Cystinosin deficiency causes podocyte damage and loss associated with increased cell motility

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The involvement of the glomerulus in the pathogenesis of cystinosis, caused by loss-of-function mutations in cystinosin (*CTNS*, 17p13), is a matter of controversy. Although patients with cystinosis demonstrate glomerular lesions and high-molecular-weight proteinuria starting from an early age, a mouse model of cystinosis develops only signs of proximal tubular dysfunction. Here we studied podocyte damage in patients with cystinosis by analyzing urinary podocyte excretion and by *in vitro* studies of podocytes deficient in cystinosin. Urine from patients with cystinosis presented a significantly higher amount of podocytes compared with controls. In culture, cystinotic podocytes accumulated cystine compatible with cystinosin deficiency. The expression of podocyte specific genes *CD2AP*, podocalyxin, and synaptopodin and of the WT1 protein was evident in all cell lines. Conditionally immortalized podocyte lines of 2 patients with different *CTNS* mutations had altered cytoskeleton, impaired cell adhesion sites, and increased individual cell motility. Moreover, these cells showed enhanced phosphorylation of both Akt1 and Akt2 (isoforms of protein kinase B). Inhibition of Akt by a specific inhibitor (Akti inhibitor 1/2) resulted in normalization of the hypermotile phenotype. Thus, our study extends the list of genetic disorders causing podocyte damage and provides the evidence of altered cell signaling cascades resulting in impaired cell adhesion and enhanced cell motility in cystinosis.

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Podocytes are highly specialized glomerular epithelial cells that are essential for maintaining the glomerular filtration barrier. Various glomerular diseases such as lupus nephritis,[1] IgA nephropathy,[2] idiopathic focal and segmental glomerulosclerosis,[3] diabetic nephropathy, and pre-eclampsia are associated with an increased urinary excretion of podocytes (podocyturia),[4–6] In some pathologic conditions, the degree of podocyturia might represent a valuable prognostic marker reflecting the severity of the glomerular damage.[7–9] Podocyte injury is a complex process involving changes in actin cytoskeleton, altered cell adhesion and motility, and enhanced cell death.[10–12] Recently, the importance of endocytosis in podocyte homeostasis was established.[13]

Nephropathic cystinosis (MIM 219800) is a prototype disease with impaired endocytosis.[14] This autosomal recessive disorder is caused by mutations in the cystinosin (*CTNS*) gene encoding a lysosomal cystine transporter, cystinosin.[15,16] Cystinosin expression is especially high in the kidney, although the protein is ubiquitously expressed.[17]

The most severe infantile form of nephropathic cystinosis is typically associated with mutations resulting in a complete loss of function of the transporter,[18] whereas the milder juvenile form is caused mostly by mutations allowing the residual function of cystinosin.[19] Among whites from Northern Europe, the most prevalent mutation is a 57-kb deletion affecting the first 10 exons of the *CTNS* gene.[20] The initial clinical manifestation of cystinosis is renal Fanconi syndrome, a generalized proximal tubular dysfunction.[21,22] Cystinosin-deficient proximal tubular cells in a mouse model of cystinosis ($Ctn^{-/-}$) and in patients with cystinosis presented with impaired receptor-mediated endocytosis associated with cell dedifferentiation, enhanced cell death, and altered lysosomal degradation.[14,23,24] In parallel with the Fanconi syndrome, some patients with cystinosis have high-molecular-weight proteinuria starting at an early age, highlighting the potential contribution of glomerular proteinuria to the renal damage in nephropathic cystinosis.[25] We therefore hypothesized that the deficiency of cystinosin causes podocyte dysfunction.

In this study, we demonstrate an increased urinary loss of podocytes in children with cystinosis and report successful establishment of podocyte lines from the exfoliated cells. We show cytoskeletal changes and increased motility in

cystinosin-deficient podocytes associated with enhanced phosphorylation of Akt kinase, which lies at the heart of the complex signaling cascade that regulates cell metabolism, adhesion, and motility.

RESULTS

Patients with cystinosis present with podocyturia

We quantified podocytes in fresh urine samples of 9 healthy donors (aged 9.3 ± 4.3 years) and 14 patients with cystinosis (aged 11 ± 5.2 years) and observed a significantly increased number of podocytes normalized to creatinine in the urine of patients with cystinosis in comparison with controls (Table 1; Figure 1a). Specific immunostaining for podocytes and proximal tubular cells of fresh urine sediments showed more positive cells in cystinotic urine than in control samples (Figure 1b; Supplementary Figure S1 online). No correlation was registered between the severity of proteinuria, estimated glomerular filtration rate, and the number of excreted podocytes.

Characterization of conditionally immortalized podocytes from patients with cystinosis

Podocyte lines from 3 controls (aged 10.2 ± 0.2 years) and 10 patients with cystinosis (aged 10 ± 3.9 years) having different mutations in the *CTNS* gene were established (Table 1). Cystinotic podocytes accumulated high amounts of cystine (up to 25.6 nmol/mg of protein) compared with control podocytes (0.3 nmol/mg). We used 2 clonal cystinotic podocyte lines bearing different mutations in the *CTNS* gene: a homozygous 57-kb deletion (patient 1 with infantile cystinosis [Table 1]) and a 57-kb deletion + c.198–218 deletion (patient 2 with juvenile cystinosis [Table 1]). The expression of podocyte-specific markers was analyzed by different methods (Figure 2) and was evident in all cystinotic podocyte lines. As expected, the *CTNS* gene expression was not detectable in the homozygous 57-kb del cell line (cystinosis 1) and was decreased in the compound heterozygous cell line (cystinosis 2) (Figure 2b).

Table 1 | Clinical characteristics of patients with cystinosis included in the study

Patient	Age (yr)	Sex	Mutation of the *CTNS* gene	Bedside Schwartz ml/min/1.73 m^2	Protein/ creatinine (mg/mg)
1	17	M	hom 57kb del	41	3.25
2	14	M	57kb del + c.198-218del	92	2.09
3	8	F	57kb del + c.926 dup	86	1.63
4	22	M	hom 57kb del	41	0.79
5	16	M	57kb del + c.926 dup	48	2.17
6	11	F	57kb del + c.18_21del	81	2.60
7	10	M	hom 57kb del	96	2.57
8	7	M	Not done	56	0.67
9	3	M	het 57kb del + c.1015G>A	113	0.95
10	7	F	57 kb del + c.926dup	71	1.42
11	13	M	hom 57kb del	76	1.09
12	13	M	hom 57kb del	108	0.71
13	12	M	hom 57kb del	60	2.30
14	12	M	57kb del + exon 5del	68	0.43

CTNS, cystinosin.

Cystinosin-deficient podocytes have altered cytoskeleton and impaired cell adhesion

In order to test for possible alterations of podocyte cytoskeleton associated with cystinosin deficiency, we performed a co-localization study of actin and actin-associated protein α-actinin-4. Although some cystinotic podocytes had abnormal membrane protrusions with apparent dissociation of the proteins, the co-localization study demonstrated no statistically significant difference in actin cytoskeleton organization between control and cystinotic podocytes (Supplementary Figure S2 online). However, cystinosin deficiency did cause a profound disturbance of focal adhesion sites. Down-regulation of *CTNS* resulted in impaired adherence of cells to glass substrate, with a decreased number of attached cells both at early (1 hour) and late (6 hours) time points in comparison with the control (Figure 3a). Immunostaining revealed perturbed paxillin-positive focal adhesion sites in cystinosin-deficient cells in comparison with controls. The adhesion sites appeared to be less structured, suggesting a negative effect of cystinosin absence on focal adhesion stability (Figure 3b).

Cystinosin-deficient podocytes are characterized by enhanced motility

In a wound-healing assay, patient-derived podocytes and *CTNS* knockdown podocytes (*CTNS* KD) presented a significant increase in the number of migrated cells after 16 hours in comparison with control cells (Figure 4a–c).

Individual cell motility was analyzed using time-lapse imaging with subsequent tracking of single cells. Patient-derived and *CTNS* KD podocytes had significantly increased cell motility, with average traveled distances being 63 ± 7 μm for control, 112 ± 14 μm for cystinosis 1, 96 ± 39 μm for cystinosis 2, and 100 ± 22 μm for *CTNS* KD podocytes (Figure 4d). There was also a shift in the distribution of cells by motility, which could be observed by plotting a histogram: cystinosis 1 and 2 and *CTNS* KD podocytes presented an elongated histogram shape that included a subset of long-traveling cells (distances >100 μm), which was absent in control podocytes and may represent podocytes with a pathologic hypermotile phenotype (Figure 4e).

Increased motility of cystinosin-deficient podocytes is associated with increased phosphorylation of Akt kinases

Western blot analysis of urine-derived podocytes from patients with cystinosis showed increased phosphorylation of both Akt1 and Akt2 compared with controls (Figure 5a). Moreover, the increased Akt1 and Akt 2 phosphorylation in cystinotic podocytes was observed during starvation and