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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 aminopeptidase N (CD-13) and megalin, confirming their proximal tubular origin. Cystinotic cell lines exhibited elevated cystine levels (0.86 ± 0.95 nmol/mg versus 0.09 ± 0.01 nmol/mg protein in controls, $p = 0.03$). Oxidized glutathione was elevated in cystinotic cells (1.16 ± 0.83 nmol/mg versus 0.29 ± 0.18 nmol/mg protein, $p = 0.04$), while total glutathione, free cysteine, and ATP contents were normal in these cells. In conclusion, elevated oxidized glutathione in cystinotic proximal tubular epithelial cell lines suggests increased oxidative stress, which may contribute to tubular dysfunction in cystinosis.

Keywords: Cystinosis; Fanconi syndrome; Proximal tubular cells; Glutathione; ATP

Cystinosis is an autosomal recessive disorder caused by mutations in the cystinosis gene, *CTNS* (17p13), encoding the lysosomal cystine transporter protein cystinosin [1,2]. The hallmark of cystinosis is the lysosomal accumulation of cystine due to a defective exodus into the cytosol [3]. Patients with the infantile form of cystinosis manifest with severe generalized proximal tubular dysfunction (Fanconi syndrome), generally appearing during the first year of life [3]. When untreated with cystine depleting agent cysteamine, they develop end stage renal disease around the age of 10 years.

Renal proximal tubular reabsorption of solutes is performed by symporters on the brush border membrane (BBM) of proximal tubular epithelial cells (PTEC). The driving force of this transport is a transmembrane sodium gradient established by Na,K-ATPase. Based on studies in proximal tubular cells loaded with cystine dimethyl ester (CDME), mimicking lysosomal cystine accumulation in cystinosis, it was hypothesized that reduced ATP production in cystinotic cells resulting in decreased sodium gradient causes a dysfunction of sodium-dependent apical transporters [4–9]. Thus far this mechanism has not been demonstrated in human cystinotic material. Furthermore, the possible link between lysosomal cystine accumulation and altered mitochondrial function is not clarified.

Recently, we and others suggested that disturbances in γ-glutamyl cycle, which accounts for glutathione (GSH) synthesis, might link lysosomal cystine accumulation and ATP depletion in cystinosis [10,11]. Insufficient cellular defense against reactive oxygen species (ROS) due to glutathione deficiency can alter mitochondrial oxidative phosphorylation [12]. Furthermore, disturbed glutathione status might reflect an increased mitochondrial ROS production, as it has been demonstrated in mitochondrial disorders [13]. Increased rate of apoptosis, demonstrated in

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cystinotic fibroblasts and proximal tubular cells loaded with CDME, is an alternative proposed pathogenetic process in cystinosis [14].

Studies of the pathogenesis of proximal tubular dysfunction in cystinosis are hampered by an unavailability of human renal cystinotic material. This limitation has been overcome by developing the technique of proximal tubular cell culture deriving from urine of patients with cystinosis [15,16].

In the present study, we established and characterized PTEC cell lines immortalized with human papilloma virus E6/E7 genes (HPV 16 E6/E7) from urine of nine cystinotic 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 (223*g*) 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) [17]. The suspension was transferred to 25 cm^2 tissue culture flask and placed at 37 °C in a 5% CO_2 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 [18].

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) [18]. 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 [19], 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 [20]. 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 0.1×10^6 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 (0.25×10^6) 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) [21]. 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 [22].

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 [11]. 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 5.0×10^6 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 (Fig. 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) (Fig. 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 (Fig. 1D) and aminopeptidase N (Fig. 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).

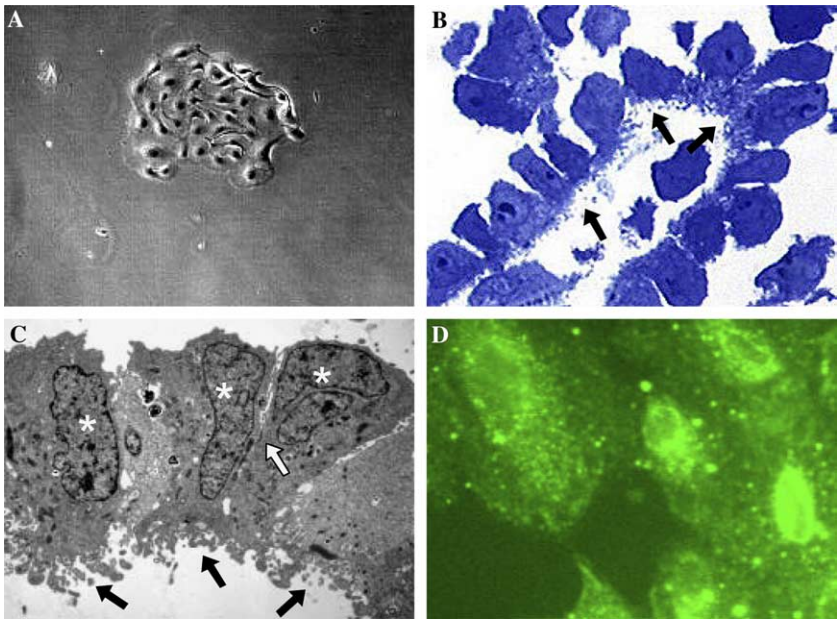

Fig. 1. (A) Phase contrast image of primary cultures showing cobblestone morphology (original magnification 100×). (B) Light microscopy of cultured cells stained with methylene blue after scraping from tissue culture flask showing bipolar orientation (original magnification 400×). (C) Transmission electron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

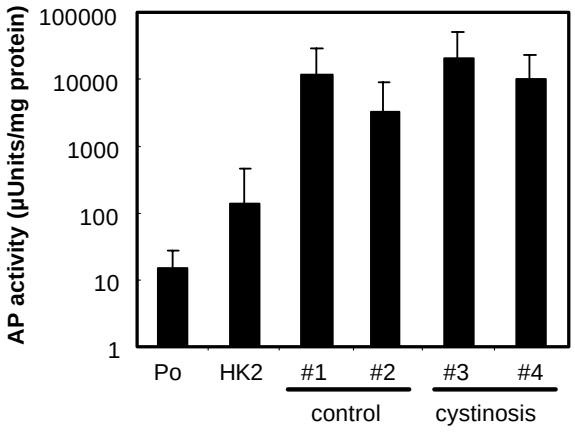

Fig. 2. Alkaline phosphatase activity in cell suspensions. Activities (mean values of at least two independent experiments) quantified using shrimp alkaline phosphatase. Data of control PTEC (#1 and #2) and cystinosis PTEC (#3 and #4) are representative for total study population. Po, conditionally immortalized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

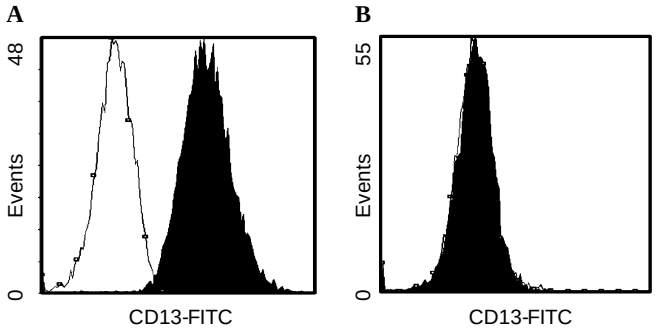

Fig. 3. Fluorescence intensity of mouse-anti-human CD-13-FITC (aminopeptidase N) antibody analyzed by flow cytometry. (A) Cultured PTEC cell lines derived from urine and (B) conditionally immortalized podocytes. Unlabeled cells are presented in white, labeled cells in black.

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 (Fig. 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 $0.29 \pm$

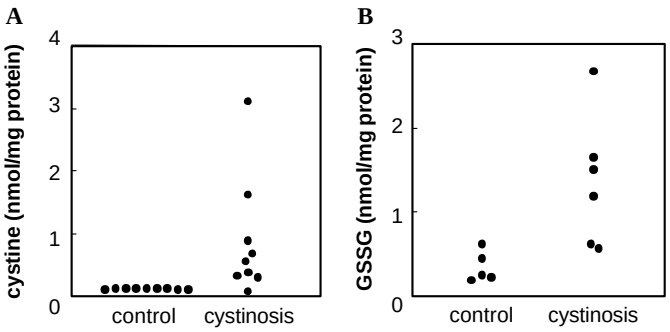

Fig. 4. (A) Cystine content (nmol/mg protein) in control ($n = 9$) versus cystinotic ($n = 9$) PTEC cell lines, $p = 0.03$. (B) GSSG content in control ($n = 5$) versus cystinotic ($n = 6$) cell lines, $p = 0.04$.

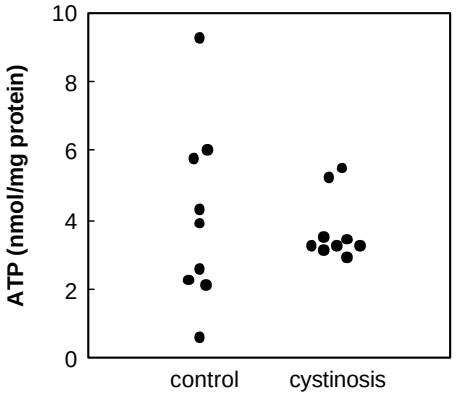

Fig. 5. ATP concentration in PTEC cultured from urine of controls ($n = 9$) and cystinotic patients ($n = 9$). Values are expressed as nmol/mg protein, $p > 0.05$.

0.18 nmol/mg protein, $p = 0.04$) (Fig. 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, $p = 0.558$) (Fig. 5). There was no correlation between cystine and ATP contents and between ATP and glutathione contents in cystinotic and control PTEC.

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 [15,16], 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 [23]. 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 [4–9], 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 [3]. 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 [24].

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 [11]. Concordant with our data, Chol et al. [10] 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 PKCδ [25] and might play a role in increased apoptosis demonstrated in cystinotic fibroblasts [14]. 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.

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