Calcium phosphate and calcium oxalate crystal handling is dependent upon CLC-5 expression in mouse collecting duct cells

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Abstract

Defects in an intracellular chloride channel CLC-5 cause Dent’s disease, an inherited kidney stone disorder. Using a collecting duct model, mIMCD-3 cells, we show expression of dimeric mCLC-5. Transient transfection of antisense CLC-5 reduces CLC-5 protein expression. Binding of both calcium phosphate (hydroxyapatite) and calcium oxalate monohydrate (COM) crystals overlaid onto mIMCD-3 cultures was affected by altered CLC-5 expression. Calcium phosphate crystal agglomerations (>10 \mu m) were minimal in control (9%) and sense (13%) CLC-5-transfected cells, compared to 66% of antisense CLC-5-transfected cells (\(P<0.001\)). Small calcium phosphate crystals (<10 \mu m) were found associated with 45% of sense CLC-5-treated cells, of which the majority (11/14 cells) appeared to be internalised within the cell. Calcium oxalate agglomerations (>10 \mu m) were also largely absent for controls or sense mCLC-5 transfectants (11% and 9% of cells, respectively) with COM crystal agglomerates predominating in antisense CLC-5 transfectants (66%, \(P<0.0001\)). We conclude that collecting duct cells with reduced CLC-5 expression lead to a tendency to form calcium crystal agglomeration, which may help explain the nephrocalcinosis and nephrolithiasis seen in Dent’s disease.

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1. Introduction

Concentration of urine occurs along the collecting duct resulting in supersaturation of the tubular luminal fluid with respect to calcium and anions, including phosphate and oxalate. This leads to calcium phosphate and calcium oxalate microcrystal formation [1], the retention of which is required for stone formation [2]. Both calcium phosphate and calcium oxalate crystals are injurious to renal epithelial cells [3,4]. Studies in a number of renal epithelial cell lines show that calcium phosphate (hydroxyapatite) [5,6] and calcium oxalate monohydrate (COM) crystals [6,7] may bind to the apical cell surface. Retention of the crystal at the cell surface in vivo is likely to act as a nucleus for further crystal growth, ultimately leading to nephrolithiasis and physical blockade of the duct [1]. To protect against this, urine flow will flush the epithelial surface, therefore limiting crystal attachment. In addition, internalisation of calcium phosphate (hydroxyapatite) and COM crystals via endocytosis is known to occur in cultured renal epithelial cells [8,9] and is a postulated physiological clearance mechanism [10].

Dent’s disease is a complex X-linked disorder resulting from inactivating mutations in the CLCN5 gene encoding an intracellular chloride channel CLC-5 [11]. Dent’s disease is characterised in males by low molecular weight proteinuria, phosphaturia and aminoaciduria with hypercalciuria, nephrocalcinosis and nephrolithiasis, leading to progressive renal failure [12,13]. Both calcium phosphate and calcium oxalate stones are commonly seen in male patients [13]. CLC-5 is a member of the CLC-family of chloride channels, which act as voltage-gated anion channels and have a conserved structure [14], with the ability to form homodimers [15]. CLC-5 mRNA and protein are expressed in multiple nephron segments including the proximal tubule, medullary thick ascending limb and collecting duct cells [16–19].

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An endocytic role for CLC-5 in proximal tubule (PT) cells has been established, where CLC-5 colocalises with acidic endocytic compartiments and facilitates receptor-mediated endocytosis of filtered proteins [16,20]. In mouse CLC-5 knockout models, the endosomal trafficking defect within proximal tubular epithelia [14,21,22] leads to secondary knockout models, the endosomal trafficking defect within endocytosis of filtered proteins [16,20]. In mouse CLC-5 endocytic compartments and facilitates receptor-mediated has been established, where CLC-5 colocalises with acidic endosomal compartments [29].

The stone [24,25], suggesting that calcium phosphate crystal [23], calcium phosphate is frequently found at the core of stones contain calcium oxalate as the major component formation. Indeed, calcium phosphate was found to be present as the initial plaque lesion in stone-forming patients with either idiopathic hypercalciuric patients or hyperoxaluria secondary to intestinal bypass [26]. As Dent’s disease patients present with both species of stone the cell surface crystal handling in a collecting duct cell model (mIMCD-3 cells) [27]). These well-characterised epithelial cells [28] endogenously express CLC-5 [29], which colocalises intra-cellularly with acidic endosomal compartiments [29]. mMCD-3 cells are also endocytically active [10] and readily bind calcium crystals [10].

2. Materials and methods

2.1. Tissue culture

mIMCD-3 cells were provided by Dr. S. Gullans [27] and were cultured in 50:50 v/v HAMs-F12/DMEM with 2 g/1 glucose, 10% v/v FCS and 2 mM l-glutamine as previously described [29].

2.2. Molecular reagents

Sense CLC-5 construct (full-length murine CLC-5), antisense CLC-5 construct (full-length reverse orientation mCLC-5), and mCLC-5-GFP (mCLC-5 with a GFP fusion protein) were prepared as previously described [29].

2.3. Western blotting

mMCD-3 cultures were incubated in protein lysis buffer (2% Nonidet P-40, 0.2% SDS, 1 mM DTT in PBS, protease inhibitor mix (Complete Mini, Roche Diagnostics Ltd.)). The lysate was centrifuged (1000 × g, 10 min) treated with NuPAGE reducing agent, (containing 0.5 M DTT (Invitrogen Life Technologies)) and heated for 10 min at 70 °C. More stringent reducing conditions ((l-mercaptoethanol), heating and sonication were used in parallel experiments. Protein concentrations were determined using the Bradford method [30].

After SDS-PAGE (4–12% Bis–Tris) proteins were transferred to PDVF membranes. Blots were blocked in Tris-buffered saline containing 0.1% Tween (TBS-T) and 5% milk powder. Blots were incubated with rabbit anti-CLC-5 antibody PEP5A1 (directed against peptide CKSRD RDRHREITNKS-amide) diluted (1:500) in blocking solution at 37 °C for 1 h then at 4 °C for 24 h. For peptide/antigen competition experiments, the peptide CKSRD RDRHREITNKS-amide was pre-incubated in excess (100 μg) with primary antibody. The mixture was then centrifuged (10,000 × g, 1 min) and the supernatant used in parallel to primary antibody. Anti rabbit-HRP conjugate (1:20,000 in TBS-T) was used for secondary detection. Reacting proteins were detected using an ECL plus system (Amersham Pharmacia Biotech) and photographic film (hyperfilm ECL, Amersham Pharmacia Biotech). Membranes were stripped and re-probed using primary antibody rabbit anti-actin antibody (Sigma) and secondarily detected using anti-rabbit HRP conjugate (as above). Band densitometry was performed on digitized gel images by Alpha Image software.

2.4. Transient transfection

mMCD-3 cells were transfected using either Effectene (Qiagen) or Lipofectamine 2000 (Life Technologies) reagents. For Western blot analysis, 75 cm² flasks of sub-confluent cells were transiently transfected with sense or antisense CLC-5 plasmid DNA and protein lysate harvested 48 h post transfection. For localisation experiments, cells were seeded onto 13 mm glass coverslips (30,000/cm²) the day prior to transfection. mCLC-5-GFP was transiently transfected 24 h prior to counter staining.

2.5. Calcium phosphate and COM crystal experiments

Calcium phosphate (hydroxyapatite) crystals were formed from a solution of 2.5 M CaCl₂, 500 μl of 1/10
TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 7.6) and 1 ml of 2 × HEPES solution (140 mM NaCl, 1.5 mM Na₂HPO₄, 50 mM HEPES, pH 7.05 at 23 °C). Media was aspirated from sub-confluent cells transiently transfected (24 h) with pcDNA3.1/GFP or co-transfected with pcDNA3.1/GFP and sense CLC-5/antisense CLC-5 using a μg ratio of 3:1 and cells were overlaid with 500 μl of calcium phosphate solution for 30 min at 37 °C.

COM crystals were prepared as described by Lieske et al. [31] by vapour diffusion from diethyloxalate into calcium-containing buffer (155 mM NaCl, 5.4 mM KCl, 100 mM glucose, 50 mM CaCl₂, 90 mM Tris). Rather than culture cells in the presence of diethyloxalate vapour, as we have previously shown [10], 200 μl aliquots of the resulting COM crystals were removed and used to overlay sub-confluent transiently transfected cells (in a directly comparable way to that of calcium phosphate crystals) and incubated for 30 min.

Coverslips were imaged by CLSM (Leica TCS-NT) using FITC and transmitted light filter sets with a ×100 oil immersion lens (NA 1.4) to allow optical sectioning. Z-series images were collected and merged using Adobe PhotoShop software. Positive transfectants were examined and analysed in a blinded fashion. Cell surface crystal attachments were measured by analysis of transmitted light z-series images, allowing the maximum diameter of crystal attachment to be calculated. Crystal attachment measuring >10 μm were considered to be a crystal agglomeration.

To control for transfection-induced toxicity, transfected cells were assayed with ‘Live/Dead Cytotoxicity Kit’ (Molecular Probes) using colocalisation of DEAD Red ethidium homodimer-2 nuclear stain and GFP. Transfection with CLC-5 and GFP cDNA constructs did not increase cell staining compared to untransfected controls.

In separate experiments, CLC-5-GFP-transfected cells were overlaid with calcium phosphate and calcium oxalate crystals (prepared as above) before imaging and analysis as described above.

2.6. Statistics

For comparisons of size of crystal attachments and agglomerations between sense- and antisense-treated cells, the Chi-square test was used.

3. Results and discussion

3.1. CLC-5 protein expression and reduction of CLC-5 protein following antisense CLC-5 transfection

Fig. 1 confirms endogenous CLC-5 expression in whole kidney and mIMCD-3 cells. A band of 83 kDa as predicted for CLC-5, is readily detected in murine whole kidney (Fig. 1A, lane 1). Two additional bands are seen, a diffuse ~90 kDa band and a faint high molecular weight band, corresponding to ~166 kDa. The relative intensity of the high molecular weight band to that at ~83 kDa remained unchanged after gel electrophoresis was repeated in non-reducing conditions (data not shown). With mIMCD-3 protein, a prominent band is present at ~166 kDa (Fig. 1A, lane 2) in addition to a number of faint bands between 83 and 166 kDa. Preabsorption of PEP5A1 antibody with peptide (CKSRDRDRHREITNKS-amide) resulted in disappearance of the prominent 83 kDa band in whole kidney and the 166 kDa band in mIMCD-3 protein (Fig. 1A, lanes 3 and 4). More stringent reducing conditions applied to mIMCD-3 protein failed to remove the 166 kDa band in favour of 83 kDa (not shown). Given the specificity of the
PEP5A1 antibody, it is likely that CLC-5 protein is present in dimeric form in mIMCD-3 cells. Diffuse 83 kDa bands with high molecular components have previously been shown, but not discussed, using comparable and different anti-CLC-5 antibodies [32,33] in Western blots for CLC-5 protein. The crystal structure of the CLC family, confirmed in two bacterial CLCs elucidated to 3 Å, indicates a two-pore homodimer [15]. The demonstration by Western blot analysis of expression of mostly CLC-5 monomer in whole kidney and almost exclusively dimer in mIMCD-3 cells is interesting. mIMCD-3 cells are a distinct cell population derived from the terminal portion of murine collecting duct [27] accounting for only a small fraction of renal mass as opposed to the heterogeneous mix of cells constituting whole kidney, and this may account for the observed difference in expression. Certainly the dimeric form of CLC-5 was resistant to reduction, implying a highly stable quaternary structure. We speculate that the difference in ratios of monomeric form to dimeric form in renal tissue may be an indication of rapid turnover of CLC-5 within

Fig. 2. Antisense CLC-5 transfection causes calcium phosphate crystal agglomeration. Comparison of mIMCD-3 cells transiently transfected with (A) pcDNA3.1/GFP alone, (B) co-transfected with pcDNA3.1/GFP and sense CLC-5, and (C) co-transfected with pcDNA3.1/GFP and antisense CLC-5 when overlaid with calcium phosphate crystals. Scale bar = 10 μm. Quantification of crystal attachment in (D) control (E) sense CLC-5 (F) and antisense CLC-5 treated cells. Data shown are from six separate experiments. Control and sense CLC-5 transfectants demonstrated either no crystal attachment or small crystal attachments (up to 10 μm in diameter). The remaining control and sense CLC-5 cells (2/23, 4/31) showed adhesion of larger (11 – 50 μm) crystal agglomerates. For antisense CLC-5 transfectants, the majority of cells (21/32, P<0.001) were associated with crystal agglomerates (>10 μm). (For color see online version).
renal tubules with a large pool of monomeric CLC-5 available within the biosynthetic pathway to form into dimers necessary for specific function.

Fig. 1B shows that transient transfection with antisense CLC-5 plasmid results in a ~50% reduction in the density of the mCLC-5 specific ~166 kDa band compared to control and sense CLC-5 treated cells (relative intensities: 1.0, 0.9, 0.5 arbitrary units, respectively). This provides confirmatory evidence as to the identity of the ~166 kDa band. This Western blot evidence of knock-down of CLC-5 protein is consistent with our previous data, where we demonstrate by immunocytochemistry that transient transfection of antisense CLC-5 reduces intracellular expression of CLC-5 in mIMCD-3 cells [10].

3.2. Antisense CLC-5 transfectants result in agglomeration of calcium phosphate and oxalate crystals

Since crystal attachment and internalisation may represent an intrinsic mechanism for prevention of stone forma-
tion in the renal tubule [8,9], we exposed mIMCD-3 cells to pre-formed calcium phosphate and calcium oxalate crystals.

For cells exposed to calcium phosphate, both pcDNA3.1/GFP control transfectants and those co-transfected with pcDNA3.1/GFP and sense CLC-5 were comparable (Fig. 2A,B). These cells had predominantly an absence of crystals (10/23 and 13/31, respectively) or single small crystal attachments up to 10 µm in diameter (11/23 and 14/31 cells, respectively). Larger crystals (11–50 µm) were seen in 2/23 and 4/31 cells, respectively (Fig. 2D,E).

In contrast, pcDNA3.1/GFP and antisense CLC-5 co-transfectants demonstrated a marked increase (21/32 cells, P < 0.001) in the tendency to form large (>10 µm) crystal agglomerations (Fig. 2C,F) when compared to control or sense transfectants. There was also a corresponding fall in the number of cells having single crystal attachments (2/32 cells) (Fig. 2F).

For cells overlaid with calcium oxalate, control and sense CLC-5-transfected cells behaved similarly (Fig. 3A,B), with either absence of crystals (34/44 and 38/44 cells, respectively) or limited small single crystal attachment (5/44 and 2/44 cells, respectively) (Fig. 3D,E). Crystal agglomerations greater than 10 µm were rarely observed (5/44 and 4/44 cells, respectively). After antisense mCLC-5 transfection, the majority of antisense CLC-5 transfectants (33/50, P < 0.0001) were associated with crystal agglomerations at the cell surface (Fig. 3C,F).

Adherence of small calcium phosphate and calcium oxalate crystals often appeared to be accompanied by rapid crystal endocytosis. Of the 14 sense CLC-5-transfected cells associated with calcium phosphate crystals less than 10 µm in diameter (Fig. 2E), in 11 showed evidence of crystal internalisation. When the focal plane was at a mid or base section, small crystals were visualised but when the focal plane was at the cell surface, crystals were not evident (Fig. 4A–C). This finding of small crystal internalisation was confirmed by the use of CLC-5-GFP constructs, where mid-cell xy-sections demonstrate internalisation of small calcium phosphate crystals associated with CLC-5, which appears to surround the crystal (Fig. 4D). In the calcium oxalate experiments, small crystals less than 10 µm in diameter were less frequently seen associated with sense CLC-5 transfectants. Only two cells had this finding (Fig. 3E), but analysis of these cells revealed that in each case, there was evidence of crystal internalisation from the surface to within the cell (Fig. 4E–G). CLC-5-GFP also appeared to surround these internalised calcium oxalate crystals (Fig. 4H).

These data demonstrate that both calcium phosphate (hydroxyapatite) and calcium oxalate (COM) crystals may attach and be endocytosed from the surface of mIMCD-3 cells. Although the crystal attachment experiments were performed in sub-confluent cells, which have been shown to be more susceptible to crystal binding [34], we have confirmed that both calcium phosphate and calcium oxalate crystal attachment and internalisation also occurs when these cells were grown to confluence (data not shown). mIMCD-3 cells behave, in terms of crystal adherence, in a similar manner to the LLC-PK1 cell line [35,36]. It is of note that the RCCD1 and MDCK-I cell lines with distal and collecting duct phenotype failed to endocytose calcium oxalate crystals [36], confirming this process is likely to depend upon specific cell receptors and is not uniform along
the nephron. Mechanisms of crystal attachment and internalisation are thus complex and it is also likely that specific crystal species will be handled differently.

Recent data suggests that calcium phosphate crystals may be the initiating event of both interstitial calcification and intratubular calcium oxalate stone formation [26]. Patients with idiopathic calcium oxalate stone formation developed interstitial crystal deposits, which led to calcium apatite plaque formation [26]. Subsequent penetration of this plaque would provide a surface for calcium oxalate adhesion and stone formation. Examination of histological section of collecting ducts in hyperoxaluric patients (secondary to intestinal bypass surgery) revealed apical attachment of calcium apatite crystals, which led to intraluminal obstruction and epithelial damage [26].

The crystal–epithelial cell interaction appears dramatically different in CLC-5 knockout cells, compared to cells expressing wild-type CLC-5. Both calcium phosphate and calcium oxalate crystals more readily adhere to the CLC-5 knockout cells, which then appear to act as a nucleus for crystal agglomeration. This process is relatively rapid and it is likely that either CLC-5 knockout cells are expressing increased levels of crystal adhesion molecules, decreased levels of crystal inhibitors or are unable to process and dissolve (by endocytosis) such large adherent crystals. Several recent studies suggest that calcium microcrystals bind to specific receptors on the cell surface [5,31,36–38]. It is of note that annexin II, a COM binding protein, colocalises with caveolin-I and is involved in exocytosis and endocytosis pathways. It now needs to be determined whether CLC-5 is regulating plasma membrane expression of such adhesion molecules. In cells deficient in CLC-5, we speculate that inhibition of endocytosis and of traffic or recycling of early endosomes alters the apical membrane complement of surface proteins and of secreted molecules regulating crystal adherence, e.g. osteopontin [39], hyaluronan [38,40] and annexin II [37]. This would then lead to the increased binding and attachment of initial calcium microcrystals. It is also possible that antisense CLC-5 transfection may induce a degree of endoplasmic reticulum stress, secondary to accumulation of misfolded proteins, thereby promoting the expression of hyaluronan [41]. This glycosaminoglycan would then promote crystal interactions at the plasma membrane. Inhibition of endocytosis, as previously demonstrated in CLC-5-deficient cells [10] may also lead to prolonged retention time of crystals at the epithelial surface, allowing enhanced agglomeration of crystals. Direct cytotoxicity of the antisense cDNA transfection was excluded prior to crystal adhesion by measurements of ethidium bromide homodimer-2 (Dead Red) in positive transfectants. We are unable, however, to dismiss the possibility that increased crystal attachment and agglomeration would result in downstream cell signalling leading to cell activation and further crystal deposition. COM crystal attachment is known to stimulate down-stream signalling events including p38 MAP kinase activation in LLCPK1 cells [42]. Clearly, a key feature is the increased residence time of COM crystal on the apical surface. This then acts as a nucleus for further crystal adhesion, which as these data show, occurs much more readily in CLC-5-deficient cells.

We conclude that an endocytic role for CLC-5 in the collecting duct and disrupted cellular handling of calcium crystals may account for the unexplained phenotype of nephrocalcinosis and renal stone formation in Dent’s disease.

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