Urinary Protein Excretion Pattern and Renal Expression of Megalin and Cubilin in Nephropathic Cystinosis

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Background: Nephropathic cystinosis is the most common cause of inherited renal Fanconi syndrome, caused by mutations in lysosomal cystine carrier cystinosin that result in lysosomal cystine accumulation throughout the body. How defects in cystinosin cause proximal tubular dysfunction is not known. We hypothesized that cystine accumulation could cause disturbed proximal tubular endocytosis by megalin and cubilin.

Study Design: Megalin, cubilin, and their ligands were studied in kidney tissue by means of immunohistochemistry. Urinary protein excretion pattern was evaluated.

Setting & Participants: Kidney tissue from a patient with cystinosis was compared with minimal change nephrotic syndrome tissue, end-stage renal disease tissue, and control renal tissue. Urine from 7 patients with cystinosis was compared with 6 control samples.

Results: Expression of megalin, cubilin, and ligands (transferrin, albumin, vitamin D–binding protein, α_1-microglobulin, retinol-binding protein, and β_2-microglobulin) in convoluted proximal tubules of cystinotic kidney was similar to that in other kidney specimens. In straight tubules, low-molecular-weight proteins were present in only cystinotic kidney samples. Next to low-molecular-weight proteins and albumin, urinary excretion of immunoglobulin G was increased in patients with cystinosis with Fanconi syndrome compared with controls. This was already observed at an early age, suggesting enhanced glomerular permeability in patients with cystinosis.

Limitations: This study is essentially observational, and immunohistochemical data are based on 1 cystinotic kidney.

Conclusion: Our findings indicate that low-molecular-weight proteinuria in patients with cystinosis is not caused by decreased megalin and cubilin expression, and glomerular damage might already be present at early stages of the disease.

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INDEX WORDS: Cystinosis; Fanconi syndrome; proteinuria; megalin; cubilin; low-molecular-weight proteins.

Proteinuria is a common feature of patients with diverse nephropathies and generally is divided into glomerular and tubular types according to the size of proteins detected in urine. The glomerular filtration barrier almost completely restricts high-molecular-weight (HMW) proteins (>100 kDa) such as immunoglobulin G (IgG; 150 kDa) and, depending on charge and shape, allows the sieving of only small amounts of intermediate-molecular-weight (IMW) proteins such as albumin (67 kDa). Increased permeability of the filtration barrier allows these proteins to enter the glomerular ultrafiltrate and results in glomerular proteinuria. Glomerular proteinuria may be selective or nonselective. Selective proteinuria is characterized by the predominance of IMW compared with HMW proteins. Tubular proteinuria is characterized mainly by extensive excretion of low-molecular-weight (LMW) proteins (<40 kDa).[1]

In physiological conditions, LMW proteins filtered across the glomerular barrier are almost completely reabsorbed in the renal proximal tubule by receptor-mediated endocytosis. Reabsorption of proteins in proximal tubules involves initial binding to the multiligand endocytic receptors megalin and cubilin, highly expressed at the brush-border membrane of proximal tubules collaborating in the uptake of their ligands. Ligands

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are dissociated from the receptors in intracellular vesicles and degraded further by lysosomal enzymes to LMW fragments and amino acids. Megalin and cubilin are recycled to the apical membrane.[2,3]

Patients presenting with generalized proximal tubular dysfunction, so-called De Toni-Debré-Fanconi syndrome, have LMW proteinuria and albuminuria, together with increased sodium, potassium, and bicarbonate excretion; aminoaciduria; glucosuria; hypercalciuria; hyperuricosuria; and phosphaturia. Nephropathic cystinosis is the most common cause of inherited renal Fanconi syndrome in children, progressing toward renal failure caused by interstitial fibrosis.[4] This autosomal recessive disorder is caused by mutations in the *CTNS* gene (17p13.3) encoding the lysosomal cystine carrier cystinosin, which leads to lysosomal accumulation of cystine in all tissues.[5] Although the genetic defect of cystinosis has been elucidated, the cellular pathways involved in defective proximal tubular reabsorption and, subsequently, end-stage renal disease (ESRD) are enigmatic.

The degree of proximal tubular dysfunction in patients with nephropathic cystinosis is variable. Patients with the most severe infantile cystinosis (Online Mendelian Inheritance in Man [OMIM] identifier 219800) develop full-blown Fanconi syndrome during the first year of life and mainly have truncating mutations in the *CTNS* gene, whereas patients with the intermediate or juvenile form of the disease (OMIM 219900), characterized by less severe proximal tubular dysfunction and slower disease progression rate, generally have milder mutations, allowing some cystinosin activity.[6] In a recent study, 8 of 14 patients with noninfantile cystinosis had Fanconi syndrome, whereas proteinuria was present in all patients.[7]

Defective endocytosis of proteins in proximal tubules was shown to cause LMW proteinuria and albuminuria in several clinical and experimental conditions[8-11] and was documented most extensively in patients with Dent disease.[12,13] This disorder (OMIM 300009) is caused by mutations in the *CLCN5* gene encoding the endosomal chloride-proton exchanger CLC5.[14] Similar to cystinosis, Dent disease manifests with Fanconi syndrome and is characterized further by nephrolithiasis and nephrocalcinosis.[15] Patients with Dent disease and *Clcn5*$^{-/-}$ mice have decreased megalin and cubilin expression at the brush border of renal proximal tubules, which is suggested to be the cause of LMW proteinuria and albuminuria.[13,16] Furthermore, urinary megalin deficiency in patients with Dent disease and *Clcn5*$^{-/-}$ mice suggest a defect in the recycling pathway of megalin.[17]

Remarkably, decreased urinary excretion of megalin also was shown in patients with Lowe syndrome.[17] This syndrome (OMIM 309000) is caused by mutations in the OCRL1 (*OCRL*) gene encoding phosphatidylinositol 4,5-biphosphate-5-phosphatase. Patients with Lowe syndrome also develop renal Fanconi syndrome combined with severe psychomotor delay and congenital cataract.[18]

Because proteinuria is a predictor of progression in patients with various renal diseases,[19] we were interested in unraveling the mechanism of proteinuria in patients with cystinosis with progressive tubulointerstitial damage leading to renal failure.[20] We hypothesized that alterations in megalin and cubilin expression could, analogous to Dent disease and Lowe syndrome, cause proteinuria in patients with cystinosis. To investigate this hypothesis, we examined renal tissue and urine from patients with cystinosis for the presence of megalin, cubilin, and their ligands by means of immunohistochemistry and immunoblotting.

METHODS

Patients and Controls

Cystinotic renal tissue was obtained 2 months after renal transplantation from nephrectomized kidney of an 8-year-old boy with infantile nephropathic cystinosis caused by homozygous insertion of a G after nucleotide 922 in the *CTNS* gene (NM_001031681.2:c.922_923insG). Nephrectomy of his native kidney was performed because of persisting Fanconi syndrome with excessive loss of fluid and electrolytes. Before nephrectomy, urinary protein excretion was 83 mg/dL. The renal graft of this patient with cystinosis was lost because of chronic rejection and nephrectomized 5 years after transplantation with signs of ESRD. We used this ESRD graft for immunohistochemistry in this study.

Control renal tissue was obtained from a healthy man aged 43 years. Additionally, renal tissue from an 8-year-old boy with minimal change nephrotic syndrome (MCNS) was examined. At the time of biopsy, he had proteinuria (protein of 98 mg/dL) similar to the degree of proteinuria in the patient with cystinosis.

After obtaining informed consent, urine was collected from 7 patients with nephropathic cystinosis and 6 healthy controls (all participants $<$ 16 years old). The diagnosis of

cystinosis was made in all patients by measuring increased cystine content in polymorphonuclear cells by using high-performance liquid chromatography[21] and confirmed by molecular analysis of the *CTNS* gene in 6 patients. The common 57-kilobase (kb) deletion[22] was present in 5 of 7 patients. All patients except patient 3 had the infantile form of cystinosis with full-blown renal Fanconi syndrome. Patient 3 had juvenile cystinosis with proteinuria and mild proximal tubular dysfunction. Patient 7 had ESRD, native kidneys in situ, and was on peritoneal dialysis therapy. Additional clinical data for patients are listed in Table 1.

Biochemical Urine Examination

Standardized biochemical laboratory methods were used to analyze freshly collected urine samples from patients and controls. Creatinine was analyzed enzymatically, levels of total protein (milligrams per milligram of creatinine) were analyzed using the method of Lowry, amino acids (micrograms per milligram of creatinine) were measured on Jeol JLC-500/V AminoTac equipment (Jeol Ltd, Tokyo, Japan) using lithium buffers for separation according to the ion exchange column chromatography principle.[23] Phosphate levels in serum and urine were determined by using the ammonium molybdate method followed by calculation of tubular phosphate reabsorption. Glucose (micrograms per milligram of creatinine) was measured using liquid chromatography followed by mass spectrometry. Albumin (milligrams per milligram of creatinine) levels were determined by using the bromcresol purple method, and α_1-microglobulin (α_1m; micrograms per milligram of creatinine) and total IgG levels (milligrams per milligram of creatinine) were determined by using nephelometric assay.[24] Accordingly, creatinine clearance (milliliters per minute per 1.73 m^2 [to convert to milliliters per second per 1.73 m^2, multiply by 0.01667]) was calculated using the Schwartz formula.[25] Additionally, the selectivity index of proteinuria was calculated (renal clearance of IgG divided by renal clearance of transferrin) and considered selective for values less than 0.1 and aselective for values greater than 0.2.[26]

Morphological Analysis of Cystinotic Kidney

Subsequent to nephrectomy, the cystinotic kidney specimen was sectioned and imbedded in paraffin. Ultramorphological structures were analyzed by using electron microscopy.

Antibodies

The primary antibodies used in this study were polyclonal sheep anti–rat megalin,[27] rabbit anti–rat cubilin,[28] rabbit anti–human transferrin (Dako, Glostrup, Denmark), rabbit anti–human albumin (Dako), rabbit anti–human vitamin D–binding protein (DBP; Dako), rabbit anti–human α_1m (Dako), rabbit anti–human retinol–binding protein (RBP; Dako), and goat anti–mouse β_2-microglobulin (β_2m; Research Diagnostics, Concord, MA). Secondary antibodies used in this study were goat antirabbit, goat antisheep, or rabbit antigoat peroxidase-conjugated antibodies purchased from Dako.

Immunohistochemistry

Semithin (2-μm) paraffin sections were obtained from cystinotic, MCNS, ESRD, and control kidneys and preincubated with phosphate-buffered saline and glycine (50 mmol/L), pH 7.4, supplemented with bovine serum albumin (1%) or milk (0.1%) when incubated further with albumin antibodies. Subsequently, sections were incubated for 1 hour at room temperature with primary antibodies at the following dilutions: megalin (1:5,000), cubilin (1:500), transferrin (1:10,000), albumin (1:40,000), DBP (1:4,000), α_1m (1:10,000), RBP (1:8,000), and β_2m (1:4,000). After incubation with peroxidase-conjugated secondary antibodies, staining was visualized using diaminobenzidine with hydrogen peroxide (0.03%).

Urine Sample Collection and Sample Preparation

After collection, urine samples were immediately stored at −80°C until further determinations. Aliquots were used to determine creatinine and protein levels according to standardized biochemical analysis as mentioned. After thawing samples on ice, the protease inhibitors phenylmethanesulfonyl fluoride (0.5 mmol/L) and leupeptin-hydrogen chloride (1 μmol/L) were added together with sodium azide (3 mmol/L), and samples were extensively mixed by vortexing. All samples were normalized for creatinine values, and volumes were adjusted accordingly using isolation buffer (sucrose, 0.25 mmol/L; tri-ethanolamine, 10 mmol/L; phenylmethanesulfonyl fluoride, 0.5 mmol/L; and leupeptin-hydrogen chloride, 1 μmol/L, pH 7.4) to a final concentration of 3.4 μg/dL of creatinine. Subsequently, urine was centrifuged at 15,500*g* for 15 minutes at 4°C. The supernatant was saved and stored for a maximum of 2 weeks at −20°C until further processing.

For determination of megalin and cubilin in urine, samples were pretreated as mentioned. Subsequently, a volume of samples was dialyzed and lyophilized with equivalent creatinine amounts.[17] Samples were reconstituted in 200 μL of isolation buffer and immediately processed further.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting

Urine samples were separated using nonreducing 12% (albumin, DBP, and β_2m) or 3% to 16% (megalin, cubilin, transferrin, α_1m, and RBP) sodium dodecyl sulfate–polyacrylamide gel electrophoresis after adding Laemmli loading buffer. Samples were normalized for creatinine concentrations, and the final amount of protein was proportional to 0.34 μg of creatinine/lane (3 nmol). Immunoblots with lyophilized samples were loaded with 28.3 μg of creatinine/lane (250 nmol) for detection of megalin and cubilin. Proteins were blotted onto a nitrocellulose membrane and incubated overnight with primary antibodies (megalin [1:5,000], cubilin [1:2,000], transferrin [1:2,000], albumin [1:1,000], DBP [1:500], α_1m [1:1,500], and RBP [1:500]). After incubation with peroxidase-conjugated secondary antibodies, proteins were visualized using enhanced chemiluminescence.

Table 1. Clinical Data for Patients With Cystinosis

Patient No.	Age (y)	Mutation *CTNS* Gene	Creatinine Clearance (mL/min/1.73 m^2)	Protein-Creatinine Ratio (mg/mg)	Aminoaciduria/Creatinine (μg/mg)			Tubular Phosphate Reabsorption (%)	Glucose-Creatinine Ratio (μg/mg)	Albumin-Creatinine Ratio (mg/mg)	α_1m-Creatinine Ratio (μg/mg)	IgG-Creatinine Ratio (mg/mg)	Selectivity Index
					Gly	Ala	Leu						
1	1	57-kb del/c.926_927insG	104	4.77	2,931	2,708	455	30	26,060	1.08	1,040	0.21	0.78
2	1	c.[681G→A (+) 1015G→A]	122	1.95	5,989	2,803	240	41	30,260	0.53	920	0.17	0.78
3*	9	57-kb del/c.198_218del23	85	2.3	242	91	7	92	185	1.7	30	0.11	0.38
4	9	Homozygous 57-kb del	28	8.84	1,302	1,122	791	17	60,521	3.66	700	0.23	0.44
5	11	Homozygous 57-kb del	79	3.18	1,362	925	132	33	20,704	1.04	340	0.12	0.52
6	13	Homozygous 57-kb del	23	6.01	968	700	75	14	5,485	3.76	500	0.37	0.5
7†	15	Not detected	9	2.74	1,024	1,016	245	8	9,290	1.25	460	0.14	0.6
		References	0-18 y	<0.18				>90		0.04-0.11		<0.09	<0.1
			1-2 y		73-236	32-102	3-20		10-734		<148		
			7-10 y		56-157	13-51	3-19		16-67		<19		
			10-13 y		42-109	17-49	3-16		16-67		<27		
			13-18 y		29-115	13-54	2-13		16-67		<27		

Note: Proteins and solutes measured according to standardized laboratory protocols as described in Methods. Creatinine clearance calculated using the Schwartz formula. To convert creatinine clearance from mL/min/1.73 m^2 to mL/s/1.73 m^2, multiply by 0.01667.

Abbreviations: α_1m, α_1-microglobulin; IgG, immunoglobulin G; gly, glycine; ala, alanine; leu, leucine; ND, not determined; kb, kilobase; del, deletion.

*Patient 3 has late-onset cystinosis.

†Patient 7 is on dialysis therapy, and mutations were not detected in the coding region of *CTNS*.

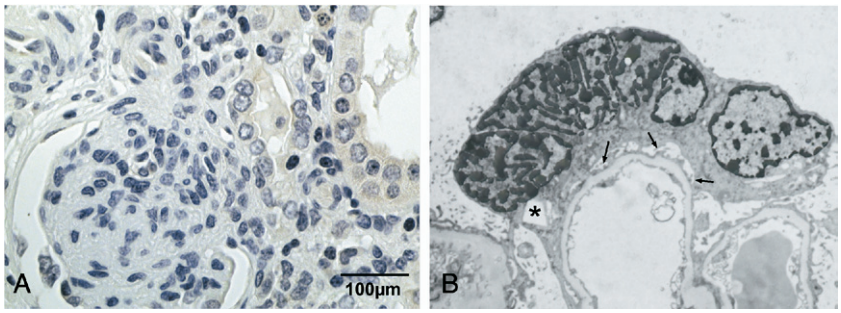

Figure 1. Morphological characteristics of the cystinotic kidney. (A) Some regions of interstitial fibrosis are observed. (B) Electron microscopic image shows giant multinuclear podocytes with effacement of podocyte foot processes (arrows). *Cytoplasmatic cystine crystal.

RESULTS

Biochemical Urine Examination

All patients with cystinosis had proteinuria and albuminuria, including patient 3 without full-blown Fanconi syndrome (Table 1). The presence of pronounced aminoaciduria, glucosuria, LMW protein α_1m levels, and decreased tubular phosphate reabsorption confirmed full-blown renal Fanconi syndrome in patients 1, 2, and 4 to 7. Furthermore, HMW protein IgG level was increased in all patients, even patients at 1 year of age. The selectivity index was greater than 0.2 in all patients, indicating that patients had nonselective glomerular proteinuria.

Morphological Characteristics of Cystinotic Renal Tissue

By means of light microscopy, some glomeruli appeared normal, but most contained peculiar giant multinucleated podocytes. Tubules were moderately atrophic, and some regions of interstitial fibrosis were observed (Fig 1A). Electron microscopy showed irregular thickening of glomerular basement membrane and effacement of podocyte foot processes (Fig 1B). Remarkably, contours of a cytoplasmic cystine crystal dissolved by the routine tissue processing are seen.

Three different regions of the cystinotic kidney with intact glomeruli and surrounding tubules were selected for additional immunohistochemical examination of megalin and cubilin expression and the presence of their ligands. In the cortex of the kidney, both convoluted proximal tubules in the cortical labyrinth and straight proximal tubules in the medullary rays were examined.

Immunohistochemical Studies of Cystinotic Renal Tissue

Staining for megalin and cubilin showed abundant brush-border expression of these endocytic receptors in intact regions of both cystinotic and control convoluted and straight proximal tubules (Fig 2). Positive staining for both megalin and cubilin could be observed in all 3 studied regions of the cystinotic kidney.

Immunohistochemical studies using antibodies against the ligands of megalin and cubilin (transferrin, albumin, DBP, α_1m, RBP, and β_2m) showed the presence of these ligands in apical endocytic vesicles and at the brush-border membrane of convoluted tubules in the cortical labyrinth of both control and cystinosis kidney sections (Fig 2; staining of DBP, α_1m, and RBP not shown). In straight proximal tubules, staining for all ligands was enhanced in cystinotic tissue, but absent (DBP, α_1m, RBP, and β_2m) or less abundant (transferrin and albumin) in medullary rays of control tissue. The presence of the endocytic receptors megalin and cubilin and all examined ligands in different segments in control and cystinotic kidney tissue was scored by 2 independent observers and is listed in Table 2.

Immunohistochemical Study of MCNS and ESRD Kidney Samples

To distinguish changes attributed to proteinuria from those attributed to cystinosis, we compared results of cystinotic tissue with those of a kidney with increased glomerular permeability caused by a known glomerular disease, MCNS, with protein-

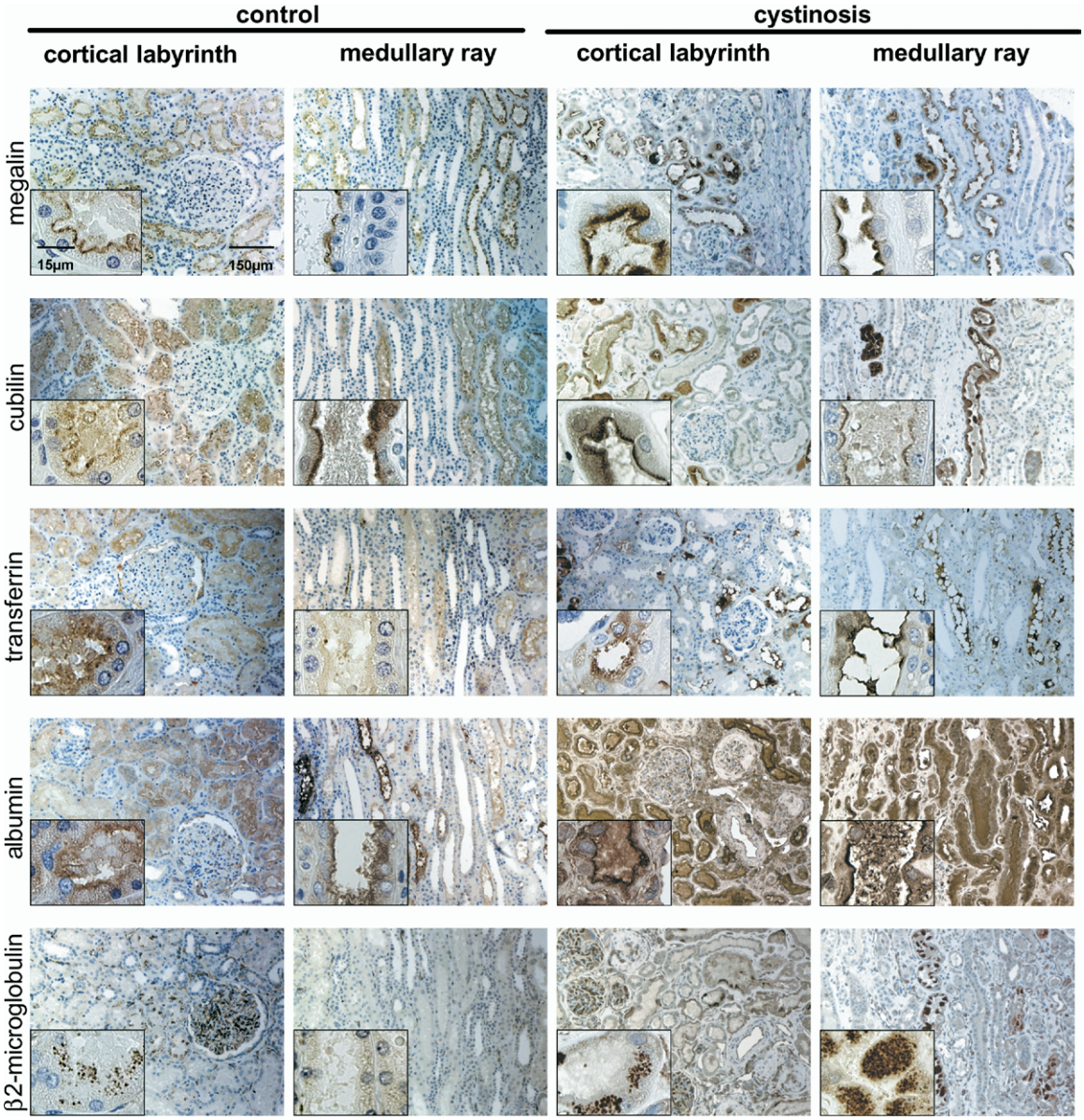

Figure 2. Immunohistochemical staining of control and cystinotic renal tissue. Semithin sections are treated with antibodies against megalin, cubilin, transferrin, albumin and β_2-microglobulin (β_2m). Both the receptors megalin and cubilin are expressed on the brush border of the convoluted and straight proximal tubules in control and cystinotic tissue. In convoluted proximal tubules of the cortical labyrinth, the ligands transferrin, albumin, and β_2m are present in apical vesicles in both control and cystinotic kidney. Staining for the ligands in straight proximal tubules in the medullary ray is more abundant in cystinotic tissue.

uria (protein, 98 mg/dL) at the time of biopsy within the same range as for the patient with cystinosis (83 mg/dL) at the time of nephrectomy. The rejected kidney after transplantation in the patient with cystinosis was included in this study to distinguish between ESRD and cystinosis. Immunohistochemical examination showed that megalin, cubilin, and all their examined ligands were located in convoluted tubules in the cortical labyrinth of both kidneys, similar to control and cystinotic kidneys (Fig 3; Table 2). Surprisingly, in straight tubules, no endocytic vesicles containing DBP, α_1m, RBP, or β_2m were present in the MCNS kidney specimen, similar to the control kidney but

Table 2. Qualitative Evaluation of the Presence of Megalin, Cubilin, and Their Ligands in Control, Cystinotic, MCNS, and ESRD Kidney Tissue

	Control		Cystinosis		MCNS		ESRD	
	Convoluted	Straight	Convoluted	Straight	Convoluted	Straight	Convoluted	Straight
Megalin	+	+	++	++	+	+	+	+
Cubilin	+	+	++	++	+	+	+	+
Transferrin	++	+/−	++	++	+	+	++	+
Albumin	++	++	+++	+++	+++	++	++	++
DBP	+	+/−	++	++	+/−	−	ND	ND
α_1-Microglobulin	+	−	++	++	+	−	ND	ND
RBP	+	−	++	++	+/−	−	ND	ND
β_2-Microglobulin	+	−	++	++	+	−	+/−	+/−

Note: Intensity scored by 2 independent observers for both convoluted tubules in the cortical labyrinth and straight tubules in the medullary ray.

Abbreviations: MCNS, minimal change nephrotic syndrome; ESRD, end-stage renal disease; DBP, vitamin D–binding protein; RBP, retinol-binding protein; ND, not determined.

different from the cystinotic kidney. Remarkable were the dense albumin vesicles located basolateral in proximal tubules in both the convoluted and straight tubules of the MCNS kidney sample. In contrast to the cystinotic kidney sample, no endocytic vesicles containing albumin, transferrin, or β_2m were observed in straight tubules of the ESRD kidney sample.

Immunoblot of Urine Fractions

After normalizing urine samples for creatinine concentration, urine was centrifuged and the supernatant was analyzed further. Proteins were separated by using nonreducing polyacrylamide gel electrophoresis and immunoblotted by using antibodies against the ligands transferrin, albumin, DBP, α_1m, and RBP. Large quantities of transferrin, albumin, and DBP were observed in samples from all patients with cystinosis. The LMW proteins α_1m and RBP were abundantly present in samples from all patients with renal Fanconi syndrome. Both ligands were only slightly increased compared with 6 controls in patient 3 with juvenile cystinosis (Fig 4). Small amounts of albumin and transferrin and almost no LMW proteins were detected in control urine specimens. After loading the equivalent of 0.34 μg of creatinine, megalin and cubilin could not be found in patient or control urine samples (data not shown). Subsequent to dialysis and lyophilization of urine samples, the equivalent of 28.3 μg of creatinine was loaded and megalin was detected in both control and cystinotic urine samples (data not shown).

DISCUSSION

Nephropathic cystinosis is characterized by LMW proteinuria, albuminuria, and various degrees of defective sodium ion–coupled transport. Assuming the similarity with other proximal tubular disorders, such as Dent disease and Lowe syndrome, we hypothesized that deficient function of the endocytotic receptors megalin and cubilin could be responsible for enhanced urinary loss of proteins in patients with cystinosis. Interestingly, the present study shows abundant megalin and cubilin expression at the brush border of proximal tubules in cystinotic renal tissue. Furthermore, we found the presence of megalin and/or cubilin ligands (transferrin, albumin, DBP, α_1m, RBP, and β_2m) in intracellular vesicles of cystinotic proximal tubules, suggesting that these ligands are reabsorbed through megalin-cubilin–mediated endocytosis. These results are distinct from those in patients with Dent disease and Lowe syndrome, indicating a different pathogenic mechanism leading to proteinuria in patients with cystinosis. This is emphasized further by the finding of relatively normal megalin levels in urine of patients with cystinosis, similar to patients with the autosomal dominant form of renal Fanconi syndrome, but contrasting to the decrease in megalin levels in urine of patients with Dent disease and Lowe syndrome.[17]

Because renal biopsies are not performed to establish the diagnosis of cystinosis, renal tissue of patients with cystinosis generally is not available. Our histological findings are based on ex-

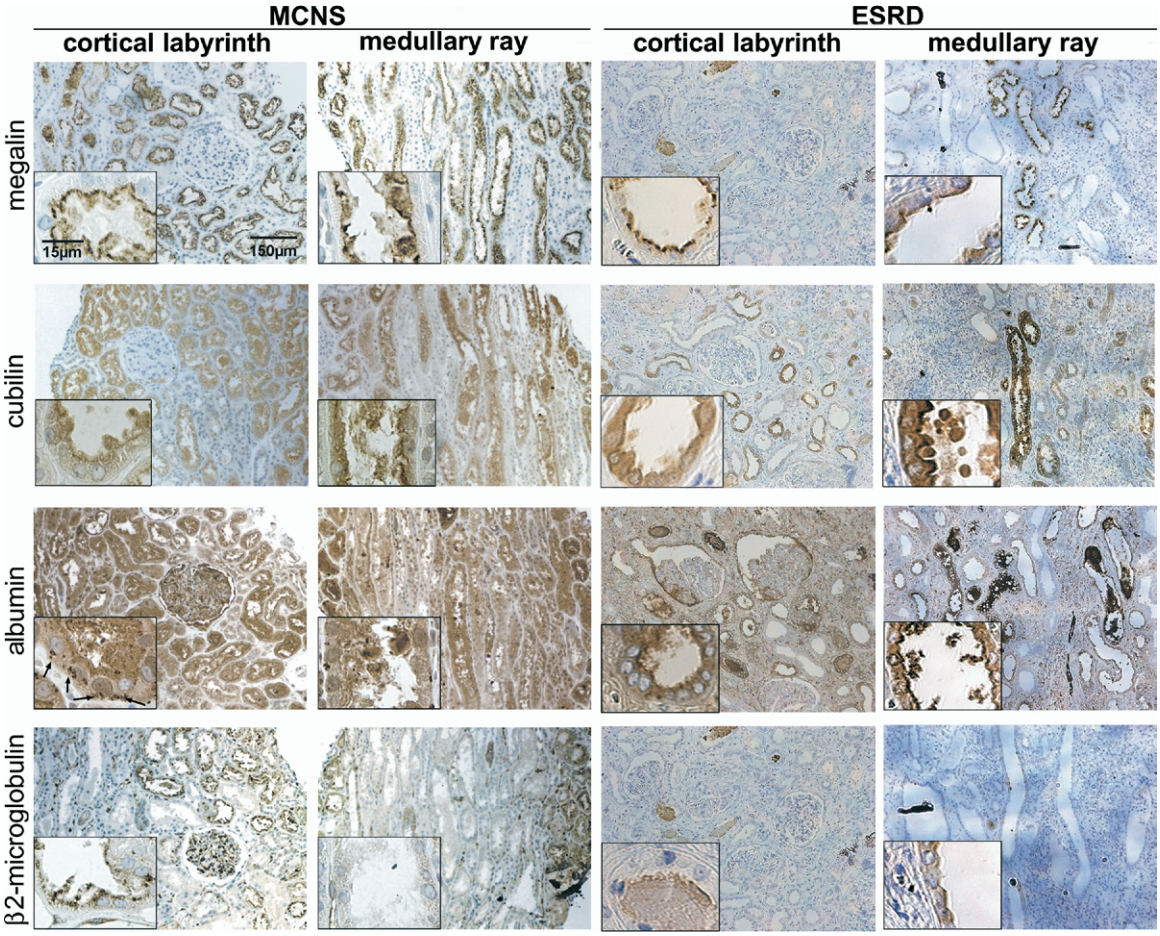

Figure 3. Immunohistochemical staining of minimal change nephrotic syndrome (MCNS) and end-stage renal disease (ESRD) kidney samples. Sections are incubated with antibodies against megalin, cubilin, albumin, and β_2-microglobulin (β_2m). In the cortical labyrinth, all antibodies show positive apical staining. β_2m is absent in straight proximal tubules of MCNS kidney specimens, whereas megalin, cubilin, and albumin are abundantly observed in both kidney specimens. Remarkably, albumin also is present in basal vesicles of convoluted proximal tubules of MCNS kidney specimens (arrows).

amination of the nephrectomized native kidney from a transplant recipient. The examined kidney was still functioning after renal transplantation, which was the reason for nephrectomy. Immunohistochemical staining was performed in selected intact kidney regions without obvious signs of pronounced tubulointerstitial and glomerular damage and therefore reflects changes that can be attributed to cystinosis, rather than ESRD, indicated further by observations in the ESRD kidney that did not have the cystinotic phenotype.

To evaluate further the mechanism of proteinuria in patients with cystinosis, we extensively studied urinary protein excretion patterns in patients with cystinosis with different degrees of renal damage. Surprisingly, we found enhanced urinary loss of the HMW protein IgG in children with cystinosis starting from an early age with normal glomerular filtration rate. Low amounts of IgG can be found in urine of healthy persons and patients with proximal tubular disorders because a small fraction of IgG can cross the glomerular filter[29] and is partially reabsorbed in proximal tubules.[30] IgG levels found in patients with cystinosis (range, 0.11 to 0.37 mg/mg creatinine) were increased compared with controls and values found by Norden et al[31] in patients with the tubular disorders Dent disease (0.049 mg/mg creatinine) and Lowe syndrome (0.054 mg/mg creatinine). The observation of increased levels of the HMW protein IgG suggests in-

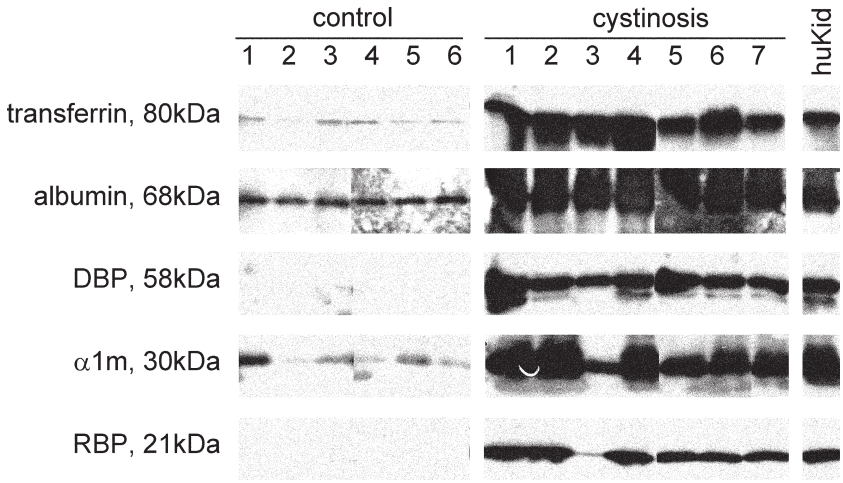

Figure 4. Western blotting of urine fractions from controls and patients with cystinosis. Transferrin, albumin, vitamin D–binding protein (DBP), α_1-microglobulin (α_1m), and retinol-binding protein (RBP) are detected in urine from patients with cystinosis, but not or less abundantly in control samples. Low-molecular-weight excretion is lower in patient 3 (with juvenile cystinosis). All samples are normalized for creatinine concentrations (0.34 μg of creatinine/lane). Human kidney (huKid) homogenate is simultaneously loaded as a control.

creased glomerular permeability in patients with cystinosis. Our previous finding of successful treatment using the angiotensin-converting enzyme inhibitor enalapril for decreasing albuminuria in patients with cystinosis also suggests the glomerular origin of proteinuria in patients with cystinosis.[32] Since patients with juvenile cystinosis (such as patient 3 in this study) present pronounced proteinuria without overt proximal tubular dysfunction, it could be argued that glomerular disease in cystinosis is independent from proximal tubular damage. Another argument for glomerular damage in patients with cystinosis is the morphological changes observed in the present and previous studies by others.[20] In cystinotic kidney specimens, podocyte foot-process effacement and the presence of characteristic multinucleated podocytes were observed, suggesting that cystine accumulation causes podocyte dysfunction, which can result in glomerular proteinuria. This idea is supported further by earlier deterioration of glomerular filtration in patients with cystinosis compared with patients with Dent disease.[15]

Increased excretion of LMW proteins in patients with pure glomerular disease such as MCNS indicates that an "overload" albumin can inhibit the reabsorption of LMW proteins in renal proximal tubules.[33-35] The presence of aselective proteinuria in patients with cystinosis, indicative of greater clearances of HMW proteins compared with IMW proteins, might point to a more pronounced defect in the filtration barrier in patients with cystinosis compared with MCNS.

To definitively resolve the question of whether glomerular permeability increases in patients with cystinosis, filtration of HMW proteins in patients with cystinosis should be studied further by determining Ficoll sieving coefficients.[36] However, performing these tests in young patients with cystinosis poses ethical problems. The recently developed *Ctns*$^{-/-}$ mice could be used to study glomerular permeability more extensively, although these mice do not manifest with Fanconi syndrome.[37]

Although megalin and cubilin are expressed at the brush border of proximal tubules in patients with cystinosis, our data cannot exclude that LMW proteinuria is caused by decreased recycling rates of these endocytic receptors. One could argue that cystine accumulation in lysosomes has an impact on the rate of the endocytic mechanism. The observation of vesicles containing megalin and cubilin ligands in straight proximal tubules in cystinosis kidney tissue, but not MCNS and ESRD kidney tissue with a similar grade of proteinuria, is in favor of this theory. Additionally, the presented data indicate increased intracellular vesicles containing ligands of megalin and cubilin, suggesting a blockade in

the recycling mechanism in the cystinotic kidney. However, abundant expression of the receptors megalin and cubilin at the brush-border membrane do not support a recycling defect. Another possible explanation of these findings could be altered lysosomal enzyme activity in cystinotic proximal tubules. Recent data indicated that proteinuria itself can be the reason for this observation. A study by Nielsen et al[38] showed that circulating lysosomal enzymes such as cathepsin B are filtered in glomeruli and reabsorbed by endocytosis. Intracellular, these hydrolases are transported to lysosomes and are functionally active for protein degradation. In case of proteinuria, competition of proteins for reabsorption in proximal tubules could result in insufficient uptake of hydrolytic enzymes. Our finding of basally located albumin vesicles in kidney tissue of a patient with MCNS supports this theory. That we could not detect ligands of megalin and cubilin in straight proximal tubules of MCNS kidney tissue suggests that enhanced glomerular filtration does not exceed the reabsorption capacity in convoluted proximal tubules at this range of proteinuria.

In conclusion, our findings indicate that LMW proteinuria in patients with cystinosis is not caused by decreased megalin and cubilin expression, and glomerular damage already might be present at early stages of the disease.

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