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.

Keywords: cystinosis; fibroblasts; glutathione; glutathione disulfide; polymorphonuclear cells

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. Extra-renal 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 [1]. Mutations in CTNS have been demonstrated in all three clinical forms of cystinosis [2].

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.
dimethyl ester, mimicking cystine accumulation in cystinosis [3]. 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 [1]. Theoretically, the intralysosomal cystine accumulation in cystinosis can result in the cystolic deficit of cysteine. Cysteine together with glutamate and glycine 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 rate-limiting 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 γ-glutamylcysteine synthetase (γ-GCS), resulting in the formation of γ-glutamylcysteine (γ-GC). 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) [4]. 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 [5]. Additionally, the presence of amino-aciduria and metabolic acidosis in patients with a deficiency of γ-GCS suggests that GSH deficiency might result in renal proximal tubular dysfunction [4].

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 H₂O₂ and lipid peroxides, defending cells against reactive oxygen metabolites. Glutathione reductase, using the NADPH oxidase system, is necessary to regenerate GSH [6].

GSH depletion results in mitochondrial dysfunction in several cellular and animal models [7,8] 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 [9]. An increased superoxide production has been demonstrated by chemiluminescence assay in isolated polymorphonuclear (PMN) cells and mononuclear phagocytes from cystinotic children [10].

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 (Figure 1).

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/1.73 m² (median 57) and eight patients had a functioning renal graft (GFR 20–160 ml/min/1.73 m², 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 custom-made 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 [12]. Approximately 5 × 10⁶ 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 N₂ and stored at −80°C.
Glutathione status in cystinotic cells

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 N₂ and stored at −80°C [12].

Determination of thiol-amino acids and small thiol-peptides

Preparation of cell extracts was performed as described previously [12]. Briefly, frozen cell pellets were resuspended in ice on sodium phosphate buffer (pH 7.2) with 1 mM N-ethylmaleimide (NEM) (oxidized thiol) or without NEM (total thiol) followed by sonication. Using NEM, binding all free -SH groups, does not allow us to distinguish between cystolic disulfides and thiol 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) [11]. 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 cysteine/mg protein (as some centres express cystine as 1/2 cystine/mg protein, we provide a conversion factor: 1/2 cystine/mg protein = 2 × 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 μmol/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).

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).

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).

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 [13,14]. 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., who found decreased GSSG in cystinotic fibroblasts [14]. The possible reason for this discrepancy is 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. who detected a modest decrease of GSH in three cystinotic conditionally immortalized fibroblast cell lines compared with three controls [15], 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) [1].

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. [13]. 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 [16]. 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 [17]. 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 [1], 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.

Fig. 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) in cultured skin fibroblasts (n=9) (filled circles) (r=-0.82, P 0.01) with cystinosis.
in order to investigate the activities of individual enzymes of the \( \gamma \)-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 [18]. 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 \( \text{H}_2\text{O}_2 \) by inducible Mn-superoxide dismutase (Mn-SOD) and, when released to the cytoplasm, can change the redox state to the rest of the cell [18]. Increased free radical production can be responsible for increased apoptosis, demonstrated in cystinotic fibroblasts and renal tubular epithelial cells [19,20]. 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. [15]. 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 [20]. 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.

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Conflict of interest statement. None declared.

References


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