research should be focused on reactive oxygen species in cystinotic cells.

In conclusion, normal ATP content in cystinotic proximal tubular epithelial cell lines questions the hypothesis of altered ATP synthesis as a keystone in the pathogenesis of cystinosis. Elevated oxidized glutathione suggests increased oxidative stress, possibly playing a role in cellular dysfunction in cystinosis.

Acknowledgments

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CHAPTER 12

Summary, general discussion

Cystinosis is a rare lysosomal storage disease whose hallmark is the accumulation of cystine within the lysosomes. The disease generally manifests with proximal tubular dysfunction, known as Fanconi syndrome, and progresses towards end stage renal disease (ESRD). Besides the kidneys, lysosomal cystine accumulation in virtually all tissues causes multi-organ damage, making cystinosis a systemic metabolic disease. An amino thiol cysteamine is the only available cystine depleting drug, which retards the deterioration of the renal function and postpones the occurrence of extra-renal complications. In patients with cystinosis, cysteamine therapy should be initiated as soon as possibly and must be continued life-long. Even after renal transplantation cysteamine is required to protect the extra-renal organs. Despite significant progress made in the last 3 decennia, most patients with cystinosis, although delayed compared to untreated patients, still develop ESRD and extra-renal complications. This could be partially related to the fact that intracellular events downstream to lysosomal cystine accumulation remain unknown, thus hampering therapeutic interference into the disease pathogenesis. Furthermore, non-compliance with cysteamine treatment might be responsible for therapy failure in some patients.

In our work described in this thesis we primary aimed improving the clinical care of cystinosis patients (**Chapters 2-7**). Next, we performed cell biological studies providing new insights towards understanding the pathogenesis of cystinosis (**Chapters 8-11**).

Improving clinical care of cystinosis patients

Diagnosis of cystinosis

Cystine measurement

For the early initiation of cysteamine therapy the early diagnosis of cystinosis is mandatory. The detection of an elevated cystine content in patient's tissue is obligatory for the diagnosis. Corneal cystine crystals are a pathognomonic marker of the disease, but usually can be detected by an experienced ophthalmologist only after the first year of life.

Blood cells are easily available for cystine measurements and are generally used for making the diagnosis. Accurate cystine measurements are also required for the monitoring of cysteamine therapy. Cystine measurements might be used for the detection of the heterozygotes mostly having a slightly elevated cystine content compared to healthy persons. Historically cystine has been measured in mixed white blood cells (ML), despite the fact that it preferentially accumulates in polymorphonuclear leukocytes (PMN) and monocytes, but not in the lymphocytes [Schulman et al. 1970]. The reason for this is that ML

are easier to isolate from whole blood. Because cystine content is expressed in nmol per mg protein, values in ML, containing large amounts of lymphocytes, can be expected to be lower compared to values measured in PMN. Additionally, any change in differential count of white blood cell populations would influence the cystine value, making the comparison between different measurements in the same patients less accurate. In **Chapter 2** we describe our study comparing cystine content of ML and PMN measured simultaneously by HPLC in 4 patients at diagnosis, 12 patients undergoing cysteamine therapy, 15 obligate heterozygotes and 8 healthy controls. As it could be theoretically predicted, intracellular cystine was significantly elevated in PMN compared to ML in patients at diagnosis and under cysteamine therapy. Furthermore, the overlap between heterozygotes and healthy controls was smaller when cystine was measured in PMN, which was already demonstrated earlier [Smolin et al. 1987]. In controls ML and PMN values were comparable due to very low cystine content close to the detection limit of our assay: 0.15 μmol/l cystine. In all patients under cysteamine therapy the dose of cysteamine had to be increased because their PMN cystine was above 0.5 nmol/mg protein (90th percentile of cystine value measured in the heterozygotes, accepted as an upper limit in patients under cysteamine therapy). In two patients at diagnosis cystinosis could be missed due to cystine values in ML within the heterozygote range, while it was clearly above the heterozygote range when measured in PMN. It could be expected that patients whose therapy is monitored by cystine detection in PMN cells would have better renal function survival compared to those whose cystine is measured in ML. Further studies have to prove it.

In conclusion, we consider it essential to measure cystine in PMN and not in ML, because it increases the sensitivity of cystine detection for the diagnosis of cystinosis and provides better target concentration during the monitoring of the cysteamine treatment.

Molecular analysis of the cystinosis gene

In our opinion the diagnosis of cystinosis has to be confirmed in all patients with another method than cystine detection in PMN. Inaccurate procedure of PMN isolation or transport could result in false increase of cystine value due to the oxidation of intracellular cysteine, which is ~100 times more abundant in the cytosole of white blood cells cell compared to cystine [de Graaf-Hess et al. 1999]. Initially we used cystine detection in primary skin fibroblasts for the confirmation of the diagnosis, as molecular analysis of the cystinosis gene (*CTNS*) was not available. In 1998 the *CTNS* gene, encoding lysosomal cystine transporter, cystinosin was identified by positional cloning strategy and mapped to the short arm of chromosome 17 (17p13) [Town et al. 1998]. In **Chapter 3** we describe an

improved double multiplex PCR method for the detection of the common in European population 57-kb deletion spanning exons 1 to 10 plus a large amount of upstream sequence. In all tested patients (n=11) 2 separate PCR were performed. The first one used LDM1 forward and reverse primers up- and downstream the deletion [Anisker, Lucero et al. 1999]. In the presence of 57-kb deletion, the amplification with these primers resulted in a fragment of 423 bp. To discriminate between heterozygous and homozygous deleted patients a second separate PCR using primers to identify the presence of D17S829 marker, which is situated in intron 3 of *CTNS* gene, located in the deletion interval, should be performed. In the presence of D17S829, 266 bp band is produced. For both PCRs an amplification of 635 bp fragment of the housekeeping gene β-actin was used as an internal control. Thus, patients with homozygous 57 kb deletion will have 423 and 635 bp PCR products, patients having heterozygous 57 kb deletion: 266, 423 and 635 pb products and patients without the deletion: 266 and 635 bp products. This simple and accurate method allowed us to identify the 57 kb deletion in 59% (13/22) of all tested Dutch alleles, 5 patients were homozygous and 3 were heterozygous for the deletion. The resting alleles were submitted to the genomic sequencing, which revealed other previously reported mutations in all but 3 alleles. The unidentified mutations might be located in untested regions of the gene such as the promoter or intronic sequences. In our small patient population no correlation between the genotype and the severity of the phenotype (age at diagnosis, decline of renal function, age at start of thyroid hormone replacement) could be detected. The analysis of larger patient's groups revealed the correlation of the genotype with the clinical form of cystinosis: complete deletion or truncating mutations with a loss of functional protein were generally detected in patients with most severe infantile form of cystinosis, while patients with juvenile and ocular form had mutations allowing residual cystinosin activity [Attard et al. 1999, Anikster et al. 2000, Thoene et al. 1999].

Actually molecular analysis of *CTNS* is used in all Dutch patients for the confirmation of the diagnosis of cystinosis and can be applied for prenatal diagnosis and heterozygote detection.

Treatment of cystinosis

Multidisciplinary approach

Cystinosis is a multisystem disorder, initially affecting kidneys and cornea. When renal replacement therapy was not available, patients with cystinosis generally died before the age of 10 years and extra-renal organ involvement was largely unknown. Longer survival

of cystinosis patients due to the possibility of renal transplantation revealed endocrine organ damage (hypothyroidism, diabetes mellitus, male hypogonadism), muscular atrophy, retinopathy, central and peripheral neural system damage, hepatic and exocrine pancreas involvement, usually developing after the first decade [Gahl et al. 2002]. This means that physicians treating older cystinosis patients (generally "adult" nephrologists) should be aware of the extra-renal complications of cystinosis. Because cystinosis is a rare disorder (for example in the Netherlands there are in total 40 cystinosis patients and among them 10 adults), nephrologists treating adult patients are not able to get experienced with treatment of these patients. The situation is relatively different for paediatric patients concentrated within few children university nephrological centres, each of them treating at least 3-5 patients. To evaluate the clinical care of 10 Dutch adult patients with cystinosis we reviewed their medical records (**Chapter 4**). Strikingly, ophthalmic control was never performed in two patients, thyroid function was not checked in another two patients and two more patients did not get glycemia control. Only seven Dutch adult patients were treated with cysteamine (three of them received the drug in 2 or 3 doses daily, while 4 times daily are obligatory). Cystine content in white blood cells was within the recommended range only in three patients. Thus, rather inadequate medical care of Dutch cystinosis patients was observed. This motivated us to organise a multidisciplinary cystinosis outpatient clinic for all adult Dutch patients, functioning in UMC St Radboud over the last 2 years. In one day the patients are examined by a physician experienced in metabolic diseases, an ophthalmologist, a neurologist (for those with neurologic complaints) and a clinical genetician (a single counselling per patient). Additionally an aid of a social worker is proposed to all patients. Blood for cystine detection is taken 5-6 hours after cysteamine ingestion and is proceeded immediately for PMN isolation. The recommendations for medical care are provided for the nephrologists who remain responsible for the medical care of their patients. During the last 2 years we have identified 2 unknown adult patients with cystinosis and one patient in whom the diagnosis of cystinosis could be rejected after almost 20 years of cysteamine therapy.

Treatment of proteinuria

The deterioration of the renal function in patients with cystinosis is associated with the progressive development of tubulo-interstitial lesions, including tubular atrophy and interstitial fibrosis [Gubler et al. 1999]. The pathogenetic mechanisms by which cystine accumulation leads to renal failure are enigmatic. Albuminuria invariably present in patients with cystinosis might be one of the contributing factors. Whether albuminuria in cystinosis originates from increased glomerular filtration, decreased proximal tubular

reabsorption or the combination of both is unknown. In **Chapter 5** we demonstrate in 5 patients with Fanconi syndrome that albiminuria could be decreased with ~ 40% under treatment with angiotensin converting enzyme (ACE) inhibitor enalapril (0.15 mg/kg once daily). Interestingly, treatment with enalapril did not change the increased urinary excretion of low molecular weight protein α-1 microglobulin, supporting the glomerular origin of albuminuria. Decrease of albuminuria might improve the survival of the renal function in cystinosis as it has been demonstrated in various other renal diseases [Ruggenenti et al. 2001]. Not surprisingly, treatment with enalapril was accompanied by a decrease of systolic blood pressure and complaints of hypotension in 2/5 patients. Creatinine clearance diminished from median 48 to 45 ml/min/1.73 m^2 and returned to previous values after the discontinuation of enalapril. These side effects of enalapril indicate that ACE inhibitors should be used with caution in patients with cystinosis, suffering from sodium losses and polyuria. It is not excluded that angiotensin II receptor blockers, possibly having less pronounced hypotensive effect [Gansevoort et al. 1999], could be better tolerated by cystinosis patients.

Improving the effectiveness of cysteamine therapy

Cysteamine can deplete white blood cell cystine content with 80-90%, but is unable to prevent the deterioration of the renal function and the occurrence of extra-renal disease in the majority of the patients. Two studies described in **Chapters 6 and 7** aimed to improve the effectiveness of cysteamine therapy.

Our first hypothesis was that cysteamine being a small molecule (MW 77.14) could be lost in urine of cystinosis patients suffering from Fanconi syndrome. As shown in **Chapter 6** it was not the case as less than 1% of the ingested dose was excreted into urine.

Secondly, we suggested that inappropriate cysteamine administration might be responsible for therapy failure in some patients (**Chapter 7**). To test this idea we asked Dutch patients with cystinosis to fill in a questionnaire concerning their cysteamine dose regimen. This questionnaire revealed that only 5/22 patients took the drug every 6 hours as it is recommended according to the pharmacodynamic data. Most of the patients ingested cysteamine only during the wake time resulting in a night pause (between the last evening and the first morning dose) of ~ 9 hours. Subsequently, we investigated whether morning cystine PMN value was elevated in patients after 9 hours night pause compared to 6 hours night pause. In 11 compliants patients mean morning PMN cystine value was indeed above the recommended value of 0.5 nmol/mg protein 9 hours after the last evening cysteamine ingestion (0.73 nmol cystine/mg protein) versus 6 hours after

the last evening cystemine dose (0.44 nmol cystine/mg protein, $p<0.05$). Although it is unknown whether PMN cystine content adequately reflects cystine accumulation in the other tissues, we strongly advise cystinosis patients to ingest cysteamine every 6 hours including the night as it provides better metabolic control of cystinosis.

In summary, for optimal clinical care of cystinosis patients we recommend:

1. As early as possible diagnosis of cystinosis using cystine detection in PMN leukocytes in order to initiate cysteamine treatment as early as possible.
2. Use of PMN leukocytes for the monitoring of cysteamine therapy (5 to 6 hours after the last cysteamine dose PMN cystine value should be below 0.5 nmol cystine/mg protein). ML should not be used.
3. Confirmation of the diagnosis of cystinosis by molecular analysis of the cystinosis gene.
4. Multi-disciplinary approach for medical care of cystinosis patients, in particular adults, suffering from extra-renal complications of the disease.
5. Administration of ACE inhibitors or angiotensin II receptor blockers in patients with albuminuria under strict control of blood pressure and serum creatinine (requires further study).
6. Administration of cysteamine treatment every 6 hours including the night.

New insights into the pathogenesis of cystinosis

The second major part of our work (**Chapters 8-11**) included cell biological studies exploring pathogenic mechanisms involved in cellular dysfunction in cystinosis.

A case report described in **Chapter 8** demonstrated a gradual deterioration of proximal tubular dysfunction starting with mild aminoaciduria detected at the age of 3 weeks towards full-blown Fanconi syndrome at the age of 6 months. The differential sensitivity of apical proximal tubular transporters to cystine accumulation is in our opinion in contrast to the generally accepted hypothesis of reduced ATP generation being a keystone in the pathogenesis of cystinosis. This because in cystine dimethyl ester (CDME) loading model of cystinosis immediate ATP depletion and direct inhibition of different transporters was demonstrated [Baum 1998].

Study of energy metabolism in cystinosis

Because no animal or cell model of cystinosis was previously available, others have studied the pathogenesis of the disease by loading proximal tubular cells with CDME. CDME loading resulted in inhibition of proximal tubular transport, mimicking Fanconi syndrome in cystinosis. In this model a pronounced decrease of mitochondrial ATP synthesis was demonstrated, which was postulated to be an underlying pathogenetic mechanism in cystinosis [Foreman et al. 1987, Salmon et al. 1990, Coor et al. 1991, Ben-Nun et al. 1993, Sakarcan et al. 1992, Foreman et al. 1995, Baum 1998]. Until now an altered energy metabolism has not been confirmed in human cystinotic material.

We performed an extensive study of ATP metabolism in primary cultured fibroblasts of patients with cystinosis with known genetic defects of the *CTNS* gene (**Chapter 9**). The choice of fibroblasts was based on their relative easy availability by skin biopsy and the extensive cystine accumulation in these cells. Furthermore, our group is experienced in studying the pathogenesis of mitochondrial disorders in primary fibroblasts, displaying altered ATP production and deficient oxidative phosphorylation (OXPHOS) activity [Visch et al. 2004, Loeffen et al. 2000, Ugalde et al. 2004]. Patient's fibroblasts accumulated marked amounts of cystine (median, range: 4.3 (2.7-5.5) versus 0.2 (0.1-0.3) nmol/mg protein in controls, $p<0.001$), consistent with their cystinotic phenotype. Strikingly cystinotic fibroblasts contained significantly decreased amount of intracellular ATP compared to control fibroblasts (median, range: 37.3 (26.9-55.0) versus 51.5 (44.7-58.5) nmol/mg protein, $p<0.05$).

To confirm the alteration of intracellular ATP status in cystinosis, we additionally measured intracellular ATP content in PMN cells immediately derived from patients and controls. Comparable to cystinotic fibroblasts, intracellular ATP was also decreased in cystinotic versus control PMN cells (median, range: 317 (149-1141) versus 557 (207-1101) nmol/mg protein, $p<0.05$).

Intracellular ATP content is the sum of ATP produced by cytosolic glycolysis and mitochondrial OXPHOS minus intracellular ATP consumption as presented by a simplistic equation: Cellular ATP content = (ATP production by OXPHOS + ATP production by glycolysis) - ATP consumption.

Decreased ATP production, enhanced consumption or a combination of both would result in diminished ATP content. We primary concentrated our efforts on studying the mitochondrial ATP generating capacity because a possible alteration of mitochondrial complex I was demonstrated in CDME model of cystinosis [Foreman et al. 1995].

Remarkably, overall energy generating capacity, activity of respiratory chain complexes in mitochondrial enriched cell fractions and bradykinin-stimulated maximal mito-

chondrial ATP production in intact cells were all normal in cystinotic fibroblasts. The reason for this observation could be the fact that cystinotic fibroblasts preferably use cytosolic glycolysis to generate ATP, which was also observed in our study. Interestingly, after the inhibition of the glycolysis by 2-deoxyglucose (DOG) or sodium iodoacetate (SIA) no difference in intracellular ATP content between cystinotic and control fibroblasts could be detected anymore, suggesting an alteration of the glycolytic pathway in cystinosis.

Because PMN cells are also glycolytic, no study of mitochondrial ATP generation was performed in PMN.

To investigate whether decreased ATP content had functional consequences in fibroblasts, we studied the activity of Na,K ATP-ase by Rb^+ influx in maximally stimulated conditions. This study also revealed no differences between cystinotic and control cells, meaning that despite decreased ATP levels in cystinosis, the ATP content was still sufficient for normal Na,K ATP-ase pump function.

In conclusion, our study demonstrating diminished ATP content in cystinotic cells failed to confirm the alteration of mitochondrial ATP generating capacity postulated in CDME model of cystinosis. The questions whether deficient glycolysis or increased ATP consumption were responsible for decreased ATP remain unanswered and should be further investigated.

Study of glutathione metabolism

Next to altered ATP metabolism we searched for alternative pathogenetic mechanisms in cystinosis.

Theoretical considerations and some clinical data indicated that a disturbed glutathione metabolism might play a role [Rizzo et al. 1999]. Cystine is carried by cystinosin to the cytosol where it is reduced to cysteine by cytosolic reducing systems [Gahl et al. 2001]. Theoretically the intra-lysosomal cystine accumulation in cystinosis can result in the cytosolic deficit of cysteine, which together with glutamate and glycine are needed for glutathione (GSH) synthesis. Cytosolic deficiency of cysteine might limit GSH synthesis. In a clinical study, comparable to the patients with GSH synthetase deficiency [Larsson et al. 2000], elevated 5-oxoproline excretion has been demonstrated in cystinotic patients with Fanconi syndrome, untreated with cysteamine, in contrast to normal 5-oxoproline excretion in patients with idiopathic Fanconi syndrome. Interestingly, urinary 5-oxoproline excretion normalized under cysteamine treatment. Thus, this observation suggests disturbed GSH homeostasis, specific for cystinosis patients untreated with cysteamine [Rizzo et al. 1999]. We studied intracellular GSH status in cultured cystinotic fibroblasts and

PMN cells immediately isolated from blood (**Chapter 10**) and found that total GSH and free cysteine were both normal in cystinotic cells. Strikingly, cystinotic fibroblasts and PMN cells demonstrated a significantly elevated amount of glutathione disulfide (oxidized GSH or GSSG) (median, range in fibroblasts: 0.7 (0.5-1.7) nmol/mg protein versus 0.3 (0.2-0.9) in controls; in PMN: 0.9 (0.3-1.8) nmol/mg protein versus 0.3 (0.2-0.4) in controls). Subsequently elevated GSSG was also detected in proximal tubular cells derived from urine of cystinotic patients (**Chapter 11**). The same change in GSH status has been demonstrated in immortalized cystinotic fibroblast cell lines by Chol et al. [2004]. Thus, increased GSSG is a consistent finding in 3 cystinotic cell types and might reflect a pathogenetic mechanism in cystinosis. The explanation of altered GSH status is speculative. GSH is the most abundant cellular thiol, functioning as an important redox buffer. In addition, GSH defends cells against reactive oxygen species (ROS), serving as a cofactor for the GSH peroxidase family of enzymes, which metabolize H_2O_2 and lipid peroxides, resulting in the formation of GSSG. Glutathione reductase, using NADPH oxidase system, is necessary to regenerate GSH [Uhlig et al. 1992]. Thus, elevated GSSG might reflect aberrant ROS handling and altered redox status present in cystinotic cells. Importantly, an increased superoxide production has been earlier demonstrated by chemoluminescence assay in isolated polymorphonuclear cells and mononuclear phagocytes from cystinotic children [Pintos Morell et al. 1985]. GSH binds various toxic substances to form GSH conjugates, that are subsequently exported out of the cells by specific transporters [Lash 2005]. Inhibited transport in cystinotic cells can be responsible for the decreased efflux of GSSG or GSH conjugates and might explain the altered GSH status in cystinosis.

In our opinion altered ratios between total and oxidised GSH in cystinotic cells might not only reflect increased oxidative stress and/or deficient GSSG transport, but itself can cause further cell damage. Oxidised glutathion (GSSG) can activate pro-apoptotic protein kinase C δ [Chu et al. 2003] and might be a pro-apoptotic stimulus in cystinosis [Park et al. 2002].

In conclusion, altered GSH status with increase of GSSG is demonstrated in cystinotic fibroblasts, PMN and proximal tubular cells. Further studies should investigate the origin and the consequences of this observation.

Development of proximal tubular cell model of cystinosis

Cystinotic fibroblasts and PMN cells, although extensively accumulating cystine, do not represent an ideal model for studying the pathogenesis of cystinosis for two reasons.

First of all, in patients with cystinosis fibroblasts and PMN cells display no overt signs

of the disease. Secondly, these cell types *in vivo* and *in vitro* preferentially use anaerobic glycolysis for ATP synthesis [Robinson 1996, Yang et al. 2004]. Cystinotic renal proximal tubular epithelial cells (PTEC), which are initially affected in cystinosis and *in vivo* dependent on oxidative metabolism would be a much better *in vitro* cell model for cystinosis research. Generally renal tissue in cystinosis is unavailable as renal biopsy is not required for the diagnosis. Therefore attempts have been made to cultivate renal cells exfoliated into urine [Racussen et al. 1995, Laube et al. 2005].

We succeeded to obtain proximal tubular cell cultures from 9 patients with cystinosis and 10 healthy controls (**Chapter 11**). As primary PTEC stop to proliferate after 4-6 passages, an immortalization step is required to obtain sufficient material for extensive metabolic studies.

We used human papilloma virus E6/E7 genes, which was previously used for the immortalization of well characterized human proximal tubular cell line HK-2 [Ryan et al. 1994]. Obtained cell lines demonstrated baso-lateral polarization, enzymatic activity of alkaline phosphatase, positive staining with anti-aminopeptidase N and anti-megalin antibody, confirming their proximal tubular origin. Unfortunately, immortalized cystinotic PTEC accumulated cystine at a much lesser extent compared to cystinotic renal tissue *in vivo*, probably due to continuous cell proliferation (0.8±0.9 nmol cystine/mg protein versus 0.1±0.01 in controls ($p<0.05$) in contrast to >15 nmol cystine/mg wet tissue in the patients).

Interestingly, immortalized cystinotic PTEC also displayed an increased GSSG content, which was found in cystinotic fibroblasts and PMN cells (1.2+0.8 nmol/mg protein versus 0.3±0.2 in controls, $p<0.05$). Because intracellular ATP content was normal in cystinotic PTEC, we performed no studies of energy generating capacity in these cells. Our current understanding of the pathogenesis of the disease is summarised in Figure 1.

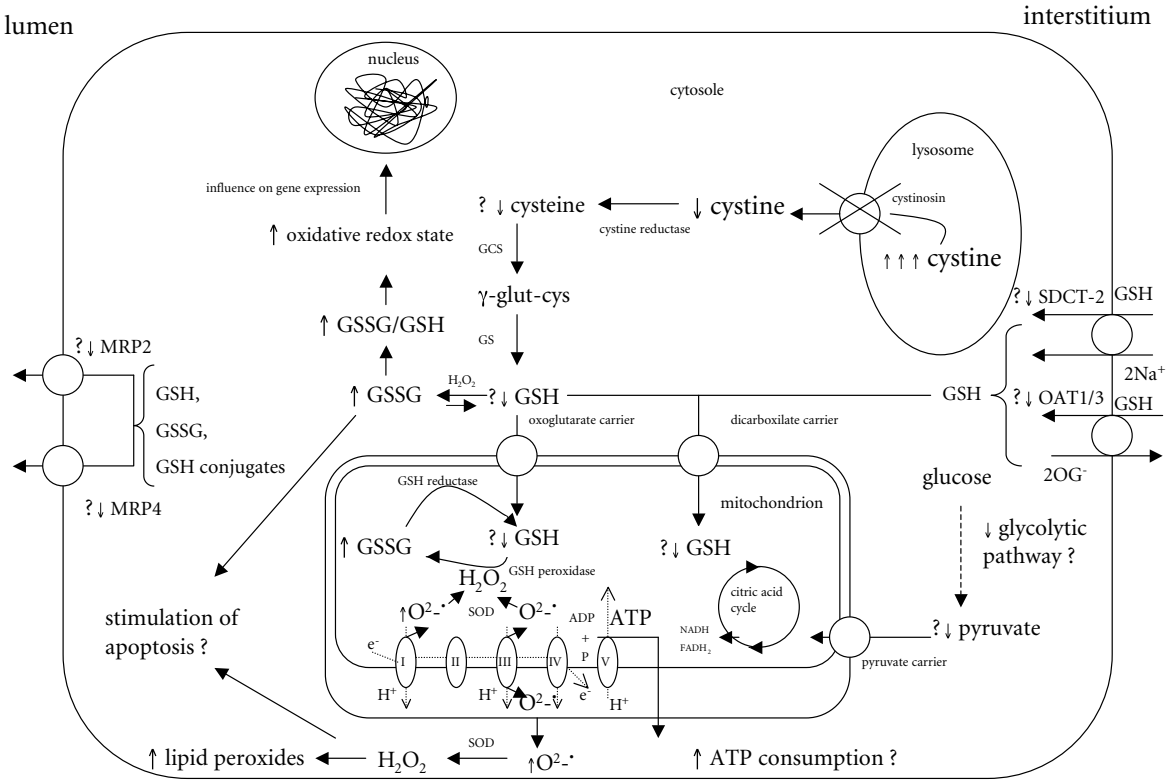

Figure 1 Speculative mechanisms of cellular damage in cystinosis.
Defective cystine exit out of the lysosomes possibly disturbs cytosolic supply of cysteine, which is a limiting step for GSH synthesis. Although GSH and cysteine deficiencies were not detected in our study in rest conditions, it can occur in conditions of increased oxidative stress, when increased ROS production or decreased ROS elimination would cause cell damage (stimulate apoptosis or necrosis). Increased GSSG might be a consequence of increased ROS generation or diminished GSSG efflux out of the cells. Elevated GSSG/GSH ratio reflects changed redox status in cystinotic cells which could influence gene expression and stimulate apoptosis. Decreased ATP content in cystinotic cells might result from increased ATP consumption (due to increased oxidative stress) or diminished glycolytic activity.
Abbreviations: GSH, glutathione; GSSG, glutathione disulfide or oxidized GSH; GCS, γ-glutamylcysteine synthetase; GS, GSH synthetase; γ-glut-cys, γ-glutamylcysteine; MRP2/MRP4, multidrug resistance proteins 2 and 4; OAT1/3, organic anion transporters 1 and 3; $2OG^-$, 2-oxoglutarate; SOD, superoxide dismutase; SDCT-2, sodium-dicarboxylate transporter 2; ?↓, putative decrease; ?↑, putative increase; ?, uncertain mechanism.

Directions for future research

Due to our extensive efforts resulting in improved care of cystinosis patients and more insights into the pathogenesis of the disease, we suggest the following directions for the future studies.

Further unraveling the pathogenesis of cystinosis

Proximal tubular cell model of cystinosis requires further optimalization. Recently with the help of the group of Bristol (Dr. M. Saleem, Dr. P. Mathiesson) we were able to solve the problem of insufficient cystine accumulation by using for cell immortalisation the human Simian Virus containing temperature allele (SV40 T antigen), which was previously used for the immortalisation of human podocytes [Pavenstadt et al. 2003]. Cells transfected with SV40 T vector proliferate at permissive temperature of 33°C, but stop to proliferate at 37°C. Our preliminary studies showed a spectacular cystine accumulation in cystinotic PTEC after 10 days differentiation at 37°C (up to 8 nmol cystine/mg protein). Because PTEC cultured from urine might be contaminated by glomerular podocytes and tubular cells from distal nephron, obtained cell lines should be subcloned.

In obtained cystinotic and control PTEC lines energy generating capacity should be studied. To force the cells to use OXPHOS, glycolytic pathway should be inhibited. Our preliminary data demonstrated a significant increase of mitochondrial ATP production after incubation of the cells with 2-deoxyglucose.

ROS generation and handling and GSH redox cycle enzymes activity should be investigated at basal conditions and during increased oxidative stress. Exploring influence of altered GSH status and impaired ROS handling on apoptotic pathways is also an attractive direction of research. GSH synthesis can be approached by measuring the activity of the enzymes of γ-glutamyl cycle with stable isotopes.

Transport of GSSG and GSH conjugates out of cystinotic PTEC is another intriguing research direction, which could possibly explain the alteration of GSH status in cystinosis.

Further improving medical care of patients with cystinosis

Treatment with cysteamine is difficult to follow because it should be taken every 6 hours and due to its side-effects such as bad breath odor and gastro-intestinal discomfort. The development of slow release cysteamine preparation or cysteamine pro-drug would solve the problem of frequent administration. The origin of halitosis induced by cysteamine

requires further studies to find pharmacological or cosmetic solutions. Gastro-intestinal complaints can be successfully treated by proton pump inhibitors (PPI) [Dohil et al. 2003], however, whether PPI do not affect cysteamine pharmacokinetics is unknown.

Factors involved in the development of interstitial fibrosis in cystinosis are another subject for further studies. Cystinotic PTEC should serve for this purpose. The identification of the mechanisms responsible for the interstitial damage leading to renal failure in cystinosis might allow new therapeutic strategies.

Solving the mechanisms linking lysosomal cystine accumulation and cellular dysfunction will lead to new treatment modalities in cystinosis.

CHAPTER 13

Samenvatting

Tijdens de laatste decennia werd qua kennis veel vooruitgang geboekt, en toch blijft bij cystinose het Fanconi syndroom irreversibel en leidt op de lange duur tot terminale nier insufficiëntie bij de meeste patiënten ondanks cysteamine behandeling. Het feit dat de pathogenese van de verstoorde cel functie veroorzaakt door cystine stapeling nog onbegrepen is, maakt effectieve behandeling moeilijk. Cysteamine is de enige momenteel bestaande behandeling welke de progressie van de nierziekte bij cystinose kan vertragen en de extra-renale organen kan beschermen, maar helaas kan cysteamine cystinose patiënten niet genezen.

Het doel van ons onderzoek beschreven in dit proefschrift was tweevoudig. Wij hebben bijgedragen aan een betere klinische zorg van cystinose patiënten (**Hoofdstukken 2-7**). Tegelijkertijd hebben wij celbiologische studies verricht om nieuw inzicht in de pathogenese te verkrijgen (**Hoofdstukken 8-11**).

Verbetering van de klinische zorg van cystinose patiënten

Diagnose van cystinose

Cystine meting

Vroege diagnose van cystinose is voor patiënten van belang omdat de behandeling met cysteamine dan vroegtijdig kan starten. De diagnose is gebaseerd op de klinische symptomen (meestal een uiting van het renaal Fanconi syndroom) en het meten van een verhoogd cystine gehalte in het weefsel van de patiënt. De meeste centra gebruiken de totale witte bloedcellen voor cystine bepaling terwijl cystine met name in de granulocyten stapelt [Schulman et al. 1970]. De reden hiervoor is dat de totale witte bloedcellen gemakkelijker dan de granulocyten uit bloed geïsoleerd kunnen worden. In **Hoofdstuk 2** beschrijven wij ons onderzoek dat heeft gedemonstreerd dat de meting in de granulocyt de gevoeligheid van de cystine bepaling verbetert, wat van groot belang is voor het stellen van de diagnose en voor het instellen van de behandeling. Bij twee patiënten was bij het onderzoek de diagnose van cystinose bijna gemist omdat de cystine gehaltes in de totale witte bloedcellen binnen de spreiding van de heterozygoten lagen, terwijl ze duidelijk verhoogd waren in de granulocyt.

Ook bij de heterozygoten was de gevoeligheid voor de detectie hoger, wanneer cystine in de granulocyt werd gemeten, zoals al eerder werd aangetoond door Smolin [1987]. Het is mogelijk dat de prognose van patiënten bij wie cysteamine therapie wordt bijgesteld aan de hand van het cystine gehalte in de granulocyt beter zal zijn in vergelijking met degene bij wie cystine in de totale witte bloedcellen wordt gemeten.

Moleculaire analyse van het cystinose gen

Wij zijn van mening dat de diagnose van cystinose bevestigd moet worden bij alle patiënten met een tweede methode naast het meten van cystine in de bloedcellen. Onnauwkeurige isolatie, bewaring of transport van de witte bloedcellen kan tot een valse verhoging van het cystine gehalte leiden tengevolge van de oxidatie van intracellulaire cysteine, waarvan de concentratie in de cel ~ 100 hoger is dan die van cystine [de Graaf-Hess et al. 1999]. In **Hoofdstuk 3** beschrijven wij een verbeterde PCR methode voor de detectie van de binnen de Europese populatie meest voorkomende mutatie van het *CTNS* gen: de grote 57 kb deletie, die bij 59% van alle onderzochten allelen werd gevonden. Het verrichten van 2 aparte PCR reacties bij elke patiënt laat een directe indeling toe tussen patiënten met een homozygote, heterozygote of geen deletie. Bij patiënten met een heterozygote deletie of geen deletie werd een sequentie analyse van het *CTNS* verricht, die andere al eerder beschreven mutaties van het *CTNS* heeft aangetoond. In onze kleine patiënten populatie (n=11) kon geen genotype-fenotype correlatie aangetoond worden. De analyse van de grotere patiënten groepen liet wel de correlatie tussen het genotype en de klinische vorm van cystinose zien [Attard et al. 1999, Anikster et al. 2000, Thoene et al. 1999]. Momenteel wordt standaard moleculaire analyse van het *CTNS* gen uitgevoerd voor de bevestiging van de diagnose, eventuele prenatale diagnostiek en heterozygoten detectie.

Behandeling van cystinose

Multidisciplinaire aanpak

Cystinose is een multi-orgaan ziekte, die klinisch eerst de nieren en cornea beschadigd. De aandoeningen van de extra-renale organen uiten zich in hypothyroïdie, diabetes mellitus, mannelijk hypogonadisme, spieratrofie, retinopathie en zenuwstelselschade en komen meestal voor na de eerste decade. Dit betekent dat de artsen die volwassen cystinose patiënten behandelen op deze extra-renale complicaties bedacht moeten zijn. Omdat cystinose een zeldzame aandoening is (in Nederland zijn er in totaal 40 cystinose patiënten bekend, waarvan 10 volwassenen), hebben de internisten weinig gelegenheid om ervaring te verwerven met de behandeling van cystinose patiënten. Wij hebben de klinische zorg geëvalueerd van 10 volwassen cystinose patiënten (**Hoofdstuk 4**) en konden vaststellen dat bij een aantal patiënten de extra-renale organen niet regelmatig of zelfs nooit werden gecontroleerd. De behandeling met cysteamine werd bij de meeste patiënten ook inadequaat voorgeschreven (of 2 tot 3 keer per dag, terwijl 4 keer per dag aanbevolen is; of de dosis werd niet aangepast op basis van een verhoogd cystine gehalte in de witte bloedcellen).

Resultaten van deze evaluatie hebben ons gemotiveerd om een multidisciplinaire polikliniek voor cystinose patiënten in UMC St Radboud te organiseren. Deze polikliniek functioneert momenteel sinds 2 jaar en wordt op prijs gesteld door de patiënten en de behandelende nefrologen, die de eindverantwoordelijkheid over hun patiënten blijven dragen.

Behandeling van proteïnurie

De achteruitgang van de nierfunctie bij cystinose wordt geassocieerd met het ontwikkelen van toenemende tubulo-interstitiële schade [Gubler et al. 1999]. Het mechanisme waardoor cystine stapeling nierinsufficiëntie veroorzaakt is onbekend. Albuminurie, die bij alle cystinose patiënten aanwezig is, kan bij het ontstaan van de interstitiële fibrose een rol spelen. In het **Hoofdstuk 5** hebben wij bij 5 patiënten met Fanconi syndroom aangetoond dat de albuminurie met ~40% verlaagd kan worden door de behandeling met een ACE-remmer enalapril (0,15 mg/kg eenmaal daags). De excretie van het lage moleculair gewicht eiwit α-1 microglobuline onder enalapril bleef onveranderd. Dit ondersteunt de opvatting dat albuminurie bij cystinose (gedeeltelijk) glomerulair is. Vermindering van de albuminurie kan de achteruitgang van de nierfunctie bij cystinose vertragen zoals het bij verschillende andere nierziekten werd aangetoond [Ruggenenti et al. 2001]. Enalapril veroorzaakte het dalen van systolische bloeddruk bij alle patiënten en klachten van hypotensie bij 2/5 patiënten. De kreatinine klaring daalde van mediaan 48 tot 45 ml/min/1,73 m^2, maar steeg terug naar de oorspronkelijke waarde na het stoppen van enalapril. Het is mogelijk dat de behandeling met de angiotensine II receptor antagonisten een minder uitgesproken bloeddrukdalend effect zou hebben en daarom beter door cystinose patiënten zal worden verdragen [Gansevoort et al. 1999].

Verbetering van de effectiviteit van cysteamine behandeling

Cysteamine kan het cystine gehalte in de granulocyt bij cystinose met 80-90% verlagen, maar helaas kan dit medicijn bij de meeste patiënten de achteruitgang van de nierfunctie en het optreden van de extra-renale complicaties niet voorkomen. In **Hoofdstukken 6 en 7** hebben wij de mogelijke redenen van het onbevredigende effect van cysteamine behandeling onderzocht.

Eerst hebben wij vermoed dat cysteamine als klein molecuul (MW 77,14) bij patiënten met het Fanconi syndroom met de urine verloren kan gaan. Zoals beschreven in **Hoofdstuk 6**, was dit niet het geval, gezien minder dan 1% van de ingenomen dosis met urine werd uitgescheiden.

Verder hebben wij verondersteld dat de inadequate cysteamine toediening verantwoordelijk kan zijn voor het falen van de behandeling bij sommige patiënten (**Hoofdstuk 7**). Om deze hypothese te testen, hebben wij Nederlandse cystinose patiënten gevraagd om een vragenlijst over hun dagelijkse schema van cysteamine toediening in te vullen. De meeste patiënten (17 van 22) nemen cysteamine alleen overdag met een nachtpauze van ~ 9 uur, terwijl volgens de pharmacodynamische gegevens een toediening elke 6 uur aanbevolen is. Bij 11 patiënten hebben wij het ochtend cystine gehalte in de granulocyt gemeten 6 uur en 9 uur na de laatste cysteamine dosis 's avonds. Na 9 uur nachtpauze was het cystine gehalte in de granulocyt significant hoger in vergelijking met 6 uur nachtpauze (0,73±0,81 versus 0,44±0,52 nmol/mg eiwit, $p<0,05$). Ondanks het feit dat het nog niet bekend is of het cystine gehalte in de granulocyt de stapeling van cystine in de andere weefsels adequaat weerspiegelt, raden wij alle cystinose patiënten aan om cysteamine elke 6 uur in te nemen, ook gedurende de nacht.

Samenvattend, naar aanleiding van de resultaten van onze studies, adviseren wij als optimale zorg van cystinose patiënten:

1. Een zo vroeg mogelijk diagnose van cystinose door het bepalen van het cystine gehalte in de granulocyt en het onmiddellijk starten van cysteamine behandeling na het stellen van de diagnose.
2. Het gebruik van de granulocyt voor de monitoring van cysteamine behandeling (5 à 6 uur na de laatste cysteamine inname moet het cystine gehalte in de granulocyt < 0,5 nmol/mg eiwit zijn).
3. Bevestiging van de diagnose van cystinose door de analyse van het *CTNS* gen (dit is mogelijk in het laboratorium van DNA diagnostiek, UMC St Radboud, Nijmegen).
4. Multidisciplinaire aanpak voor de behandeling van met name volwassen cystinose patiënten, die behalve nierziekte ook extra-renale orgaan aandoeningen vertonen.
5. Toediening van de ACE inhibitoren of angiotensine II receptor antagonisten bij patiënten met albuminurie onder controle van de bloeddruk en kreatinine klaring (vereist verdere studies).
6. Toediening van cysteamine elke 6 uur inclusief 's nachts.

Nieuwe inzichten in de pathogenese van cystinose

In het tweede deel van ons werk (**Hoofdstukken 8-11**) hebben wij via celbiologische experimenten nieuwe inzichten in het mechanisme van celschade bij cystinose proberen te verkrijgen.

Een patiënt beschreven in het **Hoofdstuk 8** heeft een geleidelijke achteruitgang van proximale tubulus functie vertoond met alleen milde aminoacidurie op de leeftijd van 3 weken en een compleet Fanconi syndroom op de leeftijd van 6 maanden. De verschillende gevoeligheid van de apicale proximale tubulus transporters voor cystine stapeling is mogelijk in strijd met de algemeen aanvaarde hypothese van ATP synthese tekort als het belangrijkste pathogenetisch mechanisme bij cystinose.

Studie van energie metabolisme in cystinose

Omdat geen dier- of celmodel van cystinose beschikbaar was, werd de pathogenese van de ziekte voorheen bestudeerd in proximale tubulus cellen geladen met cystine dimethyl ester (CDME). CDME lading resulteerde in de inhibitie van proximale tubulus transport vergelijkbaar met een Fanconi syndroom bij cystinose. In dit model werd een uitgesproken daling van de mitochondriële ATP productie aangetoond. Er werd aangenomen dat ATP tekort is het onderliggende mechanisme was van verstoorde renale absorptie [Foreman et al. 1987, Salmon et al. 1990, Coor et al. 1991, Ben-Nun et al. 1993, Sakarcan et al. 1992, Foreman et al. 1995, Baum 1998]. Tot nu toe werd de storing van energie metabolisme in humane weefsels van cystinose patiënten niet aangetoond.

Wij hebben het energie metabolisme in de fibroblasten van cystinose patiënten met een bekende mutatie in het *CTNS* gen uitvoerig bestudeerd (**Hoofdstuk 9**). De fibroblasten werden gekozen als cel model omdat zij makkelijk beschikbaar zijn via een huidbiopt en zij tevens in celkweek cystine stapelen. Bovendien heeft onze groep een uitgebreide ervaring met het bestuderen van mitochondriële functies in primaire fibroblasten, die bij patiënten met mitochondriële ziekten verstoorde ATP productie en deficiënte oxidatieve phosphorylatie (OXPHOS) vertonen [Visch et al. 2004, Loeffen et al. 2000, Ugalde et al. 2004]. Het intracellulaire ATP gehalte was significant verlaagd in fibroblasten van cystinose patiënten (mediaan (range): 37,3 (26,9-55,0) versus 51,5 (44,7-58,5) nmol/mg eiwit bij controles, $p<0,05$). Omdat het ATP gehalte in de fibroblast door de celkweek beïnvloed kon worden, hebben wij het intracellulair ATP ook bepaald in de granulocyten direct afkomstig van cystinose patiënten en gezonde controles. In de granulocyten van patiënten was het ATP gehalte significant verlaagd in vergelijking met controles (mediaan, range: 317 (149-1141) versus 557 (207-1101) nmol/mg eiwit, $p<0,05$).

Het intracellulaire ATP gehalte is het resultaat van de ATP synthese door glycolyse en/of mitochondriële OXPHOS en ATP verbruik. Verminderde productie, verhoogd verbruik of beide factoren kunnen de verlaging van het ATP gehalte veroorzaken. Wij hebben onze studies geconcentreerd op de mitochondriële ATP productie omdat in het CDME ladingsmodel van cystinose een mogelijk defect van complex I werd aangetoond [Foreman et al. 1995]. De activiteit van de mitochondriële ademhalingsketen enzymcomplexen in mitochondriële verrijkte celfracties en bradykinin- gestimuleerde maximale ATP productie in intacte cellen waren allen normaal in cystinotische fibroblasten. ATP verlaging had geen functioneel effect op de activiteit van Na,K ATP-ase, gemeten met Rb^+ influx.

Samenvattend, heeft onze studie geen storing van energie genererend vermogen in de cystinotische fibroblasten aangetoond, wat niet overeenkomt met de hypothese gepostuleerd in het CDME ladingsmodel van cystinose. De andere mogelijke verklaringen van ATP verlaging zoals de inhibitie van de glycolyse of een verhoogd ATP verbruik moeten verder worden bestudeerd.

Studie van het glutathione metabolisme

Naast het bestuderen van de mitochondriële ATP productie, hebben wij gezocht naar een andere verklaring voor de verstoorde celfunctie bij cystinose.

De stoornis in het glutathione (GSH) metabolisme was een mogelijkheid. Glutathione is een tripeptide, gesynthetiseerd uit cysteïne, glutamaat en glycine. GSH functioneert als een belangrijke redox buffer en verdedigt de cel tegen zuurstof radicalen (ROS). Cystine stapeling in het lysosoom zou kunnen leiden tot cysteïne tekort in het cytosol, omdat cystine in het cytosol tot cysteïne wordt gereduceerd [Gahl et al. 2001].

Bij patiënten met cystinose, onbehandeld met cysteamine, werd een verhoogde excretie van 5-oxoproline aangetoond, vergelijkbaar met patiënten met GSH synthetase deficiëntie [Larsson et al. 2000]. Het starten van de cysteamine behandeling resulteerde in de normalisatie van de 5-oxoproline excretie [Rizzo et al. 1999]. Gezien 5-oxoprolinurie niet werd aangetoond bij patiënten met een idiopatisch Fanconi syndroom, lijkt deze afwijking specifiek voor cystinose te zijn. Wij hebben de intracellulaire GSH status in cystinotische fibroblasten en de granulocyten bestudeerd (**Hoofdstuk 10**) en vonden een normaal totaal GSH en vrij cysteïne gehalte in beide celsoorten. Opvallend was het verhoogd gehalte van glutathione disulfide (GSSG) in de fibroblasten en granulocyten van cystinose patiënten in vergelijking met controles (mediaan (range) in fibroblasten: 0,7 (0,5-1,7) nmol/mg eiwit versus 0,3 (0,2-0,9) bij controles; in de granulocyt: 0,9 (0,3-1,8) nmol/mg eiwit versus 0,3 (0,2-0,4) in controles). Later werd dit verhoogde GSSG ook in de proxi-

male tubulus cellen van cystinose patiënten aangetoond (**Hoofdstuk 11**). Een stijging van GSSG werd ook geobserveerd door Chol et al. [2004] in geïmmortaliseerde cystinotische fibroblast cellijnen. Het gestegen GSSG gehalte in 3 cystinotische celsoorten kan een verstoorde peroxidatie en redox status in de cellen reflecteren en wijst mogelijk op een nieuw pathogenetisch mechanisme bij cystinose dat verdere studie vereist.

In de cel reageert GSH met verschillende stoffen (zoals medicijnen en xenobiotica) die verder als glutathione-S conjugaten uit de proximale tubuluscel worden geëxporteerd [Wright et al. 2004, Lash 2005]. Geremd export van GSSG of GSH-conjugaten kan eventueel ook een verstoorde GSH status in de cystinotische cellen veroorzaken (Figuur 1, pagina 136).

GSSG kan pro-apoptotische protein kinase C δ activeren [Chu et al. 2003] en zou een pro-apoptotische stimulus bij cystinose kunnen zijn [Park et al. 2002, Park et al. 2005].

Samenvattend hebben wij een verstoorde status van GSH met een verhoging van GSSG in cystinotische fibroblasten, granulocyten en proximale tubulus kunnen vaststellen. Verdere studie moet oorzaak en consequenties van deze observatie onderzoeken.

Ontwikkelen van proximale tubulus celmodel van cystinose

Cystinotische fibroblasten en granulocyten zijn geen ideaal model voor de studie van de pathogenese bij cystinose ondanks het feit dat ze cystine stapelen. Bij patiënten met cystinose vertonen deze cellen geen duidelijke tekens van de ziekte. Bovendien gebruiken beide celsoorten *in vivo* met name anaërobe glycolyse voor de ATP productie [Robinson 1996, Yang et al. 2004]. Proximale tubulus cellen (PTEC) zijn de eerste cellen die klinische tekens van cystinose vertonen en *in vivo* bijna volledig afhankelijk zijn van het oxidatieve metabolisme. Deze cellen zouden uiteraard het meest geschikte *in vitro* cel model voor cystinose zijn. Omdat nierweefsel van cystinose patiënten niet wordt afgenomen voor het stellen van de diagnose, werd getracht om cystinotische niercellen uit urine te kweken [Racussen et al. 1995, Laube et al. 2005].

Het is ons gelukt om proximale tubulus cellen uit urine van 9 cystinose patiënten en 10 controles te isoleren (**Hoofdstuk 11**). Omdat primaire PTEC stoppen met delen na 4 à 6 passages, waren wij genoodzaakt deze cellen te immortaliseren. De cellen werden getransfecteerd met humane papilloma virus E6/E7 genen (HPV E6/E7), die vroeger reeds gebruikt werden voor de immortalisatie van de humane proximale tubulus cellijn HK-2 [Ryan et al. 1994]. De verkregen cellijnen vertoonden baso-laterale polarisatie, enzymatische activiteit van alkalische fosfatase en positieve kleuring met een antilichamen tegen aminopeptidase M en tegen de megaline, wat hun proximale tubulus oorsprong

bevestigt. Helaas was het cystine gehalte veel lager in de met HPV E6/E7 geïmmortaliseerde cystinotische PTEC, in vergelijking met *in vivo* cystinotisch nierweefsel (0,8±0,9 nmol cystine/mg eiwit versus 0,1±0,01 bij controle PTEC ($p<0,05$) versus >15 nmol cystine/mg nierweefsel bij patiënten met cystinose).

Vergelijkbaar met cystinotische fibroblasten en granulocyten, vertoonde cystinotische PTEC een verhoogd GSSG gehalte (1,2±0,8 nmol/mg eiwit versus 0,3±0,2 bij controles, $p<0,05$).

Omdat het intracellulaire ATP normaal was in cystinotische PTEC, werden geen studies van mitochondriële ATP productie in deze cellen uitgevoerd.

Concluderend, hebben wij een geringe verlaging van het ATP gehalte in de cystinotische fibroblasten en de granulocyten aangetoond. Geen defect in de mitochondriële ATP productie kon worden aangetoond in de cystinotische fibroblasten. Verstoorde GSH status met een verhoging van GSSG gehalte in cystinotische cellen wijst mogelijk op een nieuw pathogenetisch mechanisme. PTEC cellen kunnen uit urine worden verkregen. De immortalisatie met HPV E6/E7 resulteerde in geringe cystine stapeling.

Op basis van onze en andere studies, is onze huidige opvatting van de pathogenese van cystinose samengevat in (zie Figuur 1, pagina 136).

Richtingen van toekomstig onderzoek

Zoals na het lezen van mijn proefschrift duidelijk is, is met name de pathogenese van cystinose nog niet opgehelderd en moet verder onderzoek plaats vinden.

Volgende onderzoeksrichtingen kunnen worden voorgesteld:
Het proximale tubulus celmodel van cystinose moet verbeterd worden. Onlangs konden wij met de hulp van de groep uit Bristol (Dr. M. Saleem, Dr. P. Mathiesson) het probleem van onvoldoende cystine stapeling *in vitro* oplossen. De immortalisatie van primaire PTEC met een humaan Simian virus met een temperatuur gevoelig allele (SV 40 T) resulteerde in celproliferatie bij 33°C en proliferatie stop bij 37°C. Onze preliminaire studie heeft een spectaculaire cystine stapeling in cystinotische PTEC na 10 dagen differentiatie bij 37°C aangetoond (tot 8 nmol cystine/mg eiwit). Om zuivere PTEC te verkrijgen moeten geïmmortaliseerde PTEC gecloneerd worden om de contaminatie door andere niercellen zoals glomerulaire podocyten en cellen van het distale nephron te voorkomen.

In de cystinotische en controle PTEC moet het energie metabolisme bestudeerd worden. Omdat in kweek PTEC vooral via glycolyse ATP produceren [Felder et al. 2002], moe-

ten de cellen gestimuleerd worden om OXPHOS te gebruiken. In preliminaire experimenten kon dat gerealiseerd worden door de cellen met een inhibitor van glycolyse (2-deoxyglucose) te incuberen.

ROS productie samen met de activiteit van GSH redox cyclus enzymen moet bestudeerd worden in rustcondities en in condities van verhoogde oxidatieve stress. Een verstoorde GSH status kan apoptose stimuleren, wat ook verder onderzocht kan worden. De GSH synthese kan onderzocht worden door het bestuderen van de activiteit van de enzymen van γ-glutamyl cyclus via het gebruik van stabiele isotopen.

Transport van GSSG, GSH en GSH-S conjugaten in cystinotische en controle PTEC is ons inziens een andere veelbelovende onderzoeksrichting.

Verdere verbetering van de medische zorg van cystinose patiënten

Het is zeer moeilijk om de behandeling met cysteamine vol te houden, omdat de medicatie elke 6 uur ingenomen moet worden. Bijwerkingen van cysteamine zoals slechte adem en gastro-intestinale klachten hebben ook een negatief effect op de therapietrouw. Het ontwikkelen van preparaten met gereguleerde afgifte of cysteamine pro-drug zou het probleem van frequente toediening kunnen oplossen. De oorzaken van de slechte adem moet verder onderzocht worden om hiervoor oplossingen te kunnen zoeken. Gastro-intestinale klachten kunnen met waterstofionen secretie inhibitoren (PPI) behandeld worden [Dohil et al. 2003], maar het is niet bekend of deze medicijnen de opname van cysteamine beïnvloeden.

De factoren betrokken bij het ontstaan van interstitiële fibrose bij cystinose moeten verder bestudeerd worden. Cystinotische PTEC kunnen daarvoor worden gebruikt.

Wanneer het mechanisme bekend zou worden waardoor cystine stapeling een stoornis van de celfunctie veroorzaakt, verwachten wij dat er andere behandelingen naast cysteamine zullen worden toegepast.

CHAPTER 14

Обсуждение полученных результатов. Заключение

Цистиноз – редкое лизосомальное заболевание, главным проявлением которого является накопление цистина внутри лизосом. Болезнь обычно проявляется дисфункцией проксимальных почечных канальцев, известной как синдром Фанкони, и прогрессирующей к терминальной стадии почечной недостаточности (ESRD). Кроме почек, лизосомальный цистин накапливается во всех тканях, вызывая повреждение многих органов, что делает цистиноз системным метаболическим заболеванием. Аминотиол цистамин в настоящее время является единственным лекарственным препаратом, уменьшающим содержание цистина, который замедляет ухудшение почечной функции и замедляет развитие экстраренальных осложнений. У больных с цистинозом терапия цистеамином должна быть назначена как можно в раннем возрасте и продолжена в течение всей жизни, включая период после трансплантации почки (для защиты экстраренальных органов) [Gahl и др. 2001, Gahl и др. 2002, Levtchenko и др. 2004]. Несмотря на значительный прогресс в последние три десятилетия, включая возможность лечения цистеамином, у большинства больных с цистинозом всё же развивается ESRD и экстраренальные осложнения. Патогенез клеточной дисфункции, вызванный накоплением цистина, до настоящего времени остается неизвестен. В своих исследованиях, представленных в настоящей работе, мы ставили целью улучшение диагностики и лечения больных цистинозом (**Главы 2 - 7**). В то же время мы проводили биохимические и моллекулярные исследования для изучения патогенеза цистиноза (**Главы 8 - 11**).

Улучшение диагностики и лечения цистиноза

Диагноз цистиноза

Определение цистина

Для раннего начала терапии цистеамином необходима ранняя постановка диагноза цистиноза. Для этого нужно определение в тканях пациента повышенного содержания цистина. Патогномоничным является также обнаружение кристаллов цистина в роговой оболочке глаза, которые проявляются только после первого года жизни. Клетки крови наиболее доступны для определения цистина и используются для постановки диагноза. Для мониторинга терапии цистеамином также требуется определение содержания цистина в клетках крови. Традиционно цистин определяли в смеси лейкоцитов (ML), несмотря на то, что он преимущественно накапливается в полиморфноядерных лейкоцитах (PMN) и в моноцитах , а не в лимфоцитах [Schulman и др. 1970]. Так как

количество цистина выражается в nmol/mg белка, содержание в ML, содержащем значительный процент лимфоцитов, оказывается ниже, чем количество, определяемое в PMN. В **Главе 2** мы описываем наши исследования по сравнению уровня цистина в ML и PMN, произведенных одновременно методом HPLC. На основании наших данных, мы рекомендуем определять уровень цистина в PMN, а не в MN, т.к. это позволяет увеличить чувствительность определения цистина при постановке диагноза, что важно для своевременного назначения терапии цистеамином, и обеспечивает более правильную оценку содержания цистина при мониторинге лечения цистеамином.

Молекулярный анализ гена цистиноза

По нашему мнению, диагноз цистиноза следует подтверждать у всех больных дополнительным методом, кроме определения цистина в PMN. Ошибки при изоляции PMN или транспортировке могут привести к увеличению уровня цистина в результате окисления внутриклеточного цистеина, который приблизительно в 100 раз превышает таковой в цитозоле лейкоцитов по сравнению с цистином [Graaf-Hess и др. 1999]. В 1998 году был установлен ген *CTNS* (17p13), кодирующий лизосомальный белок цистинозин, транспортирующий цистин из лизозом [Town и др. 1998]. В **главе 3** [Heil и др. 2001] мы описываем улучшенный метод для определения распространенной в популяции европейцев 57-kb делеции гена *CTNS*, которая была обнаружена у 59 % (13/22) из всех тестированных аллелей. Оставшиеся аллели были подвержены молекулярному анализу и выявили другие ранее известные мутации во всех, кроме трех аллелей. В настоящее время разработанный нами молекулярный анализ используется для подтверждения диагноза цистиноза в Голландии, и может быть применен для пренатальной диагностики и подтверждения гетерозиготности.

Лечение цистиноза

Мультисистемный подход

Цистиноз является мультисистемным заболеванием, вначале клинически проявляющимся поражением почек и роговой оболочки. При невозможности диализной терапии и трансплантации почки больные цистинозом обычно умирают до 10 лет, при этом экстраренальные проявления заболевания преимущественно не обнаруживаются. Более длительное выживание больных цистинозом благодаря трансплантации почек выявило поражение эндокринных органов (гипотиреоидизм, сахарный диабет, мужской гипогонадизм, вакуолярную миопатию, ретинопатию, поражение центральной и

периферической нервной системы, поражение печени и экзокринной функции поджелудочной железы), обычно развивающееся в возрасте после 10 лет [Gahl и др. 2002]. Это означает, что врачи, занимающиеся лечением взрослых пациентов с цистинозом (нефрологи-терапевты) сталкиваются с экстраренальными осложнениями цистиноза. Поскольку цистиноз является редким заболеванием (например, в Нидерландах имеется всего 40 пациентов с цистинозом, среди них 10 взрослых), нефрологи, которые лечат взрослых пациентов, не имеют опыта в лечении этих больных. Другая ситуация складывается в детской нефрологии, которая сконцентрирована в нескольких университетских нефрологических центрах. Для того, чтобы оценить адекватность лечения десяти нидерландских взрослых пациентов с цистинозом, мы тщательно изучили их истории болезни (**Глава 4**). Было выявлено, что офтальмологическое обследование никогда не проводилось у двух пациентов, тиреоидная функция не проверялась у других двух пациентов, и еще у двух пациентов не проводился контроль сахара крови. Только 7 нидерландских взрослых пациентов лечились цистеамином (у троих из них препарат был назначен в двух или трех дозах ежедневно, в то время как необходимо получение лекарства 4 раза в сутки). Содержание цистина в лейкоцитах находилось в рекомендованных границах только у трех пациентов. Неадекватное лечение нидерландских пациентов с цистинозом [Geelen и др. 2003] явилось поводом для организации поликлинического приема для взрослых голландских пациентов, который функционирует в Медицинском Центре Радбауд Нэймегена в течение двух лет. В течение одного дня пациенты с цистинозом обследуются опытным специалистом по метаболическим заболеваниям, офтальмологом, невропатологом (те, у которых есть соответствующие жалобы), и клиническим генетиком.

Дополнительно организована помощь социального работника для всех пациентов. Кровь для определения цистина берут через 5 – 6 часов после приёма цистеамина и подвергают немедленному анализу. Рекомендации направляются нефрологу, который остается ведущим специалистом при лечении этих пациентов. В течение последних двух лет мы выявили двух до этого невыявленных взрослых пациентов с цистинозом и одного пациента, у которого диагноз цистиноза был снят после 20 лет терапии цистеамином.

Лечение протеинурии

Снижение почечной функции у больных с цистинозом сопровождается прогрессивным развитием поражения интерстициальной ткани почек, включающих атрофию почечных канальцев и фиброз [Gubler и др. 1999]. Патогенетические механизмы, путем которых накопление цистина приводит к падению функции почек, неизвестны. Альбуминурия, неизменно наблюдаемая у больных с цистинозом, может быть одним из факторов,

вызывающих интестиальное поражение почек. В **Главе 5 [**Levtchenko и др. 2003] мы показали на 5 пациентах с синдромом Фанкони, что альбуминурия может быть уменьшена на 40 % при лечении ингибитором ангиотензин превращающего фермента (ACE) – эналаприлом (0,15 mg/kg ежедневно). Интересно, что лечение эналаприлом не изменяет повышенную экскрецию низкомолекулярного белка α-1 микроглобулина, что подтверждает гломерулярное происхождение альбуминурии. Снижение альбуминурии может замедлить развитие почечной недостаточности при цистинозе, как это было показано при других заболеваниях почек [Ruggenenti и др. 2001]. Неудивительно, что лечение эналоприлом сопровождалось снижением систолического кровяного давления и жалобами на низкое давление у двух из пяти человек. Клиренс креатинина снижался со средних 48 до 45 ml/min/1.73 м^2 и возвращался к прежним значениям после отмены эналоприла.

Повышение эффективности терапии цистеамином

Цистеамин может снижать содержание цистина в лейкоцитах на 80-90 %, но только замедляет снижение функции почек и проявление экстраренальных осложнений у большинства пациентов. Два исследования, описанных в **Главах 6 и 7,** направлены на повышение эффективности терапии цистеамином.

Вначале мы предположили, что цистеамин, являясь малой молекулой (мол. вес 77.14), может выделяться с мочой у больных с цистинозом, страдающих от синдрома Фанкони. Как показано в **Главе 6** [Levtchenko и др. 2002], причина была не в этом, так как менее чем 1 % введенного цистеамина экскретировалась с мочой. Далее мы предположили, что неправильное введение цистеамина может быть причиной неуспешной терапии у некоторых пациентов (**Глава 7**). Для проверки этого предположения мы выявили режим ежедневного приема цистеамина у нидерландских пациентов. Выяснилось, что только 5 из 22 пациентов принимали лекарство каждые 6 часов, как им было рекомендовано в связи с фармакодинамическими данными препарата. Большинство пациентов принимали цистеамин только в течение дня, что приводило к ночной паузе приблизительно в 9 часов между последним вечерним и первым утренним приемом. В 11 случаях утреннее содержание цистина в PMN было выше значения 0.5 nmo/mg, т.к. прошло 9 часов после вечернего приема (0.73 ± 0.81 по сравнению с 0.44 ± 0.52 nmo цистина /mg белка через 6 часов после последнего вечернего приема цистеамина, $p < 0.05$). На основании этих данных мы настойчиво рекомендуем больным с цистинозом принимать цистеамин каждые 6 часов в течение суток.

Основываясь на результатах наших исследований, для получения оптимального результата лечения цистиноза мы рекомендуем:

1. Максимально раннее установление диагноза цистиноза методом исследования содержания цистина в полиморфноядерных лейкоцитах для немедленного назначения лечения цистеамином.
2. Использовать определение уровеня цистина в полиморфноядерных лейкоцитах для мониторинга цистеаминовой терапии (через 5 – 6 часов после последнего введения цистеамина содержание цистина в PMN кдетках должно быть ниже, чем 0.5 nmol цистина/mg белка.
3. Подтверждать диагноз цистиноза молекулярным анализом *CTNS* гена.
4. Мультидисциплинарный подход к лечению взрослых больных цистинозом, страдающих от экстраренальных осложнений этого заболевания.
5. Введение ингибиторов ACE, или блокаторов рецептора ангиотензин II больным с альбуминурией при строгом контроле кровяного давления и креатинина сыворотки крови.
6. Введение цистеамина каждые 6 часов, включая ночные часы.

Иследование патогенеза цистиноза

Вторая часть нашей работы (**Главы 8 – 11**) включает исследования патогенетических механизмов клеточной дисфункции при цистинозе.

В описании казуса, представленной в **Главе 8**, показано постепенное развитие дисфункции проксимальных почечных канальцев, начавшейся с легкой аминоацидурии, выявленной в возрасте 3 недель, и приведшей к выраженному синдрому Фанкони в возрасте 6 месяцев. Различная чувствительность транспортных белков проксимальных почечных канальцев к накоплению цистина, по нашему мнению, противоречит общепринятой гипотезе о нарушении синтеза АТФ, являющейся основным звеном в патогенезе цистиноза.

Изучение энергетического метаболизма при цистинозе

Поскольку ранее не существовало модели цистиноза на животных или клетках, патогенез заболевания изучали путем культивирования проксимальных почечных канальцев с эфиром цистина (CDME). Культивирование с CDME приводило к ингибированию транспорта в клетках проксимальных почечных канальцев, подобному синдрому Фанкони при цистинозе. На этой модели было продемонстрировано выраженное снижение синтеза АТФ в митохондриях, которое считали основным патогенетическим механизмом цистиноза [Foreman и др. 1987, Salmon и др. 1990, Coor и др. 1991, Sakarcan и др. 1992, Ben Nun и др.

1993, Foreman и др. 1995, Baum 1998]. До настоящего времени энергетический метаболизм у больных цистинозом не был исследован.

Мы исследовали синтез АТФ в первичных культивированных фибробластах от больных с цистинозом с известными генетическими эффектами гена *CTNS* (**Глава 9**). Фибробласты больных накапливали цистин (в среднем 4.3 (2.7 – 5.5) против 0.2 (0.1 – 0.3) nmol/mg белка ($p < 0.01$) и содержали сниженное количество внутриклеточной АТФ по сравнению с контрольными фибробластами (в среднем 37.3 (26.9 – 55) против 51.5 (44.7 – 58.5) nmo/mg белка, $p < 0.05$).

Для подверждения снижения внутриклеточной АТФ при цистинозе, мы дополнительно измеряли содержание АТФ в PMN клетках, выделенных из крови больных и здоровых добровольцев. Аналогично снижению содержания АТФ в фибробластах цистинозных больных, в PMN клетках больных содержание АТФ по сравнению с контрольными было также снижено (среднее: 317 (149 – 1141) против 557 (207 – 1101) nmol/mg белка, $p < 0.05$).

Уровень внутриклеточной АТФ является результатом динамического процесса продукции АТФ (гликолиз в цитозоле и митохондриальное оксидативное фосфорилирование, OXPHOS) и потребления АТФ.

Снижение продукции АТФ, увеличение ее потребления, или комбинация обоих процессов может привести к снижению уровня внутриклеточной АТФ. Наши исследования были сосредоточены на способности митохондрий продуцировать АТФ, так как возможное снижение активности митохондриального комплекса I было продемонстрировано на CDME модели цистиноза [Foreman и др. 1995]. Следует отметить, что общая способность к продукции АТФ: активность комплексов дыхательной цепи в обогащенных митохондриями клеточных фракциях и стимулированная брадикинином продукция митохондриальной АТФ в цистинозных фибробластах находились на нормальном уровне. Интересно, что после ингибированного гликолиза 2-дезоксиглюкозой (DOG), или йодацетатом натрия (SIA), не выявлено различия между внутриклеточным содержанием АТФ в цистинозных и контрольных фибробластах, что дает возможность предположить изменение гликолиза при цистинозе. Для того, чтобы исследовать, сопровождается ли сниженный уровень АТФ функциональными изменениями в фибробластах, мы исследовали активность Na,K АТФ-азы. Это исследование не обнаружило различий между цистинозными и контрольными клетками, это позволяет допустить, что снижение АТФ в цистинозных клетках оставалось достаточным для нормальной функции Na,K АТФ-азного насоса.

В заключение нужно отметить: наше исследование показало, что снижение содержания АТФ в цистинозных клетках не является результатом снижения генерирующей способности митохондрий к синтезу АТФ, которое было установлено при изучении CDME

модели цистиноза. Возможное снижение активности гликолиза или увеличенное потребление АТФ требуют дальнейшего изучения.

Изучение метаболизма глютатиона

Поскольку не было обнаружено изменений митохондриальной генерации АТФ при цистинозе, мы искали альтернативные патогенетические механизмы этого заболевания. Некоторые теоретические данные указывали на то, что нарушение метаболизма глютатиона (GSH) могут играть свою роль в этом процессе. GSH - клеточный тиол, функционирующий как важный окислительно-восстановительный буфер, защищающий клетки от повреждения свободными радикалами. Глютатион синтезируется из цистеина, глютамата и глицина. Цистин, доставленный цистозином в цитозоль, окисляется в цистеин окислительными системами цитозоля [Gahl и др. 2001]. Теоретически интрализосомальное накопление цистина при цистинозе может привести к дефициту цистеина в цитозоле, что может лимитировать синтез GSH. В клиническом исследовании повышенная экскреция 5-оксипролина была продемонстрирована у цистинозных больных с синдромом Фанкони, нелеченых цистеамином, в отличие от нормальной экскреции 5-оксипролина у больных с идиопатическим синдромом Фанкони. Это наблюдение подтверждает роль нарушения гомеостаза глютатиона, специфическое для цистинозных больных, нелеченых цистеамином [Rizzo и др. 1999]. Мы изучали содержание внутриклеточного GSH в фибробластах и PMN немедленно после выделения из крови цистинозных больных (**Глава 10)** и обнаружили, что содержание общего GSH и свободного цистеина были в пределах нормы в цистинозных клетках [Levtchenko и др. 2005]. В цистинозных фибробластах и PMN клетках показано значительное увеличение количества глютатион-дисульфида (окисленный GSH или GSSG) (среднее значение в фибробластах: 0.7 (0.5 – 1.7) nmol/mg белка против 0.3 (0.2 – 0.9) в контрольных клетках; в клетках PMN: 0.9 (0.3 – 1.8) nmol/mg белка против 0.3 (0.2 – 0.4) в контрольных клетках ($p<0.05$). Повышенное содержание GSSG было также обнаружено в проксимальных клетках почечных канальцев, выделенных из мочи цистинозных больных **(Глава 11)**. Увеличение GSSG, обнаруженное в трех типах клеток у больных цистинозом, возможно указывает на новый патогенетический механизм цистиноза. Повышенная продукция кислородных радикалов и/или нарушение транспорта GSSG из клеток может быть причиной повышения GSSG. GSSG может активировать протеинкиназу C δ [Chu и др. 2003], и являться стимулом апоптоза при цистинозе [Park и др. 2002, Park и др. 2005].

В заключение следует сказать, что причины и последствия изменения статуса GSH с

возрастанием GSSG, выявленное в цистинозных фибробластах, PMN и клетках проксимальных почечных канальцев требует дальнейшего изучения.

Разработка модели клеток проксимальных канальцев при цистинозе

Цистинозные фибробласты и PMN клетки, несмотря на свою способность к интенсивному накоплению цистина, не представляют идеальной модели для изучения патогенеза цистиноза по двум причинам. Во-первых, фибробласты и цистинозные клетки цистинозных больных не имеют отчетливых признаков этого заболевания **in vivo**. Во-вторых, эти клеточные типы ***in vivo*** и ***in vitro*** для синтеза АТФ используют преимущественно анаэробный гликолиз [Robinson 1996, Yang и др. 2004]. Эпителиальные клетки почечных проксимальных канальцев (PTEC), которые преимущественно поражаются при цистинозе и **in vivo** почти полностью зависят от окислительного метаболизма, могли бы служить более подходящей **in vitro** моделью для исследования цистиноза. Так как почечная ткань при цистинозе обычно недоступна для исследования из-за того, что почечная биопсия не требуется для постановки диагноза, делались попытки культивировать почечные клетки, выделяемые с мочой [Racussen и др. 1995, Laube и др. 2004].

Нам удалось получить культуры клеток проксимальных канальцев у 9 больных с цистинозом и у 10 здоровых человек **(Глава 11).** Так как первичные PTEC прекращают деление после 4 – 6 пассажей, то необходимо проведение иммортализации для получения достаточного количества материала. Мы заражали клетки PTEC генами вируса папиломы человека E6/E7, которые ранее использовались для иммортализации хорошо изученных клеточных линий HK-2 проксимальных канальцев человека [Ryan и др. 1994]. Полученные нами линии клеток демонстрировали базо-латеральную поляризацию, активность щелочной фосфатазы, положительную иммунофлюоресценцию с антителами к аминопептидазе N и мегалину, что подтверждает их происхождение из проксимальных канальцев. К сожалению, иммортализованные цистинозные PTEC накапливали цистин в значительно меньшей степени по сравнению с цистинозной тканью почки ***in vivo***, возможно вследствие постоянной клеточной пролиферации в результате иммортализации (0.8 ± 0.9 nmol цистина/mg белка против 0.1 ± 0.1 в контроле $p < 0.05$), в то время как ***in vivo*** более 15 nmol цистина/mg мокрого веса ткани больных). Интересно, что в иммортализованных цистинозных PTEC обнаружен повышенный уровень GSSG, который также выявлен в цистинозных фибробластах и PMN клетках (1.2 ± 0.8 nmol/mg белка против 0.3 ± 0.2 в контроле, $p < 0.05$). Так как содержание внутриклеточного АТФ было нормальным в цистинозных PTEC, мы не производили исследования энергетического обмена в этих клетках.

В заключение, мы показали выраженное снижение уровня внутриклеточной АТФ в культивированных фибробластах и PMN клетках цистинозных больных. В цистинозных фибробластах продукция митохондриальной АТФ была в прелелах нормы в отличие от результатов, полученных при изучении CDME модели цистиноза. Повышенное содержание окисленного GSH в цистинозных клетках может указывать на наличие нового патогенетического механизма цистиноза. PTEC могут быть получены из мочи цистинозных больных и здоровых людей. Иммортализация цистинозных клеток с помощью HPV E6/E7 приводит к недостаточному накоплению цистина, которое не отражает накопление цистина в почке **in vivo**. На основании наших и других результатов мы представляем современное понимание патогенеза этого заболевания в рисунке 1 (стр. 136).

Направление дальнейших исследований

Дальнейшее изучение патогенеза цистиноза

1. Модель клеток проксимальных почечных канальцев должна быть улучшена. Недавно нам удалось решить проблему недостаточного накопления цистина в цистинозных PTEC иммортализованных HPV E6/E7 путем использования человеческого Simian Virus, содержащего аллель, чувствительный к температуре (SV40 T антиген), который ранее использовался для иммортализации подоцитов человека (Pavenstadt и др. 2003). Клетки, трансфектированные SV40 T вектором, пролиферируют при температуре 33^0C, но прекращают пролиферацию при 37^0C. Наши предварительные исследования обнаружили существенное накопление цистина в цистинозных PTEC после 10 дней дифференцировки при 37^0C (до 15 nmol цистина/mg белка). Так как клетки, культивированные из мочи, могут быть содержать гломерулярные подоциты и клетки канальцев дистального нефрона, полученные линии клеток следует субклонировать.
2. Следует изучить энергетическую способность (OXPHOS и гликолиз) и потребление АТФ в линиях цистинозных и контрольных PTEC. Так как в культуре PTEC аэробный обмен переходит на путь гликолиза [Felder и др. 2002], то гликолиз следует ингибировать. Наши предварительные данные показали значительное увеличение продукции митохондриальной АТФ после инкубации PTEC 2-дезоксиглюкозой, ингибирующей гликолиз.

3. Продукция свободных радикалов кислорода и активность окислительных ферментов GSH в покое и в условиях повышенного окислительного стресса следует изучить, т.к. ROS может играть роль в патогенезе цистиноза. Изменение обмена GSH могут активировать. Дефицит синтеза GSH можно показать при изучении активности ферментов γ-глютамил цикла со стабильными изотопами.
4. Транспорт GSSG в цистинозных PTEC – другое интересное направление, которое, возможно, объяснит механизм клеточной дисфункции при цистинозе.

Дальнейшее улучшение медицинской помощи больным цистинозом

1. Лечение цистеамином затруднительно, поскольку оно требует введения препаратов каждые 6 часов. Побочные эффекты этого препарата, такие как неприятный запах изо рта и желудочно-кишечные нарушения, вызывают негативное отношение к нему у больных. Разработка препаратов с медленным высвобождением цистеамина могло бы решить проблему необходимости частого введения препарата. Причина неприятного запаха, вызываемого цистеамином, должна быть в дальнейшем изучена для того, чтобы найти фармакологические или косметические пути ее решения. Желудочно-кишечные проблемы можно успешно лечить с помощью ингибиторов протонового насоса PPI [Dohil и др. 2003], однако, влияет ли PPI на фармакокинетику цистеамина при его оральном введении, еще должно быть изучено.
2. Факторы, влияющие на развитие интерстициального фиброза при цистинозе, также должны изучаться в дальнейшем. Для этой цели могут служить PTEC больных с цистинозом. Идентификация механизмов, ответственных за интерстициальные повреждения, приводящие к почечной недостаточности у цистинозных больных, может открыть новые терапевтические перспективы.
3. Когда патогенетические механизмы, связывающие накопление лизосомального цистина с нарушением функции клеток, станут известны, то, возможно, появятся другие, кроме введения цистеамина, пути лечения больных цистинозом.

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Abbreviations

ACD	Acid-citrate-dextrose
ACE	Angiotensin converting enzyme
AT II	Angiotensin II
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
CDME	Cystine dimethyl ester
COX	Cytochrome C oxidase
CS	Citrate synthase
CTNS	Cystinosis gene
2-DOG	2-deoxyglucose
DNA	Deoxyrobonucleic acid
EDTA	Ethylenenediaminetetra-acetic acid
GCS	γ-glutamylcysteine synthetase
GFR	Glomerular filtration rate
GS	GSH synthetase
GSH	Glutathione
GSSG	Glutathione disulfide or oxidized GSH
γ-glut-cys	γ-glutamylcysteine
HPLC	High performance liquid chromatography
IDDM	Insulin-dependent diabetes mellitus
α-1 MG	α-1 microglobulin
ML	Mixed leukocytes
MRP2/MRP4	Multidrug resistant proteins 2 and 4
OAT1/3	Organic anion transporters 1 and 3
OATP1	Organic anion transporting polypeptide 1
$2\text{-}OG^-$	2-oxoglutarate
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PMN	Polymorphonuclear leukocytes
PO	Podocytes
PTEC	Proximal tubular epithelial cells
SD	Standard deviation
SDCT-2	Sodium-dicarboxylate transporter 2
SE	Standard error
SIA	Sodium-iodoacetate
SOD	Superoxide dismutase
TSH	Thyroid-stimulating hormone

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Curriculum vitae

Elena Levtchenko werd geboren op 3 april 1965 te Malachovka, Rusland. Na het afronden van het middelbaar onderwijs in 1982, begon ze hetzelfde jaar met de studie Geneeskunde aan de 2de Russische Medische Staatsuniversiteit. Het artsexamen (cum laude) behaalde ze in juni 1988 en vanaf september 1988 begon ze met de opleiding kindergeneeskunde/kindernefrologie aan het Instituut voor Kindergeneeskunde van de Russische Academie van Medische Wetenschappen (opleiders Prof. Dr. Naumova, Prof. Dr. Sergeeva). In 1991 werd ze toelaten tot het Master Programma van Medische en Farmaceutische Wetenschappen aan de Vrije Universiteit Brussel (hoofd Prof. Dr. Pipeleers). Na het verkrijgen van de graad "Master in Medische en Farmaceutische Wetenschappen" in september 1993, begon ze met een opleiding kindergeneeskunde (opleiders Prof. Dr. Loeb, Prof. Dr. Vanderplas) aan de Vrije Universiteit Brussel, welke werd afgerond in september 1999. Tijdens de specialisatie tot kindernefroloog in UMC St Radboud te Nijmegen (opleider Prof. Dr. Monnens) begon ze met het promotieonderzoek, beschreven in dit proefschrift, "Cystinose: betere behandeling en inzicht in de pathogenese" onder leiding van Prof. Dr. Monnens, Dr. Blom en Dr. van den Heuvel en gesubsidieerd door de Nierstichting Nederland. Momenteel werkt ze als kinderarts-nefroloog op de afdeling kindergeneeskunde in het UMC St Radboud te Nijmegen (hoofd Prof. Dr. de Groot). In 2004 ontving ze een Dr. Frije Stipendium van UMC St Radboud. Elena is getrouwd met Aimé van Gucht en heeft twee kinderen, Anja en Philippe.

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