Abstract

Background. Lysosomal enzymuria is usually considered to be a non-specific marker of renal injury, but little is known about lysosomal enzyme excretion in renal proximal tubular cell disorders such as the renal Fanconi syndrome (FS). We examined excretion of two lysosomal enzymes and the cation-independent mannose-6-phosphate receptor (CI-MPR) in patients with inherited FS.

Methods. The lysosomal enzyme cathepsin D was measured by ELISA and isolated by pepstatin-agarose affinity chromatography; N-acetyl-β-D-glucosaminidase (NAG) was assayed colorimetrically, as was the cytosolic enzyme lactate dehydrogenase (LDH). Cathepsin D, procathepsin D and CI-MPR were also detected by western blotting. No patient had a serum creatinine concentration > 170 µmol/L. Soluble CI-MPR, isolated from fetal calf serum and bound to agarose, was used to probe cathepsin D for mannose-6-phosphate (M6P).

Results. Increased excretion of cathepsin D (mean = 44-fold) and NAG (mean = 12-fold) was found in FS patients: Dent’s disease (n = 5), cystinosis (n = 4), Lowe syndrome (n = 3) and ‘autosomal dominant idiopathic FS’ (ADIF) (n = 2). Increased cathepsin D excretion was confirmed by western blotting; excretion of procathepsin D and LDH was not increased. When compared with control subjects, CI-MPR excretion was also increased in FS (n = 6). Thus, significantly increased excretion of lysosomal enzymes and CI-MPR was found in all cases of FS examined. Cathepsin D binding to CI-MPR-agarose was inhibited by M6P.

Conclusions. We conclude that underlying gene defects in FS may disrupt normal membrane trafficking of CI-MPR, leading to mistrafficking of lysosomal enzymes via a default pathway from the Golgi to the apical surface of proximal tubule cells rather than to lysosomes. Lysosomal enzymes are then secreted into the tubular fluid and excreted in the urine. This contrasts with the widely held view that cell necrosis is the cause of lysosomal enzymuria in renal disease. Moreover, cathepsin D in FS urine is M6P-tagged.

Keywords: cation-independent mannose-6-phosphate receptor; cathepsin D; Dent’s disease; Fanconi syndrome; N-acetyl-β-D-glucosaminidase

Introduction

The renal Fanconi syndrome (FS) is a group of disorders of the renal proximal tubule that can be inherited or acquired [1]. The most consistent finding in FS patients is ‘tubular’ proteinuria, which is characterized by an excretion profile dominated by those proteins filtered at the renal glomerulus whose reabsorption is prevented by a proximal tubular cell endocytic defect [1,2]. These proteins are made up of about one-third albumin, one-third low-molecular-weight proteins (LMWP)—like retinol-binding protein, β₂-microglobulin and α₁-microglobulin—and the remaining one-third of diverse plasma proteins [2]. Other features of FS, which are more variable than tubular proteinuria, include aminoaciduria, glycosuria, hyperuricosuria, hyperphosphaturia and hypercalciiuria [1].

Examples of inherited FS are Dent’s disease, which is X-linked and usually due to a CLCN5 mutation causing loss of function of the CLC-5 proton/chloride antipporter [3–5]; cystinosis, in which lysosomal cystine accumulates because of defective function of the lysosomal membrane cystine carrier protein cystinosin; X-linked cystine accumulates because of defective function of the lysosomal membrane cystine carrier protein cystinosin; X-linked oculocerebrorenal syndrome of Lowe (OCRL) due to an OCRL1 mutation causing inositol 3⁵′ bisphosphate phosphatase deficiency and ‘autosomal dominant idiopathic Fanconi syndrome’ (ADIF)—the genetic basis of which is still unknown [1]. In addition, recent work indicates that the clinical phenotype of Dent’s disease may also result from a mutation in OCRL1 (which usually causes Lowe syndrome) [6].

The membrane trafficking of several proximal tubular cell receptors and transporters is believed to be disrupted in Dent’s disease [3,7,8]. We hypothesized that the underlying molecular mechanisms for the defects in cell trafficking...
in Dent’s disease and cystinosis, although different, are likely to interfere with the normal intracellular transport of mannose-6-phosphate receptors (MPRs). Indeed, studies in a mouse model of Dent’s disease have detected urinary excretion of renal lysosomal hydrolases [8].

MPRs are required for delivery of lysosomal enzymes to the lysosome from the Golgi, and any disruption in receptor trafficking is likely to lead to a failure of this normal trafficking process [9]. By analogy, disruption of this process is also likely to occur in Lowe syndrome, and it has been demonstrated in vitro that OCRL1 has a role in clathrin-mediated trafficking of proteins from endosomes to the trans-Golgi network [10]. Although the molecular genetic basis of ADIF is unknown, the numerous biochemical similarities of FS in ADIF to Dent’s disease, cystinosis, Lowe syndrome, and ADIF and the adult and paediatric controls is presented in Table 1.

The two related MPRs, known as the cation-independent mannose-6-phosphate receptor (CI-MPR), also termed the IGFII/M6-P receptor, 300 kDa molecular weight, and the cation-dependent mannose-6-phosphate receptor (CD-MPR), 46 kDa, carry newly synthesized lysosomal hydrolases from the trans-Golgi network to the endosomal and lysosomal compartments [9]. Targeting of lysosomal hydrolases to the apical membrane also occurs, but this is normally a minor default pathway [9].

We hypothesized that a trafficking defect in all four of the genetic forms of FS studied would interfere with normal targeting of lysosomal enzymes and their bound MPRs to lysosomes. The enzymes would fail to enter the endosomal/lysosomal compartments and instead use the default lysosomal/lysosomal compartments and instead use the default

### Table 1. Summary of patients studied

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Dent’s disease</th>
<th>Dent’s carriers</th>
<th>Cystinosis</th>
<th>Lowe syndrome</th>
<th>Autosomal dominant idiopathic</th>
<th>Adult controls</th>
<th>Paediatric controlsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Mean age</td>
<td>32 years</td>
<td>43 years</td>
<td>8 years 9 months</td>
<td>19 years</td>
<td>42 years</td>
<td>38 years</td>
<td>10 years 11 months</td>
</tr>
<tr>
<td>Serum creatinineb (µmol/L)</td>
<td>112 (57–154)</td>
<td>73 (44–80)</td>
<td>104 (51–117)</td>
<td>86 (46–113)</td>
<td>166 (131–202)</td>
<td>83 (63–118)</td>
<td>101 (51–176)</td>
</tr>
<tr>
<td>Urinary albuminb (mg/mmol creatinine)</td>
<td>63 (14–202)</td>
<td>7.1 (4.7–18)</td>
<td>97 (47–135)</td>
<td>112 (33–145)</td>
<td>77 (37–117)</td>
<td>1.8 (0.3–2.2)</td>
<td>38 (4.1–81)</td>
</tr>
<tr>
<td>Urinary retinol-binding proteinb (mg/mmol creatinine)</td>
<td>21.3 (12.2–31.1)</td>
<td>0.38 (0.082–0.61)</td>
<td>– –</td>
<td>78 (64–93)</td>
<td>13.6 (5.1–22.1)</td>
<td>0.0078 (0.003–0.016)</td>
<td>– –</td>
</tr>
</tbody>
</table>

*a Three children with reflux nephropathy, one with glomerulocystic kidney disease and one with previous haemolytic uraemic syndrome.

*b Mean (range).

*c Not measured.

### Subjects and methods

#### Subjects

All patients or their parents and guardians gave informed consent, and the study was approved by the Cambridge Local Research Ethics Committee. A summary of the patients with Dent’s disease and carriers of Dent’s disease, cystinosis, Lowe syndrome, ADIF and the adult and paediatric controls is presented in Table 1.

#### Urine collection and storage

Details of the protocol for urine collection and storage have been given previously [7]; all urine specimens to be stored for longer than 1 month were placed in liquid nitrogen. Urine was not centrifuged prior to electrophoresis.

#### Protein degradation in acidified urine

Sodium formate, 1 mol/L, pH 3.0, was added to urine on ice to a final pH of 3.5. Aliquots were either kept on ice for 1 h or incubated at 37°C, with or without addition of pepstatin A (Sigma-Aldrich Co., Cat. No. P5318), to a final concentration of 1 µmol/L. Stock pepstatin A, 1 mmol/L, was made up in ethanol. To add albumin to a sample of control urine, albumin purified from human urine (Scipac, Cat. No. P140-0) at 20 mg/mL in water was used.

Following incubation, urine was mixed with SDS loading buffer containing 100 mM DTT, incubated in a boiling water bath for 3 min and then electrophoresed as described previously, although 12% polyacrylamide gels (Bio-Rad Co., Criterion™ Cat. No. 345-0118) were used and stained with Coomassie blue [7]. Molecular weight markers were ‘Precision Plus Protein™ Standards’ (Bio-Rad Co., Cat. No. 161-0373).

#### Measurement of cathepsin D, NAG and l-lactate dehydrogenase (LDH) in urine

Urine diluted in sample diluent was assayed for cathepsin D protein by an ELISA method (Oncogene™, Cat. No. QIA29). This was a sandwich-type immunoassay utilizing a monoclonal capture and polyclonal detector antibody.
that does not detect cathepsin B. NAG was measured by a colorimetric assay using hydrolysis of 3-cresol sulfonephthaleinyl-N-acetyl-β-D-glucosaminide (Roche Co., Cat. No. 875-406). LDH was measured by enzyme-coupled oxidation of L-lactate (Dade-Behring Co., Dimension® Cat. No. DF53A). Results of enzyme protein, or activity, were expressed per mmol urinary creatinine measured by a rate alkaline picrate method (Dade-Behring Co., Dimension® Cat. No. DF33A). Urine specimens from five carriers of Dent’s disease were available for NAG measurement, but only three for cathepsin D analysis. Urine specimens from four patients with cystinosis were available for NAG and LDH measurement, but only three for cathepsin D analysis.

**LDH stability in urine**

LDH was extracted as a crude soluble fraction from human kidney cortex by making a homogenate of 1 g wet weight cortex in 8 mL 0.05 mol/L tris hydrochloride buffer pH 7.5 and centrifuging at 100 000 × g for 20 min. To assess the stability of LDH in urine we added the supernatant to control aliquots of 0.25 mL were assayed immediately, or incubated adjusted to either pH 5.5 or 6.5 with 1 mol/L acetic acid, and LDH measurement, but only three for cathepsin D analysis.

Affinity chromatography capture of cathepsin D

Using a modification of the method of Conner [12], urine samples (5 mL adjusted to pH 3.5 as described above) were applied to 0.5 mL columns of pepstatin A-agarose (Sigma-Aldrich Co., Cat. No. P2032) equilibrated with 0.1 mol/L sodium formate pH 3.5 at 4°C, washed with the same eluent and bound protein recovered directly into Laemmli sample buffer for electrophoresis on 10–20% polyacrylamide gels (as above). As described previously [7], these gels were blotted onto PVDF membranes and probed with either rabbit anti-human cathepsin D (Calbiochem Co., Cat. No. IM-16-100UG) or non-immune rabbit serum (diluted 1:1000–1:5000), followed by adding donkey anti-rabbit F(ab')2, fragment conjugated to horseradish peroxidase (GE-Amersham Cat. No. NA9340) and visualized with ECL Plus reagent (GE-Amersham Co., Cat. No. RPN2132) and BioMax Light film (Eastman Kodak Co., Cat. No. 876-1520).

Detection of CI-MPR in urine

Urine samples in volumes containing 25 μmol creatinine were dialyzed against 0.1 mol/L ammonium hydrogen carbonate (Sigma-Aldrich Co., Fluka Cat. No. 09830) at 4°C. The volume of dialysis fluid was at least 100-fold that of the samples and a total dialysis time of 18 h was used. The dialysis fluid was changed twice during this time and the dialyze was then lyophilized. The lyophilizate was resuspended in non-reducing CriterionTM XT Sample Buffer (Bio-Rad Co., Cat. No. 161-0791), warmed to 40°C for 30 min and then electrophoresed on 3–8% Tris-acetate CriterionTM gels (Bio-Rad Co., Cat. No. 345-0131) in XT-Tricine Buffer (Bio-Rad Co., Cat. No. 161-0790). Proteins were blotted onto PVDF (as described before [7]) and probed with 1:10 000 goat anti-human CI-MPR, or as a control without the addition of the first antibody. The properties of the anti-human CI-MPR antibody used have been described by Causin et al. [13]. Detection was performed as already described, except that donkey anti-sheep IgG conjugated to horseradish peroxidase (Sigma-Aldrich Co., Cat. No. A3415) was used at 1:80 000 dilution as the second antibody, and detection sensitivity was increased by using ECL advance reagent (GE-Amersham Co., Cat. No. RPN2135). In preliminary experiments the anti-sheep antibody conjugate was found to cross-react with the goat primary antibody that was used to detect CI-MPR. Molecular weight standards were "Precision Plus All Blue" (Bio-Rad Co., Cat. No. 161-0373).

Phosphomannan-core agarose (PMC-agarose) was prepared by coupling the core oligosaccharide obtained after mild acid hydrolysis of yeast phosphomannan to cyanoagens bromide-activated agarose as described by Sahagian et al. [14]. For adsorption of urine onto PMC-agarose, urine, dialyzed and lyophilized as above, was resuspended in phosphomannan-binding buffer (PBB) comprising 50 mmol/L sodium phosphate pH 7.2, 0.15 mol/L sodium chloride, 5 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA and 1 mL/100 mL mammalian protease inhibitor (Sigma P8340). The composition of this buffer is based on that described by Sleat et al. [15]. To adsorb CI-MPR from urine, 50 μL urine in PBB was incubated overnight on a rotator with 100 μL 70% slurry of PMC-agarose in PBB. The agarose was washed with excess PBB and CriterionTM XT Sample Buffer (Bio-Rad Co., Cat. No. 161-0791); 30 μL was added prior to electrophoresis and immunoblotting for CI-MPR as described above.

CI-MPR-agarose was synthesized using CI-MPR prepared from fetal calf serum as described by Valenzano et al. [16] and coupled to CNBr-Sepharose (G.E.-Amersham 17-0430-01), as recommended by the manufacturer, using a CI-MPR concentration of ~3 mg/mL. For binding of urinary cathepsin D to CI-MPR-agarose, we incubated 0.1 mL of urine in PBB with 0.2 mL of a 50% slurry of CI-MPR-agarose in PBB, with or without M6P, at a final concentration of 2.5 mmol/L. After incubation on a rotator for 1 h at room temperature, the gel was washed three times with the cognate PBB with or without M6P and 100 μL CriterionTM XT Sample Buffer (Bio-Rad Co., Cat. No. 161-0791) containing 100 mmol/L dithiothreitol, added prior to boiling for 3 min, electrophoresing on 12% Bis-Tris gels (Bio-Rad Cat. 345-0118) and immunoblotting for cathepsin D as described above.

Results

Preliminary experiments showed that acidification of urine from a patient with Dent’s disease D1, followed by incubation at 37°C for 1 h, caused almost complete degradation of endogenous albumin and several other excreted proteins (Figure 1). Figure 1 also shows that a heterogeneous mixture of low-molecular-weight proteins and peptides was
Fig. 1. Protein degradation in urine acidified to pH 3.5 from patients with renal Fanconi syndrome (FS) compared with control urine to which albumin was added. Urine samples examined were from patients with Dent’s disease, Patient D1; control urine to which albumin was added to a concentration of 1 mg/mL; Dent’s disease, Patient D2 and Lowe syndrome, Patient L1. Each urine sample was either not incubated, incubated at 37°C for 1 h without pepstatin A or incubated at 37°C for 1 h with pepstatin A as shown. The panel on the right shows molecular weight markers. Urine samples were denatured in SDS and reduced, electrophoresed on 12% SDS gels and stained with Coomassie blue. The arrow shows the location of monomeric urinary albumin.

Table 2. Cathepsin D, N-acetyl-β-D-glucosaminidase (NAG) and l-lactate dehydrogenase (LDH) levels in the urine of patients with hereditary renal Fanconi syndrome (FS), carrier females of Dent's disease and adult and paediatric control patients

<table>
<thead>
<tr>
<th></th>
<th>Dent’s affected</th>
<th>Dent’s carriers</th>
<th>Cystinosis</th>
<th>Lowe syndrome</th>
<th>Autosomal dominant idiopathic</th>
<th>Controls</th>
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<tr>
<td>Cathepsin Dc</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
<td>14.6</td>
<td>4.0</td>
<td>13.3</td>
<td>11.5</td>
<td>50.9</td>
<td>1.53</td>
</tr>
<tr>
<td>Range</td>
<td>10.3–22.4</td>
<td>2.9–5.1</td>
<td>8.4–21.4</td>
<td>4.9–16.4</td>
<td>22.0–79.8</td>
<td>0.2–1.8</td>
</tr>
<tr>
<td>Number of patients</td>
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<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>N-Acetyl-β-D-glucosaminidase (NAG)d</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
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<td>33</td>
<td>67</td>
<td>5.9</td>
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<tr>
<td>Range</td>
<td>12–42</td>
<td>8–41</td>
<td>18–66</td>
<td>31–37</td>
<td>46–88</td>
<td>1–9</td>
</tr>
<tr>
<td>Number of patients</td>
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<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>8</td>
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<tr>
<td>l-Lactate dehydrogenase (LDH)e</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.22</td>
<td>0.31</td>
<td>0.19</td>
<td>0.3</td>
<td>0.25</td>
<td>0.28</td>
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<tr>
<td>Range</td>
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<td>&lt;0.1–0.7</td>
<td>&lt;0.1–0.4</td>
<td>&lt;0.1–0.7</td>
<td>&lt;0.1–0.4</td>
<td>&lt;0.1–0.7</td>
</tr>
<tr>
<td>Number of patients</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

aThree out of five patients studied had measurements of Cathepsin D.
bThree out of four patients studied had measurements of Cathepsin D.
cµg/mmol creatinine.
dµmol/h/mmol creatinine.
eUnits/mmol creatinine.

generated by this procedure, which could be blocked by the addition of the specific aspartic-acid-protease inhibitor pepstatin A. When albumin was added to control urine to a final concentration of 1 mg/mL, acidification and incubation in the same way produced only minor albumin degradation. Similar results to those found in patient D1 were obtained with urine specimens from a second patient with Dent’s disease, D2, and a patient, L1, with the Lowe syndrome.

Since cathepsin D is a major lysosomal protease inhibited by pepstatin A [12], we measured the levels of this enzyme in the urine of patients with FS by a specific ELISA. This showed significantly increased excretion of cathepsin D in the urine of FS patients compared with controls, and intermediate levels were found in female carriers of Dent’s disease (Table 2, Figure 2).

Neither the enzyme assay nor the ELISA can distinguish between the precursor of cathepsin D, pro cathepsin D and the mature form of the enzyme. Therefore, we used affinity chromatography of insolubilized pepstatin A to isolate both forms of the enzyme under conditions that minimize conversion of the pro-enzyme to the mature form. Using
Measurement of cathepsin D by ELISA in the urine of patients with: Dent’s disease, D1–D5 (n = 5); carriers of Dent’s disease, DC1–DC3 (n = 3); cystinosis, C1–C3 (n = 3); Lowe syndrome, L1–L3 (n = 3); ADIF, AD1 and AD2 (n = 2) and control patients (n = 8 adults and n = 4 children). Results are expressed per mmol of urine creatinine.

Affinity chromatographic purification of cathepsin D from a patient with Dent’s disease (lane 1) and from two control urine samples (lanes 2 and 3). Cathepsin D was captured from acidified urine samples by chromatography on columns of pepstatin A-agarose, a specific aspartic acid protease inhibitor. Bound proteins were eluted at neutral pH and detected by immunoblotting with anti-cathepsin D antibody (left panel) or non-immune serum (right panel).

In addition, we examined the stability of LDH in urine by adding purified enzyme to control urine samples adjusted to pH 5.5 and pH 6.5 and incubated for up to 4 h at 37°C. Residual LDH levels in duplicate samples expressed as a percentage of initial activity were in control urine (mean of duplicates): pH 6.5 at 1 h 100.1% and at 4 h 102.8%; pH 5.5 at 1 h 100.8% and at 4 h 101.1%. In FS residual LDH levels were pH 6.5 at 1 h 102.0% and at 4 h 103.6%; pH 5.5 at 1 h 103.9% and at 4 h 104.5%. Therefore, degradation of the enzyme in urine is unlikely to explain its low activity in the urine of FS patients.

The intracellular packaging of lysosomal hydrolases depends on the recognition of their phosphorylated mannose (mannose-6-phosphate, M6P) residues by MPRs [9]. Using urine concentrated by dialysis and lyophilization we tested urine for the presence of MPRs. To standardize urine samples for this experiment, we dialyzed urine taken from each type of FS and control urine, which were equivalent in their content of creatinine (25 µmol), so that after lyophilization and reconstitution in denaturing SDS buffer, the samples electrophoresed were equal in their urinary creatinine. We detected elevated CI-MPR in the urine of three patients with cystinosis, two patients with Dent’s disease and one patient with Lowe syndrome (Figure 5, left panel), when compared with control urine.

CI-MPR appears as multiple high-molecular-weight bands of around 250 kDa. The approximate intensity of staining of these bands (scaled from 1+ to 3+) was: cystinosis 3+, 2+ and 2+; Dent’s carrier 1+; Dent’s affected 1+ and 2+ and Lowe syndrome 2+; control samples were all <1+ (Figure 5). Thus, we found greatly increased excretion of CI-MPR in six patients with FS of differing aetiology. Very little CI-MPR could be detected in control urine under the conditions used; an intermediate level was found in a carrier of Dent’s disease (Figure 5). When exposure of the blot to the film was prolonged, clear bands corresponding specifically to CI-MPR were visualized in the control patients (data not shown). To confirm that the immunoreactivity detected was due to CI-MPR, we omitted the anti-CI-MPR antibody from the blotting protocol (Figure 5, right panel).
Fig. 4. Measurement of N-acetyl-β-D-glucosaminidase in the urine of patients with: Dent’s disease (n = 5); carriers of Dent’s disease (n = 5); cystinosis (n = 4); Lowe syndrome (n = 3); ADIF (n = 2) and control patients (n = 8 adults and n = 4 children). Results are expressed as µmol substrate hydrolyzed per hour per mmol of urine creatinine.

Fig. 5. Western blotting of CI-MPR from the urine of FS and control patients: cystinosis patients labelled Cy1, Cy2 and Cy3; paediatric and adult controls, PCon and ACon; Dent’s carrier, DC1; Dent’s affected male patients D1 and D2 and Lowe syndrome patient, L1. Urine of equal creatinine content was dialyzed, lyophilized and electrophoresed on 3–8% SDS gels, blotted and probed with goat anti-CI-MPR antibody followed by peroxidase-labelled anti-goat antibody and chemiluminescent detection. For corresponding control lanes the antibody against CI-MPR was omitted. The arrows give the migration position of 250 kDa and 150 kDa protein markers. Lanes on the right show duplicate urine samples from Lowe syndrome patient (L1) without ‘−’ and with ‘±’ adsorption on PMC-agarose, electrophoresed and then probed with anti-CI-MPR antibody.

Using CI-MPR, we also examined Fanconi urine before and after adsorption with yeast phosphomannan, a specific high-affinity ligand for CI-MPR (Figure 5, right panel). Omission of anti-CIMPR or phosphomannan-agarose adsorption independently eliminated the specific bands corresponding to the region of the gel in which CI-MPR would be expected to run. It is evident from Figure 5 that the proteinuria in FS causes non-specific signals from tubular proteinuria, but these are distinct from those due to CI-MPR.

Efforts to detect CD-MPR in urine using a specific anti-CD-MPR detection method were frustrated by high levels of non-specific immunoreactivity in areas of the gel in which CD-MPR is expected to migrate. Presumably, this is due to the high levels of endogenous low-molecular-weight proteins in the urine of patients with the FS, which are concentrated by the lyophilization procedure used.

To determine whether cathepsin D in urine was tagged by a mannose-6-phosphate (M6P) group, urine was adsorbed onto CI-MPR agarose in the absence and presence of M6P...
Endogenous cathepsin D can almost completely convert endogenous albumin to low-molecular-weight fragments (Figure 1). In patients with FS, the potent degradative capacity of the lysosomal system appears (at least in part) to be exported into the urine (Figures 1–4; Table 2). From the data in Figure 3, the possibility that this activity results from inappropriate secretion of precursor enzymes seems less likely. Normal serum levels of cathepsin D are <2 µg/L, which are undetectable by routine enzyme immunoassay [20], yet we have found levels in FS urine of ~100 µg/L (Table 2). The likely sieving coefficient for the serum 48 kDa heterodimer form of cathepsin D is <10^{-3} [2]; in the absence of proximal tubular cell re-uptake, a urine concentration of about 0.4 µg/L is expected. However, since the serum form of cathepsin D is largely the higher molecular weight precursor, even this may be an overestimate. Therefore, it is unlikely that glomerular filtration and defective proximal tubular cell protein uptake are the origin of the high levels of this enzyme present in FS urine; it is more likely that the proximal tubule cell’s lysosomal compartment is the source of urinary cathepsin D and NAG. When lysosomal enzymes have been measured in the plasma of patients with Lowe syndrome, modest increases of 1.6- to 2.0-fold have been found [21], which are unlikely to account for the larger increases in excretion shown in Table 2 and Figures 2 and 4.

To clarify the mechanism by which lysosomal enzymes appear in FS, we examined the urine of FS patients for the presence of the CI-MPR. CI-MPR found in serum and urine is ~10 kDa smaller than the intracellular form [13]. A very small amount of soluble CI-MPR is known to be excreted in normal human urine, but at <5.6 µg/L [22] this is below the range of reliable quantitation by current ELISA methods. Corresponding levels in normal serum are ~100-fold higher at 0.73 ± 0.61 mg/L [22]. The molecular weight of the soluble receptor is 205–250 kDa [13] (Figure 5) with a predicted glomerular sieving coefficient of ~10^{-5} [2]. In the absence of its tubular re-uptake, the concentration of CI-MPR in normal urine is ~2 µg/L, which makes it unlikely that the elevated CI-MPR levels found in urine originate from filtered plasma and defective proximal tubular protein reabsorption in FS. A more likely explanation is that the proximal tubule itself is the source of the increased excretion of CI-MPR. Further work is needed to establish whether exosomes are the source of this receptor in urine [23].

Carriers of Dent’s disease, all of whom have a normal serum creatinine and minimally low-molecular-weight proteinuria, demonstrated uniformly elevated excretion of cathepsin D and (mostly) increased excretion of NAG (Table 2, Figures 2 and 4), as well as increased CI-MPR excretion (Figure 5). These findings suggest that in FS, impairment of glomerular filtration and proteinuria are not sufficient to cause increased enzyme and receptor excretion. In patients with a variety of forms of glomerulonephritis with proteinuria (greater than generally seen in FS), a correlation between NAG excretion and proteinuria is observed [24].

Recently, it has been found in mice that the proximal renal tubule can take up the lysosomal protease cathepsin B from filtered plasma. Cathepsin B filtered by the glomerulus is thought to be the natural source of this enzyme for its delivery to lysosomes of the proximal tubule [25]. In contrast to the cathepsin B detected in the urine of the mouse model, we have found cathepsin D in human urine to have an M6P tag (Fig. 6). Indeed, in normal human urine, M6P levels are 10 kDa smaller than the intracellular form [13]. A very small amount of soluble CI-MPR is known to be excreted in normal human urine, but at <5.6 µg/L [22] this is below the range of reliable quantitation by current ELISA methods. Corresponding levels in normal serum are ~100-fold higher at 0.73 ± 0.61 mg/L [22]. The molecular weight of the soluble receptor is 205–250 kDa [13] (Figure 5) with a predicted glomerular sieving coefficient of ~10^{-5} [2]. In the absence of its tubular re-uptake, the concentration of CI-MPR in normal urine is ~2 µg/L, which makes it unlikely that the elevated CI-MPR levels found in urine originate from filtered plasma and defective proximal tubular protein reabsorption in FS. A more likely explanation is that the proximal tubule itself is the source of the increased excretion of CI-MPR. Further work is needed to establish whether exosomes are the source of this receptor in urine [23].

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urine cathepsin D has also been found to be M6P-tagged [26].

Thus, we hypothesize that the molecular defects in the various forms of FS studied lead to abnormal trafficking of CI-MPR to the apical membrane of proximal tubule cells, followed by secretion and excretion in the urine. The normal endosomal and lysosomal targeting of CI-MPR is replaced (at least in part) by the default pathway to the apical surface of the cell. Figure 7 illustrates how this might occur in proximal tubule cells in Dent’s disease. The exact mechanism of endocytic failure in Dent’s disease is unclear, in view of the recent finding that CLC-5 is an H+∕Cl− antiporter rather than a Cl− channel [27]. The consequences of impaired MPR function may be partly compensated by MPR-independent pathways, which are not shown in Figure 7.

There are precedents for such aberrant receptor trafficking along the endosomal/lysosomal compartment in Dent’s disease, including of megalin and cubilin [3,8]. Abnormal trafficking of these proteins has been demonstrated, which could explain the finding of their increased urinary excretion in the urine of patients with Dent’s disease or Lowe syndrome. Our findings are also consistent with the recent demonstration that the OCRL1 protein defective in Lowe syndrome is required for normal trafficking of CI-MPR [10]. Moreover, defective acidification of the endosomal compartment in breast cancer cells causes aberrant secretion of cathepsin D [28], and there may be similarities to defective renal endosomal trafficking. Also, in a rat model of FS, Al-Bander et al. concluded that abnormal enzyme secretion, rather than cell necrosis, explained their finding of lysosomal enzymuria following maleic acid administration [18]. Again, this interpretation is consistent with our present findings in patients with FS. However, confirmation that lysosomal enzymuria is caused by defective MPR trafficking will require cell-based in vitro studies in models of FS.

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**References**


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